Two Types of Defective Human T-Lymphotropic Virus Type I Provirus in Adult T-Cell Leukemia

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Adult T-cell leukemia (ATL), an aggressive neoplasm of mature helper T cells, is etiologically linked with human T lymphotropic virus type I (HTLV-I). After infection, HTLV-I randomly integrates its provirus into chromosomal DNA. Since ATL is the clonal proliferation of HTLV-I–infected T lymphocytes, molecular methods facilitate the detection of clonal integration of HTLV-I provirus in ATL cells. Using Southern blot analyses and long polymerase chain reaction (PCR) we examined HTLV-I provirus in 72 cases of ATL, of various clinical subtypes. Southern blot analyses revealed that ATL cells in 18 cases had only one long terminal repeat (LTR). Long PCR with LTR primers showed bands shorter than for the complete virus (7.7 kb) or no bands in ATL cells with defective virus. Thus, defective virus was evident in 40 of 72 cases (56%). Two types of defective virus were identified: the first type (type 1) defective virus retained both LTRs and lacked internal sequences, which were mainly the 5′ region of provirus, such as gag and pol. Type 1 defective virus was found in 43% of all defective viruses. The second form (type 2) of defective virus had only one LTR, and 5′-LTR was preferentially deleted. This type of defective virus was more frequently detected in cases of acute and lymphoma-type ATL (21/54 cases) than in the chronic type (11/18 cases). The high frequency of this defective virus in the aggressive form of ATL suggests that it may be caused by the genetic instability of HTLV-I provirus, and cells with this defective virus are selected because they escape from immune surveillance systems.

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MATERIALS AND METHODS

Patients. Genomic DNAs were extracted from peripheral blood mononuclear cells (PBMC) or lymph node cells of patients with acute (33 cases), lymphoma-type (21 cases), and chronic ATL (18 cases). Diagnosis was based on criteria by Shimoyama and
the Lymphoma Study Group and monoclonal integration of HTLV-I provirus was identified by Southern blot analyses using an HTLV-I probe.

**Southern blot analyses.** Genomic DNA, digested with restriction enzymes, Prs I, HindIII, EcoRI, and Stu I, was electrophoresed in an agarose gel and transferred to nylon membrane (Amersham International, Buckinghamshire, UK). The filters were hybridized with radiolabeled probes at 65°C for 12 hours, washed in buffers, and then exposed to X-ray film at ~80°C.

**Long PCR.** Long PCR was performed using XL PCR kits (Perkin Elmer, Branchburg, NJ). For amplifications, we used a Robocycler (Stratagene, La Jolla, CA). Genomic DNA (500 ng) was amplified according to manufacturer’s protocol. Briefly, primers, MgCl2 (final 1.1 mmol/L), dNTP (final 0.2 mmol/L), and XL buffer were mixed (total 20 μL), then AmpliWax (Perkin Elmer) was added. The wax was melted by incubating the reaction tube at 80°C for 5 minutes. After this step, tubes were cooled to room temperature, then, substrate DNA (20 μL), XL buffer (9 μL), and rTh DNA polymerase XL (1 μL) were added. Cycles for long PCR were as follows: denaturation at 94°C for 30 seconds, annealing and extension at 64°C for 10 minutes, and 25 or 30 cycles were run. Sequences of primers used in these experiments were as follows: primer 1: 5'-GGCTCTAAGCCCCGAGATTTGGGGGCTACATG-3'; primer 2: 5'-GGCTCTAAGCCCCGAGATTTGGGGGCTACATG-3'; primer 3: 5'-GGGGTCCCAGGTGATCTGATGCTC-3'; primer 4: 5'-GGGGTCCCAGGTGATCTGATGCTC-3'. We selected these sequences from conserved regions among different HTLV-I sequences. To amplify the entire provirus, primers 1 and 3 were used. To determine which LTR was deleted, primers 1 and 3 or primers 2 and 4 were used for PCR.

PCR products were electrophoresed in a 0.7% agarose gel. For further analyses, PCR products were subcloned into pCR II using TA cloning kits (Invitrogen Corporation, San Diego, CA) and plasmid DNAs were used for restriction mapping and sequencing experiments. Sequences were determined by the dideoxy method, using Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, OH), based on their protocol.

For cloning of type 2 defective virus, we used inverse-long PCR. At first, genomic DNA was digested with EcoRI, because EcoRI site inside the HTLV-I provirus was lacking. Then, digested DNA was ligated with T4 DNA ligase, and linearized by digestion with Mlu I, which digested HTLV-I at the pX region. This DNA served as substrate for long PCR, in which both primers (primer 1 and 3) were made in the opposite direction at pX and LTR. PCR products were subcloned in plasmid. The DNA fragment containing junction between the 5' site of HTLV-I and genomic DNA was isolated by making a comparison of the restriction map, and the junctional sequence was determined by sequencing.

**Statistical analysis.** Chi-square test was used to determine the statistical significance.

**RESULTS**

**Two different types of defective HTLV-I virus.** We first used conventional Southern blot method to analyze 72 cases of ATL. Genomic DNAs were digested with restriction enzymes (Prs I, EcoRI), and hybridized with a whole HTLV-I probe. EcoRI could not digest HTLV-I provirus, and only clonal proliferation of HTLV-I–infected cells gave bands, whereas polyclonal proliferation of infected cells showed a smear pattern. The number of bands by EcoRI digestion indicated the number of integrated provirus or number of clones. Because Pst I digested HTLV-I provirus, three (2.4, 1.6, 1.3 kb) or four bands were generated within HTLV-I provirus (Fig 1A).7 If the integrated virus was complete, two additional bands derived from integration sites should be visible (Fig 1B, lane 1); one corresponded to the 5'-genomic flanking region, 5'-LTR and a part of gag, and the other to pX, 3'-LTR and 3'-genomic flanking region. If some internal bands were missing, the internal region of HTLV-I provirus might be deleted (Fig 1B, lane 3). However, this method did not reveal if it was the defective virus or that Prs I sites within the provirus were mutated. More than three bands derived from integration sites indicated that multiple proviruses were integrated in chromosomal DNA or that several clones with different integration sites of HTLV-I were present in the same patient (Fig 1B, lane 2).

To confirm the presence of LTR, genomic DNA was digested with Prs I or Stu I, which digested inside the HTLV-I provirus, and then hybridized to the LTR probe (Fig 1C). In 18 cases, only one LTR could be detected, showing that one of LTRs was deleted (Fig 1C, lane 4). These results revealed two types of defective virus, one with two LTRs and the other with only one.

**Analyses of HTLV-I provirus with long PCR.** Because Southern blot methods could not detect small deletions of HTLV-I provirus and could not rule out the possibility of mutated Prs I sites, all the defective viruses in ATL cases would not be detected. We thus used long PCR to analyze HTLV-I provirus of ATL cells. This approach enabled amplification of the complete HTLV-I provirus. We used LTR primers (Fig 2, primers 1 and 2) to amplify HTLV-I provirus (7.7 kb) (Fig 3). Thirty-two of 72 cases contained the complete virus (Fig 3, lanes 2 through 4), and for 18 cases, smaller bands were detected (Fig 3, lanes 5 through 7), that is, defective virus with both LTR sequences. In 18 cases, which had only one LTR by Southern blot analysis, no bands were amplified by long PCR (Fig 3, lane 8). Therefore, both methods confirmed that only one LTR was present in these cases.

The pX region is conserved in most defective virus. Therefore, to determine which LTR was deleted, we performed long PCR with the 5'-LTR primer (primer 1) and pX primer (primer 3), or pX primer (primer 4) and 3'-LTR primer (primer 2) (Fig 2A). Amplification was performed at limited cycles (25 cycles) so as not to detect contaminating nonleukemic HTLV-I–infected cells, which were usually less than 1% of all cells. In case of a complete virus, both bands (5- and 3-LTR–related) should be detected. Detection of band a meant that 5'-LTR was retained, whereas the presence of band b indicated that 3'-LTR was present (Fig 2B). In most type 1 defective viruses, shorter band a and intact band b were amplified, thereby confirming that the pX region was conserved, but the 5'-region was deleted in the defective virus. Based on this approach, 16 of 18 cases retained 3'-LTR, but lost 5'-LTR (Fig 2). In two cases, neither band (a or b) was detected. It may be that the pX region, where primers existed, might be deleted in these two cases.

Thus, two types of HTLV-I provirus are present in ATL cells: the first type (type 1 defective virus) retained both
TWO TYPES OF DEFECTIVE HTLV-I PROVIRUS

A

HTLV-I provirus

Pst I sites

genomic DNA

LTR gag pol env px LTR

1.6kb 1.3kb 2.4kb 0.6kb

whole probe

LTR probe

Fig 1. (A) Structure of HTLV-I provirus. Hatched lines represent DNA fragments used for DNA probes. Vertical lines are Pst I sites inside provirus. Boxes indicate LTRs. Predicted DNA sizes when digested with Pst I are also shown. (B and C) Southern blot analyses of HTLV-I provirus from ATL patients. Genomic DNAs were digested with Pst I and hybridized with whole HTLV-I (B) and LTR (C) probes. Lane 1, ATL cell line, ED (positive control: ATL cell line with one copy of complete virus); lanes 2-4, ATL cases.

LTR and lacked internal sequences within the provirus, while the second type (type 2 defective virus), 5'-LTR and internal sequence were deleted.

For eight cases, there were multiple copies of HTLV-I proviruses, which means that one ATL cell clone had multiple copies of provirus, or that different clones were present in the same patient. In two, there were complete viruses, three with complete and type 1 defective virus, one with complete and type 2 defective virus, one with two type 1 defective viruses, and one with type 1 and 2 defective virus. However, we found different ATL clones at different time points or different sites in two ATL patients (Takemoto et al, manuscript in preparation), confirming that different clones in some patients showed multiple bands by Southern blot analysis.

Type 1 defective virus. To determine the exact structure of type 1 defective virus, products of long PCR were subcloned into plasmid and analyzed. Restriction maps of these products in two cases are demonstrated in Fig 4A, showing that the 5' portion of the provirus (gag, pol, and env) was missing in both cases of type 1 defective virus. Previous reports of defective virus showed similar defects, although all were noted in HTLV-I transformed cell lines. Sequences around deletion points revealed that splicing signals were not found at deletion sites. In case 1, a nonviral sequence (35 bp) was found between the primer binding site and the env region, a homology search of which showed that it was derived from human proline transfer RNA (tRNA). Human proline tRNA had the homologous sequence (16 bp) with the 5'-region region of HTLV-I, the primer binding
Fig 2. Determination of deleted LTR in type 2 defective virus with PCR. (A) Primers (1 and 3, 4 and 2) and generated bands (a and b) are shown. If 5'-LTR and the pX region are conserved, band a should be amplified. When pX region and 3'-LTR were retained, band b should be amplified. (B) Ethidium bromide stained gel pattern of PCR products. In the complete virus, band a (16.8 kb) and band b (10.9 kb) could be amplified. In type 1 defective virus, smaller a and the same b bands were found. In type 2 defective virus, no band was amplified by primers 1 and 3, but band b was detected.

Type 2 defective virus. To determine the structure of type 2 defective virus, we used inverse and long PCR, as described in Materials and Methods. Genomic DNA was digested with EcoRI, which did not cut the provirus, and was ligated by T4 DNA ligase. Next the formed circular DNA was digested with Mlu I for relinearization. Mlu I cut the HTLV-I provirus at the pX region, and this served as the substrate of long PCR with primers (primer 1 and 3) present in LTR and in the pX region. The PCR product was

site. Thus, reverse transcriptase probably transcribed part of the tRNA in this case. In case 2, more than half of the env region was deleted, indicating that the tax gene lost the second exon in the env region. This provirus probably could not produce Tax.
Two Types of Defective HTLV-I Provirus

HTLV-I provirus with the pX region. Although the defective virus was equally present in acute or lymphoma type ATL cells containing complete HTLV-I provirus, few defective viruses were detected (Fig 5, lanes 1 and 2), whereas many defective viruses were present in chronic and smoldering ATL with complete virus (lanes 3 and 5). Therefore, a malignant clone was predominant in acute type and lymphoma-type ATL, but nonleukemic clones with a defective virus were present in smoldering and chronic ATL. While an exact quantification was difficult, and our method detected only complete and type 1 defective virus, the majority of HTLV-I provirus was thought to be a complete virus in the intermediate state. Cells with a defective virus already existed in the intermediate state.

Fig 3. Analyses by long PCR. (A) Results for each ATL sample (lanes 2 through 8) shown on the ethidium bromide gel. HL-60 (lane 1) is used as a negative control. (B) Hybridization with an internal probe (pX region) demonstrated that the amplified bands contained HTLV-I provirus with the pX region.

Correlation between defective virus and clinical subtypes. Four clinical subtypes of ATL were proposed; acute, chronic, smoldering, and lymphoma-type.23 The relation between types of defective virus and clinical subtypes was then analyzed. Table 1 shows a summary of the clinical diagnosis and types of provirus. Although the defective virus was equally observed among each subtype of ATL, as reported by other investigators,21,22 striking differences were found in the type 2 defective virus. This type of defective virus was frequently present in acute or lymphoma type ATL (21/54 cases). On the other hand, one case of chronic ATL (1/18 cases) also had this defective virus, and there was an increased number of abnormal lymphocytes (149 x 10³/L), and hepatomegaly in this patient. She died of exacerbatation of the ATL 18 months after the diagnosis, despite intensive chemotherapy. There is a statistical significance between type 2 defective virus of aggressive form (acute and lymphoma-type) ATL and chronic ATL (P < 0.01: Chi-square test). One patient with acute ATL had a long history of leukocytosis (over 5 years), indicating that he had a chronic ATL before the crisis; here too there was a complete HTLV-I provirus.

Presence of defective virus in intermediate state and smoldering ATL. The high frequency of defective virus in ATL cells suggested that defective virus already existed in the preleukemic or early stage of ATL. In the intermediate state, there is a polyclonal proliferation of HTLV-I-infected cells, and this is also considered to be the preleukemic state.24 Long PCR analyses with LTR primers were used to detect defective virus in various clinical conditions of HTLV-I infection (Fig 5). Defective viruses were noted in the intermediate state (Fig 5, lanes 4, 6, and 7). In acute and lymphoma-type ATL cells containing complete HTLV-I provirus, few defective viruses were detected (Fig 5, lanes 1 and 2), whereas many defective viruses were present in chronic and smoldering ATL with complete virus (lanes 3 and 5). Therefore, a malignant clone was predominant in acute type and lymphoma-type ATL, but nonleukemic clones with a defective virus were present in smoldering and chronic ATL. While an exact quantification was difficult, and our method detected only complete and type 1 defective virus, the majority of HTLV-I provirus was thought to be a complete virus in the intermediate state. Cells with a defective virus already existed in the intermediate state.

Discussion

Over half of the ATL cases (56%) we analyzed carried defective provirus, type 1 lacked internal sequences, such as gag and pol, but retained two LTRs; 5'-LTR and internal sequences were deleted in the type 2 defective virus. Among clinical subtypes of ATL, type 2 defective virus was more frequently observed in aggressive forms (acute and lymphoma type) of ATL than in the chronic type.

Ohshima et al reported that defective virus was detected in 28 of 95 cases examined (29%) by Southern blot analyses.25 Deletion occurred in gag, pol, or env regions, whereas the pX region was conserved. Korber et al used semiquantitative PCR to identify defective virus and the frequency was 32%.26 The frequency of defective virus was 56% in our study. This discrepancy was thought to be caused, in part, by different methods to detect HTLV-I provirus. Long PCR can detect even small defects, such as several hundred base pairs not identifiable by Southern blot analyses. One explanation of such a high frequency of defective virus in ATL cells is that such cells can escape immunosurveillance systems. Cytotoxic T lymphocytes were identified in patients with HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP),27,28 most of which were directed against Tax. Perhaps, cells infected with a defective virus can escape CTL, hence a greater likelihood for leukemic changes.

The structure of type 2 defective virus was described for
A Type 1 defective virus

Case 1

- LTR
- gag
- pol
- env
- pX

~GGGATT~ CGAGCG ~
---TATAAG~TACTT---
TGAACCCCGGACCTCTCGCACCCAAGCGAG~~TCA
---non-viral sequences---

Case 2

- LTR
- gag
- pol
- env
- pX

~ATTAAC~ TACTCC~ ---TGTCAT~ AACTCCC---
---non-viral sequences---

B Type 2 defective virus

~CCCCTGGGGCT~
---TACCGG~CCATGT---

Fig 4. Structure of defective virus. Broken lines mean a defective region within the HTLV-I provirus. (A) Restriction map and sequence of break-point of type 1 defective provirus. In case 1, a nonviral inserted sequence (35 bp) was evident. (B) Restriction map and sequence of break-point of type 2 defective provirus. Part of 5'-LTR (30 bp of U3 region) was evident.

two cases.\textsuperscript{30,31} In both cases, 5'-LTR and the 5'-region of provirus were deleted, thereby confirming the preferential deletion of 5'-LTR. Although the short fragment of 5'-LTR (30 bp of U3 region) remained in type 2 defective virus in the former report\textsuperscript{30} and in the present study, Kubota et al\textsuperscript{31} reported a different kind of type 2 defective virus. The 5'-LTR was completely deleted in their case, and the same genomic sequences (long direct repeat) were identified at both ends of the provirus (99 bp). They speculated that this defective virus was integrated through homologous recombination between cellular and viral read-through sequences. Since we found only the 6 bp direct repeats adjacent to both ends of the defective virus, homologous recombination cannot explain the deletion in this case.

We found type 2 defective virus in 18 of 72 cases we analyzed (25%). Although the frequency of defective virus (type 1 plus type 2) did not differ among different clinical subtypes, type 2 defective provirus was more frequently observed in acute or lymphoma type ATL than in the chronic form. Genetic instability may generate type 2 defective provirus during the clinical course. Recently, it was reported that the acute form of ATL had higher frequencies of mutations or deletions of p53, p15, and p16\textsuperscript{32,33} indicating that acute ATL cells do have the genetic instability compared

Table 1. Types of HTLV-I Provirus in Each Subtype of ATL

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>Acute</th>
<th>Lymphoma</th>
<th>Chronic</th>
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<tbody>
<tr>
<td>No.</td>
<td>33</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Types of provirus</td>
<td></td>
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<td></td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Complete</td>
<td>8</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Defective</td>
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<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Type 2</td>
<td>12</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Type 2/total</td>
<td>21/54 (39%)</td>
<td>1/18 (6.6%)</td>
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</table>
with chronic ATL cells. However, it was noteworthy that 5'-LTRs were deleted in almost all type 2 defective virus. This finding meant that this deletion was not caused by random events.

5'-LTR is critical for transcription of viral genes, and the loss indicates that viral proteins, including Tax or Rex, cannot be transcribed from viral promoter in these cells. Even cells with type 1 defective provirus were found not to produce Tax in vitro. It is very important to determine whether this deletion occurs before or after integration into chromosomal DNA. Hiramatsu and Yoshikura analyzed HTLV-I provirus in vitro transmitted to a human myeloid cell line, HL-60. They found that 41% of the HTLV-I provirus was defective. Interestingly, they also identified a defective virus similar to type 2 seen in our study. They suggested that this type deletion was not generated after the establishment of proviral integration, because subclones with this type of provirus showed no alternations during long culturing.

If deletion of 5'-LTR in the type 2 defective virus was generated before integration, such a defective provirus could not transcribe viral genes from the LTR promoter after integration into chromosomal DNA. It is possible that the cellular promoter in the 5'-region of integration site transcribes the viral gene (promoter trap). Although defective viruses (shown in Fig 4) could not encode Tax, the cellular promoter trapped by defective virus could transcribe viral genes that encode proteins from the orfI and orfII or p21. These viral proteins rather than Tax might be important for leukemogenesis. If viral genes could not trap the cellular promoter and viral genes, such as tax, could not be transcribed from type 2 defective virus, viral proteins like Tax are probably not essential for leukemogenesis in the case. Since all ATL cells contain clonally integrated HTLV-I provirus, its presence likely plays an important role in leukemogenesis. Since even type 2 defective virus has 3'-LTR, insertion of LTR into chromosomal DNA may activate the transcription of some genes, which are important for T-cell activation. Recently, defective virus LTR was found to be inserted downstream of an unknown cellular gene promoter. 3'-LTR could transcribe the truncated form of this gene. Although the integration sites of HTLV-I provirus were reported to be different in two cases of ATL, it may be that integration of LTR in different sites in the chromosome activated multiple genes, as was noted in murine type-C retrovirus.

The high frequency of defective HTLV-I in ATL cells was evident in our studies using long PCR and Southern blot analyses. The presence of type 2 defective virus in ATL cells suggests that genetic instability could cause this defect, or viral proteins such as Tax are not mandatory for leukemogenesis or maintenance of leukemia. The presence of HTLV-I provirus is direct evidence of its association with HTLV-I. Analyses of defective virus will shed light on leukemogenesis of ATL.

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Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia

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