A Novel Diagnostic Method of Adult T-Cell Leukemia: Monoclonal Integration of Human T-Cell Lymphotropic Virus Type I Provirus DNA Detected by Inverse Polymerase Chain Reaction

By Shigeki Takemoto, Masao Matsuoka, Kazunari Yamaguchi, and Kiyoshi Takatsuki

Adult T-cell leukemia (ATL) is neoplasms of the mature helper T lymphocytes and human T-cell lymphotropic virus type I (HTLV-I) has been shown to be causative virus of ATL. Because HTLV-I integrates its provirus randomly into host chromosomal DNA, monoclonal integration of HTLV-I provirus indicates the clonal proliferation of HTLV-I-infected cells. Therefore, demonstration of clonality of HTLV-I proviral DNA is essential to diagnosis of ATL. Southern blot analysis was used for this purpose. We developed the novel method using inverse polymerase chain reaction (IPCR) to detect the clonality of HTLV-I proviral DNA. This method identified the clonality in all ATL cases. Diagnosis could be made within 3 days using this method. It enabled us to detect specifically the presence of minimal numbers of ATL cells with high sensitivity. It also identified the monoclonal or oligoclonal proliferations of HTLV-I-infected cells in HTLV-I carriers and the intermediate state, in which no clonality could be shown by conventional Southern blot analyses. This finding indicated that even HTLV-I carriers had monoclonal proliferation of HTLV-I-infected cells without any symptoms. This novel method is shown to be useful for the diagnosis of ATL and provides information on the natural course of HTLV-I infection.

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MATERIALS AND METHODS

Patients. Diagnosis of ATL was made from clinical and hematological data, the presence of anti-HTLV-I antibodies, and the monoclonal integration of HTLV-I proviral DNA identified by Southern blot method. Classification of ATL subtypes was according to criteria previously described. Genomic DNAs were extracted from peripheral blood mononuclear cells (5 patients with acute ATL, 5 with chronic ATL, 5 with smoldering ATL, 2 with intermediate state ATL, and 5 HTLV-I carriers) or lymph node cells (7 with lymphomatoid type ATL and 1 HTLV-I carrier with T-cell lymphoma).

IPCR. One microgram of genomic DNA was digested with

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Sau3AI or A1 (Takara, Kyoto, Japan) and then ligated with T4 DNA ligase (New England Biolabs, Beverly, MA) in diluted form (4 μg/mL) to cause self-ligation. Ligated DNA was then digested with Sac II (TOYOBO CO, LTD, Osaka, Japan) to eliminate the circular DNA originated from 5' proviral DNA, following random nicking with heating (93°C, 30 minutes). This DNA was used as a template in 50 μL of 50 mM Tris-HCl, pH 9.9/20 mM MgCl2/50 mM/L of each deoxyribonucleotide triphosphate, 1.25 U of T4 polymerase (Amersham International, UK). At first step of PCR, primer 1; 5'-AACCCCGGCAGTCAGTCGTGA-3' (residues 8946-8927) and 2; 5'-AAGTACCGGCAACTCTGCTG-3' (residues 8958-8977) were used at a final concentration of 200 mM/L. Five microliters of PCR products was then amplified in the same condition, with the nested primers 3; 5'-GAAAGGGAAGGGTGAGGAC-3' (residues 8924-8905) and 4; 5'-CCAGCGAAGCACTTGATAT-3' (residues 8986-9005). Each PCR reaction was performed in the Thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) using 50 cycles (the first PCR), or 35 cycles (the nested PCR) at 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds.

Southern blot analysis with radiolabeled oligonucleotide. PCR products were electrophoresed in a 2% agarose gel and transferred to a nylon membrane (Hybond-N Plus; Amersham). Oligonucleotide (5'-CTCCAGGAGAGAAAmAGTACAC-3'; residues 9012-9035) was used as a probe to confirm specific band and labeled at 37°C by the 5'-end labeling method with MEGALABEL labeling kit (Takara) and γ32P-ATP. The filters were hybridized with radiolabeled probes at 55°C for 12 hours, washed, and then exposed to x-ray film at −80°C.

DNA sequencing. PCR products were subcloned into pCR II with TA cloning kit (Invitrogen Corp, San Diego, CA) and these plasmid DNAs were used for sequencing experiments. Sequence was determined by dideoxy method with Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp, Cleveland, OH) according to its protocol.

**RESULTS**

IPCR to detect the monoclonality of HTLV-I-infected cells. IPCR was instrumental in determining the flanking sequences of proviral DNA of retroviruses.16,16 To detect the monoclonality of HTLV-I-infected cells with IPCR, we made the primers shown in Fig 1. Defective provirus of HTLV-I was frequently observed in genomic DNA from ATL patients (approximately 30%, Korber et al17; approximately 40%, our unpublished data, January 1994), in which sometimes 5' long terminal repeats (LTR) was deleted.18 Therefore, we decided to detect genomic DNA adjacent to 5' LTR (Fig 1). We chose Sau3AI for digestion of genomic DNA for the following reasons: (1) Sau3AI site existed only in 5' region of U5 in LTR at various HTLV-I sequences reported.19,22 (2) Four-base cutter such as Sau3AI produced the small DNA fragments (average size of DNA fragments is theoretically 256 bp) and such a small DNA fragment was suitable for amplification by PCR. For the same reasons, Alu 1 could be used to digest the genomic DNA instead of Sau3AI. Therefore, genomic DNA was digested with Sau3AI at first, and then Sau3AI-digested DNA was ligated in diluted condition to cause self-ligation. If complete HTLV-I proviral DNA was integrated, circular DNA from 5' LTR and gag sequence could be detected (302 bp) with this method as well as that from 3' LTR and flanking genomic sequences (Fig 1B). To eliminate this possibility, ligated DNA was digested with SacII, which digested the 5' portion of gag sequence and this site was conserved in HTLV-I sequences reported previously.19,22 This DNA was used as substrate for following PCR reactions. PCR was performed with primer 1 and 2 at first (Figs 1B and C). Since these primers had opposite direction on the genomic DNA, only ligated DNA should be amplified. After the first reaction, the second set of primers (primer 3 and 4) was used for nested PCR (Figs 1B and C) to enhance the sensitivity and specificity of the reaction.

IPCR method can detect the monoclonality of HTLV-I-infected cells. DNA samples from five ATL patients were analyzed with IPCR. Figure 2 shows the result of IPCR with typical ATL samples. Figure 2A is electrophoretic pattern of PCR products and Fig 2B is an autoradiogram after Southern blot analysis of PCR products.

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experiments, suggesting that this was not artifact, but derived from HTLV-I proviral DNA. Without SacII digestion, a 302-bp band derived from 5' LTR and gag sequence was detected (see Fig 1B, data not shown), but this band was not demonstrated with SacII digested samples, which indicated that SacII digestion could eliminate the circular DNA formed within HTLV-I provirus.

To confirm that these PCR products derived from 3' LTR and flanking genomic sequences, we determined the DNA sequences of these PCR products. Figure 3 shows the results of sequencing experiments from 4 samples of ATL, 1 of intermediate state, and 1 of carrier. The flanking sequences of 3' LTR and upstream of Sac3AI site were unique to each case. This showed that these sequences were derived from genomic DNAs of HTLV-I provirus integration sites and were unique to each case. It was evident that these bands were specific to integration sites. No common sequences were observed in the integration sites as reported previously.13

**Sensitivity of IPCR.** To examine the sensitivity of this assay, IPCR was performed with diluted ATL samples. Genomic DNA from acute ATL patients, which gave a distinct band by IPCR, and was confirmed to be derived from 3' LTR and genomic sequence, was mixed with DNA from HTLV-I--negative HL-60 at various proportions, and then these DNA (1 µg each) were analyzed using IPCR. This experiment showed that IPCR could detect about 1% of ATL cells in HTLV-I noninfected cells, indicating that this assay was more sensitive than Southern blot analysis. Indeed, IPCR detected the monoclonality in DNA samples, which was shown to be no monoclonality of HTLV-I--infected cells by Southern blot analysis. It showed that IPCR was not only a rapid method, but also superior to the Southern blot method in point of sensitivity.

**IPCR method in various clinical states of HTLV-I infection.** DNA samples from various clinical conditions of HTLV-I infection were analyzed by IPCR. These conditions were HTLV-I carrier, intermediate state, and smoldering ATL. Figure 4 shows the result of IPCR. In chronic ATL with two copies of HTLV-I proviruses (lane 2), two distinct bands could be also detected by IPCR, confirming two copies of proviral DNA. IPCR detected distinct bands in cases of smoldering ATL, in which the monoclonalities were detected by Southern blot analysis (lanes 3 and 4). In addition to a major band, a few faint bands could be detected in these samples, suggesting that the proliferation of HTLV-I--infected cells were oligoclonal. It was interesting to examine the intermediate state, in which the number of HTLV-I--infected cells increased, but monoclonality could not be detected by Southern blot method. It is likely that a few monoclonal HTLV-I--infected cells exist in this condition. Indeed, IPCR detected the monoclonal band in the sample from the intermediate state (lane 5). It showed that monoclonal proliferation of HTLV-I--infected cells had already occurred in the intermediate state. We also examined DNA samples from carrier conditions.
HTLV-I carriers (lanes 6 through 9), which gave no monoclonality by Southern blot analysis. In one of the cases evaluated, we could detect the oligoclonal proliferation of HTLV-I-infected cells (lane 6). Random integration of HTLV-I provirus DNA was detected in this case by Southern blot analysis. It showed that monoclonal proliferation had already occurred in the intermediate state and HTLV-I carrier with monoclonality from the viewpoint of HTLV-I infection. It is likely that an immunosuppressive state such as HTLV-I carrier could be one of the factors to cause T-cell lymphoma not associated with HTLV-I.

Table 1 summarizes the results of IPCR and Southern blot analysis for the same samples. It showed that IPCR was a reliable and sensitive method to detect the clonality of HTLV-I-infected cells. However, if a SacII site existed in 3' genomic DNA region within SmaI-digested DNA by chance, monoclonal bands could not be detected by IPCR. From our data, it is very rare, but we should be careful in such cases.

**Table 1. Comparison Between IPCR and Southern Blot Analysis**

<table>
<thead>
<tr>
<th>Clinical State</th>
<th>IPCR</th>
<th>Southern Blotting*</th>
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<tbody>
<tr>
<td></td>
<td>Clonal</td>
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<td>1/1</td>
</tr>
<tr>
<td>Carrier (II)</td>
<td>5/5</td>
<td>5/5</td>
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*Carrier (I): Polyclonal integration of HTLV-I provirus could be detected by Southern blot analysis. Carrier (II): No HTLV-I provirus could be detected by Southern blot analysis.

- The results of Southern blot analyses were shown as monoclonal integration of HTLV-I provirus (M), polyclonal integration of HTLV-I provirus (P), and no detected provirus (ND).
the peripheral blood in association with the skin involvement or respiratory lesions. IPCR could detect the monoclonal proliferation of HTLV-I–infected cells in the samples that were diagnosed to be monoclonal by Southern blot analysis (Table 1). However, monoclonal integration of HTLV-I provirus could not be identified in some patients with skin or respiratory involvement by conventional Southern blot analysis. It might be that Southern blot method could not detect the monoclonal integration of HTLV-I provirus in such a case. With IPCR, it is now possible to more precisely diagnose smoldering ATL.

Intermediate state of HTLV-I infection was proposed as a clinical condition between the smoldering ATL and HTLV-I carriers. The number of HTLV-I–infected cells increased in such patients, but the integration of HTLV-I provirus was random as detected by Southern blot method. Immune- compromised conditions such as strongyloidiasis were associated with the intermediate state. Bovine leukemia virus (BLV) is related to HTLV-I and II, but causes B-cell neoplasm in cattle. The natural course of BLV infection has been well studied and resembles that of HTLV-I. Lymphocytosis with random integration of BLV was observed as a prestage of leukemia, which is considered to be similar to intermediate state HTLV-I. It is possible that oligoclonal or monoclonal proliferations of HTLV-I–infected cells already occur in such a condition, but the number of monoclonal or oligoclonal cells is beyond the sensitivity of Southern blot analysis. We can detect the monoclonal or oligoclonal proliferation of HTLV-I–infected cells in the intermediate state and HTLV-I carriers. This indicated that clonal, but nonmalignant proliferation of HTLV-I–infected cells already occurred even in the HTLV-I carriers, and such cases could be a high-risk group for the development of ATL in the future.

Frequent detection of clonality was reported in the peripheral blood mononuclear cells from HTLV-I–associated myelopathy (HAM), tropical spastic paraparesis (TSP) patients and their family members. The same observation was reported in patients with strongyloidiasis. Monoclonality was detected with majority of random integrated HTLV-I provirus. In our study, monoclonal proliferation was detected in HTLV-I carriers in which polyclonal integration of HTLV-I had been proven by Southern blot analysis (Table 1). It suggested that some factors allowed not only the proliferations of HTLV-I–infected cells (polyclonal integration of HTLV-I provirus), but also the monoclonal proliferation. Such HTLV-I carriers with monoclonal integration of HTLV-I provirus were considered to be high-risk group for development of ATL. Pre-ATL was proposed as a designation for such a clinical condition. Although reported cases seem to contain various clinical entities such as smoldering and chronic ATL, ATL developed in obvious HTLV-I carriers with monoclonal HTLV-I provirus. It demonstrated that monoclonal HTLV-I–infected cells in HTLV-I carriers could develop into ATL.

Genomic sequences flanking 3’ LTR can be determined rapidly with IPCR (within 1 week). It is easy to make another primer in 3’ genomic region to detect case-specific sequence with LTR primer. Indeed, it enabled us to detect one ATL cell in 10⁶ peripheral blood mononuclear cells in a case of acute ATL (our unpublished data, March 1994). This PCR is highly sensitive and useful in the detection of residual ATL cells in the remission state. Prognosis for patients with ATL is still poor compared to those with other lymphoid malignancies, and its treatment should be established to increase survival rates. One possible therapy is autologous bone marrow transplantation or peripheral blood stem cell transplantation with extensive chemotherapy. In such therapies, detection of residual ATL cells in the remission bone marrow is very important for success. ATL cells are often very difficult to distinguish from non-ATL lymphocytes by morphology or immunophenotyping. Rearranged immunoglobulin sequences or fused oncogenes such as bcl-2 or bcr-abl were used for marker of leukemic cells detected by PCR. ATL cells do not have such specific oncogenes or rearrangements. PCR with primer in 3’ LTR and primer in flanking genomic sequence determined by IPCR is highly sensitive and useful in the detection of remaining ATL cells. Purging of ATL cells with monoclonal antibodies could be checked by this method. IPCR will also be a useful method in the therapy of ATL.

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NOVEL DIAGNOSTIC METHOD OF ATL


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