Rapid Communication

Homozygous Deletions of the p15 (MTS2) and p16 (CDKN2/MTS1) Genes in Adult T-Cell Leukemia

By Yoshihiro Hatta, Toshiyasu Hirama, Carl W. Miller, Yasuaki Yamada, Masao Tomonaga, and H. Phillip Koeffler

Adult T-cell leukemia (ATL) is associated with prior infection with human T-cell leukemia virus type I (HTLV-I). Twenty to 40 years often elapse from viral infection to overt ATL, suggesting that other genetic events must occur to produce frank leukemia. The p15 (MTS2) and p16 (CDKN2/MTS1) genes located on chromosome 9p have been implicated as candidate tumor-suppressor genes in several types of tumors. We examined for alterations of these genes in ATL using Southern blot and polymerase chain reaction--single-strand conformation polymorphism analyses. Both p15 and p16 genes were homozygously deleted in 4 of 23 acute/lymphomatous ATL (17%). An additional 3 (13%) and 4 (17%) acute/lymphomatous samples had hemizygous deletions in at least one exon of p15 and p16, respectively. One of 14 chronic ATL samples had a homozygously deleted p16 gene and another had a hemizygous deletion of p16. Neither homozygous nor hemizygous deletions of the p15 gene were found in chronic ATL. In total, 10 of 37 (27%) ATL samples had loss of the p15 and/or p16 genes. No point mutations of the p15 and p16 genes were found. The ATL patient with a homozygously deleted p16 in the chronic phase rapidly progressed to acute ATL and died within 6 months of the initial diagnosis. One instructive patient had no detectable deletion of the p15 and p16 genes during the chronic phase of ATL but had a homozygous deletions of both genes when she progressed to acute ATL. Our results suggest an association of p15/p16 deletions with development of acute ATL.

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Materials and Methods

Samples. Thirty-seven samples from 35 patients were examined in this study. Samples were collected predominantly from Kyushu, Japan, where ATL is epidemic. They consisted of 23 acute-phase, 14 chronic-phase, and 2 lymphomatous ATL samples. Three sequential samples in chronic and acute phase from the same patient (patient no. 19) were included in this study.

Mononuclear cells from patients with ATL were obtained by density gradient separation from the peripheral blood of chronic and acute ATL patients, lymph nodes from lymphomatous ATL samples, and pleural effusions from patients with acute and lymphomatous ATL.

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ATL. Most samples had greater than 75% CD4⁺ cells. Bone marrow DNA from a normal individual was used as a standard for normal p15 and p16 genes. DNA from the K562 cell line, which has a homozygous deletion of the p16 gene, was also used as a negative control. DNA was extracted by a standard methodology using phenol and chloroform extractions.

**Southern blot hybridization.** Southern blotting was performed for 37 tumor samples (23 in acute/lymphomatous phase and 14 in chronic phase of ATL). Five micrograms of each DNA was digested with EcoRI (GIBCO-BRL, Gaithersburg, MD), separated on 0.7% agarose gels, and transferred to nylon membranes (Hybond-N; Amersham, Amersham, UK). The p15 exon 1 was detected with a 466-bp DNA fragment that was prepared by PCR using primers 4BS0 (GGACTCCGCGACGGTCCGCA) and 4BA1 (CCTCCCGAAGACGG). The probe specific for exon 2 of p16 was a 410-bp DNA fragment that was prepared by PCR using primers 4BS1 (CTGCGCGTCTGGGGGCTGC) and 4BA1, which yielded a fragment of 150 bp. Primers 4BS2 (CCCGGCCGGCATGACGGTTGACTCC) and p16-X1AI (GCGCTACCTGATGAGTCC), which yielded a fragment of 150 bp. All the primers were synthesized by the Cedars-Sinai Research Institute Molecular Biology Core.

**PCR-SSCP.** All 37 samples were screened for exons 1 and 2 of p15 and p16 with PCR-SSCP. The exon 1 of p15 was amplified with primers 4BS1 (CTGCGCGTCTGGGGGCTGC) and 4BA1, which yielded a fragment of 150 bp.

**Table 1. Deletions of the p15 and p16 Gene in ATL**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Disease/Stage</th>
<th>p15 Exon 1</th>
<th>p15 Exon 2</th>
<th>p16 Exon 1</th>
<th>p16 Exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Acute</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Hemi</td>
</tr>
<tr>
<td>3</td>
<td>Acute</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
</tr>
<tr>
<td>6</td>
<td>Acute</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Hemi</td>
</tr>
<tr>
<td>8</td>
<td>Acute</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Hemi</td>
<td>Intact</td>
</tr>
<tr>
<td>19A</td>
<td>Acute</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
</tr>
<tr>
<td>32</td>
<td>Acute</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
</tr>
<tr>
<td>38</td>
<td>Acute</td>
<td>Intact</td>
<td>Intact</td>
<td>Hemi</td>
<td>Hemi</td>
</tr>
<tr>
<td>14</td>
<td>Lymphoma</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
<td>Homo</td>
</tr>
<tr>
<td>15</td>
<td>Chronic</td>
<td>Intact</td>
<td>Intact</td>
<td>Hemi</td>
<td>Hemi</td>
</tr>
<tr>
<td>33</td>
<td>Chronic</td>
<td>Intact</td>
<td>Intact</td>
<td>Hemi</td>
<td>Hemi</td>
</tr>
</tbody>
</table>

**Abbreviations:** Homo, homozygous deletion; Hemi, hemizygous deletion.

Each reaction contained 100 ng of sample DNA, 0.7 pmol of each primer, 5 pmol of each of the four deoxyribonucleotide triphosphates (Pharmacia, Stockholm, Sweden), 0.5 U of Taq DNA polymerase (GIBCO-BRL), and 3 μCi of [α-32P]dCTP (ICN) in 20 μL of the specified buffer. The conditions for exons 1 and 2 of p15 were 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds. The PCR condition for p16 exon 1 was 35 cycles of 95°C for 45 seconds, 52.5°C for 30 seconds, and 72°C for 60 seconds. The condition for p16 exon 2 was 35 cycles of 95°C for 40 seconds, 55°C for 30 seconds, and 72°C for 35 seconds. The MgCl₂ concentrations were 1.0 mmol/L and 0.75 mmol/L for exons 1 and 2 of p15 and 1.5 mmol/L and 1.25 mmol/L for those of p16, respectively. The PCR products were denatured and electrophoresed through a nondenaturing 5% polyacrylamide Mutation Detection Enhancement (MDE; J.T. Baker Inc, Phillipsburg, NJ) gel at 400 V for 24 hours, dried, and exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) at −80°C.

**RESULTS**

Tables 1 and 2 summarize the results. Using Southern blot analysis, we examined 23 acute/lymphomatous-phase ATL samples and 14 chronic-phase ATL samples. Four of 23 (17%) acute/lymphomatous-phase samples and 1 of 14 (7%) chronic-phase samples were homozygously deleted in both exons 1 and 2 of the p16 gene (Fig 1). The chronic ATL patient with homozygous p16 deletion progressed to acute ATL and died within 6 months (sample no. 33). All acute/lymphomatous-phase samples with homozygously deleted p16 also had homozygous deletions of p15. However, the chronic-phase ATL sample with homozygous loss of p16 had an intact p15 gene. One instructive case had no detectable deletions of the p15 and p16 genes in the ATL cells during the chronic phase of the disease (samples no. 19C1 and 19C2), but, during the acute phase of the disease, the ATL cells from the same patient had homozygous deletions of these genes (sample no. 19A; Fig 2). This sample had homozygous deletions of both p15 and p16 genes as determined by densitometry, because the intensities of signals of these genes were less than one eighth of those of the control.

Hemizygous deletion of the p16 gene in at least one exon was found in 4 of 23 (17%) acute/lymphomatous-phase sam-
transferred to nylon membranes, with various patients were digested with EcoRI, electrophoresed on 0.7% agarose gels (5 μg/lane), transferred to nylon membranes, and hybridized with either MPO cDNA (control), p16 exon 1, or exon 2 after 32P-labeling. Lanes 3 and 14 show homozygous deletions of the p16 exons 1 and 2. Lanes 2, 6, 8, and 15 (lower blot) and lanes 2, 6, and 15 (upper blot) show hemizygous deletions of exons 1 and 2 of p16, respectively. The asterisk shows the cross-hybridizing bands with p16 exon 1. Both human normal bone marrow (HNBM) and K562 cell line (K562), which has a homozygously deleted p16 gene, served as controls. Only representative samples are shown.

Fig 1. Southern blot analysis of the p16 gene in ATL. Genomic DNAs from ATL samples from various patients were digested with EcoRI, electrophoresed on 0.7% agarose gels (5 μg/lane), transferred to nylon membranes, and hybridized with either MPO cDNA (control), p16 exon 1, or exon 2 after 32P-labeling. Lanes 3 and 14 show homozygous deletions of the p16 exons 1 and 2. Lanes 2, 6, 8, and 15 (lower blot) and lanes 2, 6, and 15 (upper blot) show hemizygous deletions of exons 1 and 2 of p16, respectively. The asterisk shows the cross-hybridizing bands with p16 exon 1. Both human normal bone marrow (HNBM) and K562 cell line (K562), which has a homozygously deleted p16 gene, served as controls. Only representative samples are shown.

### DISCUSSION

The close association between ATL and HTLV-I virus has been established. Nevertheless, the mechanisms of development of ATL after HTLV-I infection have not been clarified. Although HTLV-I does not have an oncogene, it contains the tax region in addition to the genes common to all retroviruses. The Tax protein encoded from this region is required for viral replication and activates the expression of several cytokines and cytokine receptors including interleukin-2 (IL-2) and the α subunit of IL-2 receptor (IL-2Rα). The Tax-mediated induction of NF-κB protein that binds to the IL-2Rα promoter may contribute to polyclonal or oligoclonal expansion of HTLV-I–infected T cells. In addition, the overexpression of tax can transform RAT-1 cells. These in vitro results strongly implicate the involvement of Tax in the progression toward development of ATL. However, this does not explain the 20 to 40 years of latency from infection to development of ATL. In addition, ATL cells usually do not express tax in vivo. Therefore, additional genetic changes are probably required for progression to ATL.

In this study, we determined for the first time that p15 and p16 genes are altered in ATL. Four of 23 (17%) acute/lymphomatous ATL samples had homozygous deletions of both the p15 and p16 genes. Among 14 chronic-phase samples, homozygous deletions of p16 were found in 1 case (14%), but deletions of p15 were not found. This chronic ATL patient rapidly progressed to acute ATL and died, suggesting that the sample obtained from the patient in the "chronic" phase was in a "pre-acute" phase. Another instructive case had no deletions of the p15 and p16 genes in the ATL cells in the chronic phase but had homozygous deletions of both genes in the acute phase, indicating that the deletions were acquired during the transition from chronic to acute ATL. The association of these genetic events with acute but not chronic ATL suggests to us that homozygous deletions of these genes may be frequently acquired abnormalities during the development of the acute phase of ATL.

We have found that all samples with homozygous p15 deletions also had homozygous p16 deletions; however, one sample had a homozygous deletion and two samples had a hemizygous deletion of p16 with a normal appearing p15 gene. These data suggest that p16 rather than p15 may be the target of these deletion. Potentially, homozygous deletions of the p15/p16 genes coupled with point mutations of the undeleted allele could also inactivate these genes. This is the frequent mechanism of inactivation of the p53 gene. However, we were unable to detect any point mutations of the p15 and p16 genes in our samples. Because we did not analyze the promoter region and exon 3 of p16, which is only 14 bp, we do not know if this part of the gene is altered.
showing intact p15 and p16 genes. Lane 19A, which is a sample from the same patient during the acute-phase ATL shows deletions of the techniques are similar to those described in legend for Fig 1. Lanes labeled control, as determined by densitometry. Both lanes 20 and 21 are ATL samples and have intact p15 and p16 genes.

Fig 2. Southern blot analysis of p15 and p16 gene in ATL. Techniques are similar to those described in legend for Fig 1. Lanes labeled 19C1 and 19C2 are chronic-phase samples from the same patient showing intact p15 and p16 genes. Lane 19A, which is a sample from the same patient during the acute-phase ATL, shows deletions of the p15 and p16 genes. Sample 19A was determined to have homozygous deletions for both the p15 and p16 genes, because the signal intensities of these genes were less than one eighth of those of control, as determined by densitometry. Both lanes 20 and 21 are ATL samples and have intact p15 and p16 genes.

in ATL. In addition, mutations and genetic rearrangements in the second intron of p16 could potentially lead to aberrant splicing and loss of exon 3,12,13,15,38.

Because our ATL samples were contaminated with normal cells, we may have underestimated the number of cases with homozygous and hemizygous deletions. In addition, in another study, the p16 protein was not detected in some cancers with an intact p16 gene,39 suggesting that mechanisms other than genetic alteration of this gene might inactivate p16. Thus, the inactivation of p15/p16 might be more frequent than shown in this study.

Others as well as ourselves have found that p16 deletions occurred in about 75% of T-ALL and 20% of B-ALL cases (Takeuchi, manuscript in press).15 In contrast, alterations of p15 and p16 genes in both T- and B-cell lymphomas and acute and chronic myeloid leukemias are infrequent (<6%; Gombert and Nakamaki, manuscript submitted).40 Among the common hematopoietic malignancies, the reason why ALL and ATL have a high incidence of alterations of the p15 and p16 genes is unclear.

Another CDKI family consists of p21 (also known as WAF1, MDA-6, CIP1, SDI1, and CAP20) and p27 (also known as KIP1). The p21 gene had no abnormalities when over 350 primary tumors from 14 types of malignancies were studied.41 Furthermore, of 305 primary tumors from 11 types of malignancies, we have found only 1 case of ATL with a p27 mutation (Kawamata and Morosetti, manuscript in press). Taken together, these findings indirectly suggest that p15 and p16 proteins have a different function than the p21 and p27 family of CDKIs.

We have determined that certain point mutations in the ankyrin repeats of p16 result in a p16 that can no longer inactivate the CDK4 kinase activity in vitro (Yang, manuscript in preparation); furthermore, several cancers have these same point mutations such as familial melanoma12 and pancreatic cancers.13 In contrast, some tumors such as the hematopoietic malignancies, including T- and B-ALL and ATL, rarely have p15 and p16 point mutations but, instead, have deletions of these genes.16,38,41,42 Why the method of inactivation of the p15/p16 genes varies in cancers of different tissue types is unexplained at this time.

We previously reported that p53 mutations occurred in only 2% of childhood lymphoid malignancies of both B- and T-cell type,43 whereas the frequency of p53 mutation in acute ATL was about 40%.25,44,45 Studies in other tissue types have found that the p16 gene is often mutated or deleted in cell lines containing wild-type p53 and it is often intact in cell lines containing mutated p53 genes.44 Furthermore, studies in lung cancer cell lines also showed that these cells usually have a normal p16 when the Rb gene is inactivated either by mutation or by a DNA-transforming viral protein.11,39 Perhaps a cellular growth advantage leading to ATL requires inactivation of either the p15/p16, p53, or Rb gene. Further studies are required to determine if this hypothesis is correct.

Taking these data together, we hypothesize that the development of ATL may be as follows: tat expression in T cells infected by HTLV-I induces an IL-2/IL-2R autocrine stimulatory loop leading to slow polyclonal proliferation of these cells. At this stage, patients are in a pre-ATL or HTLV-I carrier state that can continue over a long period of time, generally more than 40 years. In some cases, the cells may lose factor-dependence, proliferate autonomously, become a clonal disorder, and evolve to chronic ATL. These cells probably acquire one or several genetic changes such as loss of p16 with or without loss of p15 and/or mutations of p53, resulting in acute ATL.

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