Molecular Characterization of Chronic-type Adult T-cell Leukemia/Lymphoma

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Abstract

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1–induced neoplasm with four clinical subtypes: acute, lymphoma, chronic, and smoldering. Although the chronic type is regarded as indolent ATL, about half of the cases progress to acute-type ATL. The molecular pathogenesis of acute transformation in chronic-type ATL is only partially understood. In an effort to determine the molecular pathogeneses of ATL, and especially the molecular mechanism of acute transformation, oligo-array comparative genomic hybridization and comprehensive gene expression profiling were applied to 27 and 35 cases of chronic and acute type ATL, respectively. The genomic profile of the chronic type was nearly identical to that of acute-type ATL, although more genomic alterations characteristic of acute-type ATL were observed. Among the genomic alterations frequently observed in acute-type ATL, the loss of CDKN2A, which is involved in cell-cycle deregulation, was especially characteristic of acute-type ATL compared with chronic-type ATL. Furthermore, we found that genomic alteration of CD58, which is implicated in escape from the immunosurveillance mechanism, is more frequently observed in acute-type ATL than in the chronic-type. Interestingly, the chronic-type cases with cell-cycle deregulation and disruption of immunosurveillance mechanism were associated with earlier progression to acute-type ATL. These findings suggested that cell-cycle deregulation and the immune escape mechanism play important roles in acute transformation of the chronic type and indicated that these alterations are good predictive markers for chronic-type ATL.

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1 (HTLV-1)–induced neoplasm (1, 2). Four clinical subtypes of ATL have been classified on the basis of clinical manifestation: acute, lymphoma, chronic, and smoldering (3). Among these subtypes, chronic-type ATL shows characteristic manifestations such as increased abnormal lymphocytes in peripheral blood, lactate dehydrogenase (LDH) levels up to twice the normal upper limit, and absence of hypercalcemia. Chronic-type ATL is relatively rare and its frequency is estimated to be 8% to 18% of ATL cases (3). Previous reports regard the chronic type as indolent ATL compared with acute/lymphoma types, which show an aggressive clinical course (3, 4). However, a recent study of indolent ATL demonstrated that about half of the patients with chronic-type ATL progress to acute-type ATL within approximately 18 months from diagnosis and subsequent death (4). This finding suggests that patients with chronic-type ATL also had a poor prognosis. High LDH, high blood urea nitrogen, and low albumin levels have been identified as poor prognostic factors for chronic-type ATL, and patients with chronic-type ATL with these poor prognostic factors therefore need to be treated by intensive chemotherapy as in the case of patients with aggressive ATL (5). Disruptions of CDKN2A, CDKN2B, and TP53 have been reported as candidate genes that play important roles in acute...
transformation of chronic-type ATL (6–12). However, these acute transformation–related genetic alterations have been identified only by focusing on genes that were previously shown to be involved in tumor progression of other malignancies. Therefore, these genetic alterations may be indicative of acute transformation in some cases, although the molecular mechanism of acute transformation remains to be fully elucidated. Identification of the molecular characteristics of chronic-type ATL using unbiased and genome-wide methods can provide further insights to elucidate the acute transformation mechanisms in chronic-type ATL. However, the molecular pathogenesis of chronic-type ATL has long remained unknown due to its rarity (13).

In the present study, high-resolution oligo-array comparative genomic hybridization (aCGH) and gene expression profiling (GEP) were applied to 27 cases of chronic-type ATL in an effort to determine the molecular pathogenesis. The same approaches were used with 35 cases of acute-type ATL, and we then compared the molecular characteristics of chronic- and acute-type ATL to investigate the molecular mechanism of acute transformation.

Materials and Methods

Patient samples

We collected and analyzed 27 cases of chronic-type ATL and 35 cases of acute-type ATL (Table 1 and Supplementary Table S1 in Supplementary Data). These samples were obtained from patients at Imamura-Bunin Hospital (Kagoshima, Japan), Nagasaki University School of Medicine (Nagasaki, Japan), Heart Life Hospital (Nakagusukuson, Japan), and Kyushu Cancer Center (Fukuoka, Japan). In accordance with Shimoyama criteria, the diagnoses were made by expert hematologists (A. Utsunomiya, K. Tsukasaki, Y. Imazumi, N. Taira, and N. Uike; ref. 3). Samples and medical records used in our study were approved by the Institute Review Board of the Aichi Cancer Center (Nagoya, Japan). Informed consent was obtained from all patients. DNA and RNA used in this study were extracted from purified CD4-positive cells as previously reported (14). For the cumulative incidence of acute transformation, events were defined as acute transformation or any treatment for ATL.

Copy number analysis by aCGH and GEP

We performed aCGH analysis on all samples using 400K aCGH (Agilent, Cat. # G4448A; Agilent Technologies) and 44K aCGH (Agilent, Cat. # G413A) slides (Supplementary Table S1). Thirteen acute-type cases analyzed in a previous study were included (14). Procedures for DNA digestion, labeling, hybridization, scanning, and data analyses were performed according to the manufacturer's protocols (www. agilent.com). Raw data were transferred to the Genomic Workbench v5.0 software (Agilent Technologies) for further analysis as described previously (14–16). Among these identified alterations, we focused on minimal common regions (MCR). MCRs are defined as alterations that encompass less than 3 protein-coding genes among all samples analyzed in this study (17). Copy number variations/polymorphisms (CNV) were identified using a database (HS_hg18_CNV-20120403, Agilent), which was obtained from Database of Genomic Variants (http://projects.tcag.ca/variation/) in April 2012 and then excluded from further analyses as described previously (16). We also performed aCGH analysis on matched normal DNA samples that were available and confirmed that the identified MCRs were not CNVs (Supplementary Fig. S1A).

For analysis of GEP, the Whole Human Genome 44K Oligo-microarray Kit (Agilent, Cat. # G4112F) was used for the hybridization of labeled RNA. The total RNA of 13 chronic samples and 21 acute samples was analyzed. The experimental protocol used reflected the manufacturer’s protocol (www.agilent.com) as previously reported (15, 16). Using the results of GEP, gene set enrichment analysis (GSEA) was performed as previously described (15, 16, 18).

The detailed description of these analyses can be found in Supplementary Methods. The microarray data were submitted to ArrayExpress and assigned accession numbers E-MTAB-1808 (aCGH) and E-MTAB-1798 (GEP). 

Mutation analyses of CD58 and β2-microglobulin

The exons 1–4 of CD58 and 1 and 2 of β2-microglobulin (B2M), whose mutations were identified in peripheral T-cell lymphomas (PTCL; ref. 19), were amplified from gDNA using PCR. PCR primers used are detailed in the previous study (20). Twenty-six acute-type and 26 chronic-type ATL samples, for which adequate DNA was available, were analyzed. Direct sequencing of PCR products was performed through capillary electrophoresis using the ABI3100 sequencer (Applied Biosystems).

Flow cytometry

Analysis of cell surface CD58 in ATL cell lines was performed using anti-CD58 PE antibody (AICD58, Beckman Coulter).

### Table 1. Patient information at sampling

<table>
<thead>
<tr>
<th>Subtype</th>
<th>No. of samples</th>
<th>Median age (range), y</th>
<th>Median WBC (range), u/L</th>
<th>Median LDH (range), IU/L</th>
<th>Median calcium (range), mg/dL</th>
<th>Median albumin (range), g/dL</th>
<th>Median BUN (range), mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>27</td>
<td>61 (42–81)</td>
<td>1,1400 (6,000–22,100)</td>
<td>233 (155–465)</td>
<td>9.3 (8.4–10.2)</td>
<td>4.2 (3.0–4.8)</td>
<td>15.5 (7.4–26.4)</td>
</tr>
<tr>
<td>Acute</td>
<td>35</td>
<td>57 (32–85)</td>
<td>2,1700 (4,100–22,800)</td>
<td>688 (203–2,223)</td>
<td>9.3 (7.7–17.4)</td>
<td>3.8 (2.6–4.5)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: BUN, blood urea nitrogen; NA, not available; WBC, white blood cells.
Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo Version 7.2.4 software (TreeStar). The detailed description of these analyses can be found in Supplementary Methods.

**Statistical analysis**

Frequencies of genomic alterations were evaluated using Fisher exact test, and cumulative acute transformation rates were analyzed using Kaplan–Meier method.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing; ref. 21).

**Results**

**Genomic alteration profiles of chronic- and acute-type ATL**

To evaluate the genomic alterations of chronic- and acute-type ATL, aCGH was performed for 62 patient samples (27 cases of chronic-type and 35 cases of acute-type ATL; Table 1 and Supplementary Table S1). Figure 1A shows genomic alteration profiles of chronic- and acute-type ATL. We identified 362 MCRs (230 losses and 132 gains) among the alterations. These MCRs contained 1–3 protein-coding genes, which are most likely the candidate genes of the alterations (15, 17). Frequent alterations are supposed to especially contribute to the pathophysiology of the disease. MCRs that were found in...
more than 20% of chronic- or acute-type ATL were therefore analyzed (Fig. 1B and C).

Genomic loss of CDKN2A/CDKN2B was the first most frequently altered MCR in acute-type ATL (17 of 35 cases). The second most frequently altered MCR of acute-type ATL was genomic loss of SYNCRIP (16 of 35 cases). On the other hand, genomic losses of SYNCRIP and NRK3 and gain of RRA1 were most frequently altered MCRs in chronic-type ATL (7 of 27 cases). Among these identified MCRs, the losses of CDKN2A/CDKN2B, CCDC7, and ITGB1 were significantly characteristic of acute-type ATL (Fig. 1B, P < 0.05). In addition, acute-type ATL tended to have a loss of CD58 (Fig. 1B). The frequently altered MCRs in chronic-type ATL were also found in acute-type ATL (Fig. 1B and C). Gains of chromosomes 1q, 3p, 3q, 7q, and 19p were also frequently observed in acute- and chronic-type ATL, although they did not show MCRs (Fig. 1D). Among these alterations, acute-type ATL tended to have a gain of 3q (P = 0.07).

**Frequent loss of CDKN2A/CDKN2B**

Our analysis identified loss of CDKN2A/CDKN2B located in 9p21.3 as the most frequently and specifically altered genomic region in acute-type ATL compared with chronic-type ATL. Therefore, this loss is suggested to play an important role in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL.

Seventeen of the 35 acute-type ATL samples showed loss of 9p21.3, which was also found in 5 of the 27 chronic-type ATL samples. These losses always included CDKN2A/CDKN2B (Fig. 2A). Homozygous loss of CDKN2A/CDKN2B was observed in 10 of the 17 affected acute-type ATL samples but was never observed in chronic-type ATL. The genes whose expression was affected by copy number changes are considered candidate genes in the regions of genomic alterations (15, 22, 23). We therefore evaluated the expressions of CDKN2A and CDKN2B in acute-type and chronic-type ATL with or without loss of 9p (Fig. 2B). CDKN2A expression was much lower in acute-type ATL samples with the loss of 9p than in other samples. CDKN2B expression was not reduced in accordance with the loss of 9p. Therefore, CDKN2A is a likely candidate tumor suppressor gene located in 9p21.3.

Serial samples of a patient with chronic-type ATL showing acute transformation were analyzed in detail. The DNA and RNA samples of this patient at about 19 months before acute transformation (chronic phase, C-10) and at acute transformation (acute phase, A-15) were available. Clonality analysis of T-cell receptor gamma locus showed that clones of ATL cells at chronic and acute phases were identical to each other (Supplementary Fig. S1A). Although the chronic-phase sample showed heterozygous loss of CDKN2A/CDKN2B, the acute-phase sample showed homozygous loss of CDKN2A/CDKN2B (Fig. 2C). In addition, the expression of CDKN2A was remarkably reduced in the acute phase (Fig. 2D). Analysis of these serial samples of an identical patient also indicated that CDKN2A is the most likely candidate gene located in 9p21.3 and that the loss of CDKN2A is associated with acute transformation.

**Frequently altered cell-cycle pathway in acute-type ATL**

CDKN2A contains 2 known transcriptional variants, INK4a (p16) and ARF (p14). Both of these genes are known to be negative regulators of the cell cycle. We next evaluated the distributions of genomic alterations of CDKN2A with other genes that were previously reported to affect the cell cycle (Fig. 2E; ref. 24). Our analysis revealed that losses of CDKN2A and losses of TP53 tended to be mutually exclusive events, and this pattern was also observed for losses of TP53 and gains of MDM4/RFWD2. These alterations of cell-cycle–related genes were specifically observed in acute-type ATL compared with chronic-type ATL (80% of acute-type and 56% of chronic-type ATL, P < 0.05; Fig. 2F). Among chronic-type ATL cases, those with acute transformation tended to have alterations of cell-cycle–related genes (Fig. 2G). GSEA also revealed that the cell-cycle–related gene set and genes functionally associated with proliferation were significantly enriched in acute-type ATL compared with chronic-type ATL (Supplementary Fig. S1C).

These results indicated that alterations of the cell-cycle pathway, including the genomic loss of CDKN2A, played critical roles in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL. In vitro assays showed that inductions of INK4a or ARF that are encoded by CDKN2A caused suppression of cell proliferation, cell-cycle arrest, and apoptosis in ATL cell lines with genomic loss of 9p21.3 (Supplementary Fig. S2).

**Genomic alterations of CD58 in ATL**

In addition to loss of CDKN2A/CDKN2B, we found that losses of CCDC7, ITGB1, and CD58 and gain of chromosome 3q were more frequently recognized in acute-type ATL than in chronic-type ATL. Alterations of cell-cycle–related genes, including CDKN2A, are considered important events for the transformation described above. We therefore analyzed the distributions of alterations of cell-cycle–related genes and the genes that were characteristic of acute-type ATL in each type of ATL case (Fig. 3). This analysis revealed that alterations of cell-cycle–related genes and the gene alterations characteristic of acute-type ATL mainly coexisted. A case having the loss of CD58 or gain of 3q without alterations of cell cycle existed for each type of ATL, although all cases with losses of ITGB1 and CCDC7 showed the alterations of cell-cycle–related genes.

In chronic-type ATL cases without alterations of cell-cycle–related genes, a case with loss of CD58 showed acute transformation later, although a case with gain of 3q did not exhibit the transformation without any therapy during 30 months after the diagnosis. CD58 is a gene known to be involved in activation of natural killer (NK) cells and cytotoxic T cells (CTL; refs. 25, 26). Inactivation of CD58 is reported to play an important role in the pathophysiology of diffuse large B-cell lymphoma (DLBCL) through the mechanism of escape from the immunosurveillance system (20). Recurrent mutation of CD58 has also been observed recently in PTCLs (19). We therefore further analyzed CD58 in ATL.

Analyses using aCGH revealed that 26% (9 of 35) of acute-type ATL and 7% (2 of 27) of chronic-type ATL had genomic loss of 1p13 (Figs. 1B and 4A). These losses always included CD58 and one case showed genomic loss that only included
Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including CDKN2A/CDKN2B. Heatmap analysis of 400K aCGH shows log2 ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the CDKN2A/CDKN2B locus. B, gene expression levels of CDKN2A and CDKN2B. Gene expression levels of CDKN2A and CDKN2B were analyzed in 13 chronic-type and 21 acute-type ATL cases by GEP. Average gene expressions and SDs are shown in cases grouped as indicated. CDKN2A expression was reduced only in acute-type ATL cases exhibiting loss of CDKN2A/CDKN2B. CDKN2B expression did not change in relation to genomic loss or subtype. Probes of A_23_P43484 (CDKN2A) and A_23_P216812 (CDKN2B) were used in experiments. C, genomic alteration of 9p in serial samples of a case with chronic type showing acute transformation. Left, a heatmap of the log2 ratio in the chronic phase; right, a heatmap of the ratio in the acute phase. The sample in the chronic phase indicates a heterozygous loss of the CDKN2A/CDKN2B locus and the loss changes to a homozygous loss for the sample in the acute phase. D, gene expressions of CDKN2A and CDKN2B in serial samples. CDKN2A expression was remarkably reduced in the acute phase, but CDKN2B expression was almost identical during transformation in this case. Gray, the chronic phase; black, the acute phase. E, alterations of cell-cycle–related genes in chronic-type and acute-type ATL. In the heatmap, rows correspond to the indicated alterations and columns represent individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Losses of TP53 and gains of MDM4/RFWD2 showed a similar tendency. F, alteration frequency of cell-cycle–related genes. Genetic alteration frequency of cell-cycle–related genes was significantly higher in acute-type ATL cases (80%) than in chronic-type ATL (56%; Fisher exact test; *, P < 0.05). The actual number of affected samples over the total number analyzed is shown at top of the figure. G, alteration frequency of cell-cycle–related genes among chronic-type ATL cases. The frequency of alterations of cell-cycle–related genes was higher in cases with later acute transformation than in cases without acute transformation.
CD58. Homozygous loss of CD58 was observed only in acute-type ATL samples. Furthermore, expression of CD58 was reduced in acute-type ATL cases accompanied with the genomic loss (Fig. 4B). Flow cytometric analyses also suggested that genomic loss of CD58 reduced the expression on the cell surfaces (Fig. 4C). Sequence analysis of CD58 revealed a nonsense mutation in one acute-type ATL case. This mutation indicated that the 97th position of serine changed to a stop codon (p.S97X; c.290C>G; Supplementary Table S2). Combined with the results of the genomic and mutation analyses, 29% of acute-type and 7% of chronic-type ATL had a gain of 3q without disruption of the cell cycle existed in each type of ATL. The loss of CD58 and gain of 3q were almost mutually exclusive, except for two cases of acute-type ATL.

Discussion

We have studied 27 cases of chronic-type ATL and compared with 35 cases of acute-type ATL. Until now, only a few chronic-type ATL cases had been analyzed, and the molecular mechanisms of the transformation were investigated by focusing on the well-known tumor suppressor genes (CDKN2A and TP53; refs. 6–12). In contrast, our investigation comprehensively analyzed genomic profiles, and molecular aspects were analyzed using unbiased and whole-genome methods. Our study of chronic-type ATL represents the largest study to date that has analyzed the whole-genomic status of chronic-type ATL cases.

Common genomic alterations in chronic- and acute-type ATL

Genomic alteration profiles of chronic- and acute-type ATL were found to be almost identical (Fig. 1). The number of genomic alterations was found to be higher in acute-type ATL than in the chronic type, and the frequently altered regions of chronic-type ATL were also observed in the acute type. Thus, chronic-type ATL might be a pre-acute form of the disease.

The common MCRs in chronic- and acute-type ATL included genes involved in T-cell receptor signaling, such as ITGB1 and STK (27, 28). We also identified SYNCRIP as a common MCR in both types of ATL. SYNCRIP is a gene known to be involved in maturation of mRNA (29). RXRA, which has been reported to be implicated in colorectal carcinogenesis (30), is also frequently altered in both types of ATL. In addition, our analysis suggested that gain of RXRA is involved in acute transformation of chronic-type ATL because the chronic-type ATL possessing the gain of RXRA showed earlier progression to the acute type. These MCRs may play important roles in the development of ATL coordinately with HTLV-1.

Deregulation of the cell-cycle pathway: an alteration related to acute transformation

Our analyses of genomic alterations revealed that no single genomic alteration seems to be responsible for the mechanism...
of acute transformation, and various genomic alterations and combinations of alterations exist in this mechanism (Fig. 3).

We found that deregulation of the cell cycle, including genomic loss of CDKN2A, might be an important event in the transformation. Genomic loss of CDKN2A was also reported to play a crucial role in the transformation of chronic lymphocytic leukemia known as Richter syndrome (31, 32).

Although previous studies using Southern blot analysis revealed that 11% to 17% of acute-type ATL had the homozygous loss of CDKN2A (7, 9), our analyses using unbiased and whole-genome methods were able to reveal the frequency of the loss in greater detail. We found that approximately 30% of acute-type ATL cases showed a homozygous loss of the CDKN2A/CDKN2B locus, and 50% of acute-type ATL cases...
exhibited the homozygous or heterozygous loss of this locus. Yamagishi and colleagues used high-resolution aCGH analyses and found that this loss was frequently found in ATL samples (33). We also found that 5 of 27 chronic-type ATL cases had homozygous loss of CDKN2A. Three of the 5 cases with CDKN2A loss progressed to the acute type, but 11 of the 22 cases without CDKN2A loss also showed acute transformation. Because of this finding, CDKN2A loss was not significantly associated with the earlier acute transformation in our study (Supplementary Table S3). Although previous studies revealed that approximately 5% of chronic-type had this loss (7, 9, 10), these previous studies did not show the cumulative acute transformation rate according to CDKN2A loss.

CDKN2A expression was reduced in acute-type ATL samples exhibiting genomic loss of the CDKN2A locus. A portion of acute-type ATL cases without the genomic loss showed a low expression level of CDKN2A, suggesting that methylation of the gene might affect the expression in these samples (11, 12). However, we consider that the genomic loss of CDKN2A has a greater influence on the expression of the gene than the methylation because the CDKN2A expression levels were remarkably reduced in accordance with the genomic loss (Fig. 2B and D).

Alterations of both CDKN2A and TP53 were previously reported to be mutually exclusive (34), and our results showed the same trend. In addition, loss of TP53 and gains of MDM2/BFWD2 tended to be mutually exclusive in our acute-type ATL samples. Because these genes are involved in the TP53 pathway, our findings indicate that the TP53 pathway may also play a pivotal role in the pathophysiology of acute-type ATL. In fact, 80% of acute-type ATL had the alterations of cell-cycle–related genes, including CDKN2A and TP53. On the basis of this finding, we found that the alterations of cell-cycle–related genes might be predictive factors for acute transformation in chronic-type ATL cases (Fig. 5B).

**Disruption of the immunosurveillance system in acute transformation of chronic-type ATL.**

The combined analyses of aCGH and sequencing revealed that 19% of ATL cases (7% of chronic-type and 29% of acute-type ATL) exhibited the CD58 alteration. One acute-type ATL case showed somatic mutation, and the other cases showed genomic loss of the CD58 locus. The alteration of B2M was a rare event in ATL compared with DLBCL (20). CD58 is a ligand of the CD2 receptor that is expressed on CTLs and NK cells and contributes to adhesion and activation of these cells. Previous reports showed that CTLs and NK cells could not recognize and injure target cells when treated with monoclonal CD58 antibody (35, 36). It is important to note that immune escape mechanism by CD58 inactivation was proven in DLBCL by Challa-Malladi and colleagues (20). The genomic loss and nonsense mutation of CD58 were for the first time demonstrated in ATL in this study and were suggested to be a predictive marker for acute transformation in chronic-type ATL. Therefore, the immune escape mechanism by the CD58 inactivation is likely to be involved in the pathophysiology of ATL as shown in DLBCL, although detailed analysis is needed in the future.

Administration of immunosuppressive drugs to HTLV-1 carriers is currently considered a risk factor for early development of ATL (37, 38). It has been also suggested that immune escape from CTLs is induced by inactivation of the Tax protein derived from HTLV-1 in ATL (39–41). In addition, a report also suggested that immune escape from NK cells played an important role in ATL development (42). These findings suggest the presence of an immune escape mechanism in the pathophysiology of ATL. The present result regarding the significance of CD58 alteration as a predictive factor for acute transformation in chronic-type ATL should be validated in more number of cases in the future study. Further studies are also needed regarding the protein expressions of CD58, B2M, and human leukocyte antigen class I.

In conclusion, our comparison of the molecular characteristics of chronic-type and acute-type ATL revealed that deregulation of the cell cycle and escape from the immune system are likely to be involved in acute transformation of chronic-type ATL. Development of ATL is thought to involve accumulation of several genomic alterations (43). The alterations of both pathways discovered in this study might be the late events following viral infection in the pathophysiology of ATL. These alterations could serve as biomarkers for patients with...
chronic-type ATL. Furthermore, the presence of genomic alterations related to immune escape should be considered in the development of immunotherapeutic approaches for ATL.

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

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Conception and design: N. Yoshida, A. Utsunomiya, K. Tsukasaki, A. Umino, M. Seto
Development of methodology: K. Karube
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References

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