Clinical Importance of Extraordinary Integration Patterns of Human T-Cell Lymphotrophic Virus Type I Proviral DNA in Adult T-Cell Leukemia/Lymphoma

By Yoshinori Shimamoto, Kenji Suga, Keisuke Shibata, Miwako Matsuzaki, Hiromi Yano, and Masaya Yamaguchi

The proviral DNA of human T-cell lymphotrophic virus type I (HTLV-I) is known to be integrated monoclonally in the malignant cells of adult T-cell leukemia/lymphoma (ATL), which is a peripheral T-cell malignancy caused by this virus. We studied the relationship between the integration patterns of HTLV-I and clinical characteristics in 89 patients with ATL. The proviral DNA of HTLV-I was examined by the standard Southern blot analysis using the endonucleases EcoRI and PstI. One clear band of greater than 9 kb was detected in most of the patients (83 cases) when cellular DNA was digested with EcoRI. On the other hand, extraordinary integration patterns of HTLV-I proviral DNA were detected in 6 patients; 3 of them showed two bands, while the other 3 showed one band smaller than 9 kb. When cellular DNA was digested with PstI, the band patterns of these patients were quite different from those of typical patients. The patients with the extraordinary integration patterns had clinical characteristics dissimilar to those of the other 83 patients with ATL.

Patients and Methods

Patients. Eighty-nine patients (54 men, 35 women) with ATL were admitted to our hospital between 1981 and 1993. All were born in the Kyushu district of Japan, an area where ATL is known to be endemic. The diagnosis of ATL was based on clinical features, surface marker analysis, HTLV-I antibody, and the monoclonal integration of HTLV-I proviral DNA.

Surface marker analysis. Surface marker analysis of peripheral lymphocytes was performed by flow cytometry by a direct immunofluorescence method with monoclonal antibodies (MoAbs) as previously reported. The antibodies used were OKT4A (CD4), OKT8 (CD8), and OKT26a (CD25) from Ortho Diagnostic Systems Inc, Raritan, NJ. Whole blood was collected in tubes containing EDTA. Direct immunofluorescent staining was accomplished by incubating 100 μL of whole blood with an aliquot of appropriate titer of fluorescein isothiocyanate (FITC)-conjugated MoAb or with FITC-conjugated isotypic control MoAb for 30 minutes at 4°C. Two milliliters of lysing reagent was then added to the cells for 10 minutes at room temperature until erythrocytes were completely lysed. Samples were then analyzed immediately using an Ortho Cytoron Absolute. In this system, cells flowing in a small cuvette pass through a focused 488-nm laser beam. Light scattered at narrow angles and wide angles (forward and right angle light scatter) produces signals that are used to discriminate lymphocytes from other leukocytes. Fluorescence signals from cells with lymphocyte light-scattering characteristics are recorded to produce a histogram of cells counted versus fluorescence intensity. At least 5,000 cells were assayed and the results were expressed as the percentage of positive cells after the fluorescence threshold was determined using control MoAb.

Serum test for HTLV-I antibody. The antibody against HTLV-I was screened by an enzyme-linked immunosorbent assay and a gelatin particle agglutination test, and was confirmed by Western blotting assay, as described previously.

Detection of HTLV-I proviral DNA and T-cell receptor gene recombination.

From the Division of Hematology, Department of Internal Medicine, Saga Medical School, Saga, Japan.
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Address reprint requests to Yoshinori Shimamoto, MD, Division of Hematology, Department of Internal Medicine, Saga Medical School, Nabeshima 5-1-1, Saga 849, Japan.

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\[ \text{Eco RI} \quad \text{Pst I} \]

**Fig 1.** Detection of HTLV-I proviral DNA by Southern blot analysis using the endonuclease EcoRI (E) or PstI (P) in typical ATL patients (T1 and T2), a HTLV-I-seronegative individual (C), and seropositive individual with polyclonal integration of HTLV-I proviral DNA (P).

**RESULTS**

Integration patterns of HTLV-I proviral DNA in patients with ATL. The integration of proviral DNA in tumor cells was investigated in 89 patients with ATL. After digestion of cellular DNA with the endonuclease EcoRI, one clear band of greater than 9 kb was detected in 83 cases. After digestion of cellular DNA with the endonuclease Pst I, one or more clear bands containing viral-cellular DNA were usually detected in addition to the three internal fragments of 2.5, 1.8, and 1.2 kb in typical ATL patients. Only three internal fragments were detected in the polyclonal integration of HTLV-I proviral DNA (Fig 1). Extraordinary integration patterns of HTLV-I proviral DNA were detected in 6 patients. Three patients showed two clear bands (Fig 2), and the other 3 showed one band smaller than 9 kb (Fig 3).

**Patients with two bands.** In patients with two bands after digestion with EcoRI, more than 5 bands were detected when the cellular DNA from the tumor cells was digested with Pst I. Patients 1 and 3 showed the three internal fragments, but patient 2 lacked the internal fragment of 2.5 kb (Fig 2). Rearrangement of the TCR gene was found in patients 1, 2, and 3 (Fig 4). Tables 1 and 2 show the clinical features of the 3 patients with two bands. These 3 patients had clinical characteristics similar and dissimilar to those of the other 83 ATL cases with the ordinary provirus integration pattern. Like the typical ATL patients, they had generalized lymphadenopathy and leukemic T cells with highly convoluted nuclei in the peripheral blood (Fig 5), but they simultaneously showed severe hypoxemia and extremely high levels of serum lactate dehydrogenase (LDH) at first presentation, which were not so frequent in the ordinary cases. Their clinical subtype was the acute type (mixed). Pulmonary involvements were noted in all of these patients; in addition, gastric infiltrations and skin tumor formation were found in...
and the other was greater than 2.5 kb. Rearrangement of the
TCR gene was seen in patients 4, 5, and 6 (Fig 6). These 3
patients had mild symptoms such as fatigue and anorexia at
presentation (Table 1). They had abnormal and mature T
lymphocytes with bilobulated nuclei (Fig 5), but not lymph-
adenopathy. Serum levels of LDH were within the normal
range or mildly elevated (Table 2). Only 1 of them needed
chemotherapy because of hypercalcemia and liver infiltr-
ation, but responded promptly; the other 2 patients have been
stable for more than 28 months without chemotherapy. They
were all alive 20 to 38 months after diagnosis. Their clinical
characteristics, such as the bilobulated leukemic cells and a
favorable prognosis, were uncommon in the other 83 cases
with the ordinary provirus integration pattern and the other
3 patients with integration pattern of two bands by EcoRI
digestion. The clinical subtypes of these patients were smol-
dering, chronic, and acute as defined by other investigators,3
but were all the leukemia type by our classification.5

**DISCUSSION**

The clinical importance of the integration pattern of
HTLV-I proviral DNA in patients with ATL was shown in
the present study. When the cellular DNA of fresh leukemic
cells of patients with ATL is digested with EcoRI, the
one clear band of greater than 9 kb is detected in most cases by
the standard Southern blotting method.2,3,13 However, a few
patients with ATL are reported to show extraordinary integra-
tion patterns.14,15 In fact, patients 1, 2, and 3 showed two
bands and patients 4, 5, and 6 showed one smaller band. As
the viral genome has no cleavage site for the restriction
enzyme EcoRI, the two bands indicate that the provirus was
integrated in different sites of the tumor cell DNA. Yoshida
et al10 showed that 2 of 11 ATL patients had two bands. In
our series, 3 patients showed two bands. Konishi et al14
reported a higher incidence of multiple integration of HTLV-
I and even three bands were detected in a small portion of
patients with ATL. On the other hand, the detection of one
smaller band is considered to indicate a defective provirus,
and the detection of one smaller band in 2 out of 48 patients
with ATL has been reported.14 In the present study, 3 out of
89 patients showed one smaller band, but we could not
determine the exact location of the defective portion of
HTLV-I because we used an entire probe of HTLV-I proviral
DNA. As discussed above, extraordinary integration patterns

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**Fig 3.** Detection of HTLV-I proviral DNA by Southern blot analysis
using the endonuclease EcoRI or Pst I in patients with typical ATL
(T), polyclonal integration (P), and unusual integration with one
smaller band by EcoRI digestion (4-6). Patients 4, 5, and 6 showed
one smaller band with EcoRI, and one or two bands with Pst I.

**Fig 4.** Detection of TCR-\(\gamma\) gene rearrangement with BamHI
and EcoRV in control subjects from human placenta (C), from a
typical ATL case (T), from polyclonally integrated case (P), and
from patients with integration pattern of two bands by EcoRI
digestion (1-3). Patients 1, 2, and 3 showed the rearranged bands
of TCR gene.
Table 1. Clinical Characteristics of Patients With Unusual Integration of HTLV-I Proviral DNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Symptom</th>
<th>Lymphadenopathy</th>
<th>Morphology of Leukemic Cells</th>
<th>Clinical Subtype</th>
<th>Organ Involvement</th>
<th>Response to Chemotherapy</th>
<th>Survival (mos)</th>
<th>Integration Pattern of HTLV-I Proviral DNA by EcoRI Digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42/M</td>
<td>Dyspnea</td>
<td>+</td>
<td>Highly convoluted</td>
<td>Acute (mixed)*</td>
<td>Lung, 1 retina</td>
<td>Poor</td>
<td>8</td>
<td>Two bands</td>
</tr>
<tr>
<td>2</td>
<td>75/F</td>
<td>Dyspnea</td>
<td>+</td>
<td>Highly convoluted</td>
<td>Acute (mixed)</td>
<td>Pleura</td>
<td>Poor</td>
<td>4</td>
<td>Two bands</td>
</tr>
<tr>
<td>3</td>
<td>69/F</td>
<td>Dyspnea, skin tumor formation</td>
<td>+</td>
<td>Highly convoluted</td>
<td>Acute (mixed)</td>
<td>Stomach, skin, pleura, muscle</td>
<td>Poor</td>
<td>6</td>
<td>Two bands</td>
</tr>
<tr>
<td>4</td>
<td>62/F</td>
<td>Fatigue</td>
<td>-</td>
<td>Bilobulated</td>
<td>Smoldering (leukemia)</td>
<td>None</td>
<td>Not done</td>
<td>+28</td>
<td>One smaller band</td>
</tr>
<tr>
<td>5</td>
<td>65/M</td>
<td>Fatigue</td>
<td>-</td>
<td>Bilobulated</td>
<td>Chronic (leukemia)</td>
<td>None</td>
<td>Not done</td>
<td>+38</td>
<td>One smaller band</td>
</tr>
<tr>
<td>6</td>
<td>64/M</td>
<td>Fatigue, jaundice</td>
<td>-</td>
<td>Bilobulated</td>
<td>Acute (leukemia)</td>
<td>Liver</td>
<td>Good</td>
<td>+20</td>
<td>One smaller band</td>
</tr>
<tr>
<td>Typical ATL (n = 83)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age Range</td>
<td>62</td>
<td>Dyspnea (n = 3), skin tumor formation (n = 4), fatigue (n = 22), jaundice (n = 2)</td>
<td>+</td>
<td>Highly convoluted</td>
<td>Acute (n = 51), smoldering (n = 5), chronic (n = 14), skin (n = 23)</td>
<td>Lung (n = 5), pleura (n = 9), stomach (n = 7), skin (n = 15)</td>
<td>Poor (n = 24), Good (n = 9)</td>
<td>&lt;8</td>
<td>Ordinary one clear band</td>
</tr>
</tbody>
</table>

* The clinical subtype proposed by the Lymphoma Study Group, and the subtype proposed by the authors in parentheses.
† These findings appeared during their clinical course.

Table 2. Laboratory Findings of Patients With Unusual Integration of HTLV-I Proviral DNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>WBC (x10^3/μL)</th>
<th>Lymphocytes (%)</th>
<th>Abnormal cells (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD25 (%)</th>
<th>Serum LDH (IU/L)</th>
<th>Serum Ca (mEq/L)</th>
<th>PaO₂ (Torr)</th>
<th>Rearrangement of TCR gene</th>
<th>Integration pattern of HTLV-I proviral DNA by EcoRI Digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.5</td>
<td>15.5</td>
<td>65.0</td>
<td>93</td>
<td>2</td>
<td>32</td>
<td>2,406</td>
<td>4.2</td>
<td>67.5</td>
<td>+</td>
<td>Two bands</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
<td>11.0</td>
<td>47.0</td>
<td>89</td>
<td>4</td>
<td>52</td>
<td>3,294</td>
<td>4.4</td>
<td>48.3</td>
<td>+</td>
<td>Two bands</td>
</tr>
<tr>
<td>3</td>
<td>5.1</td>
<td>21.0</td>
<td>25.0</td>
<td>87</td>
<td>5</td>
<td>92</td>
<td>7,156</td>
<td>4.3</td>
<td>65.5</td>
<td>+</td>
<td>Two bands</td>
</tr>
<tr>
<td>4</td>
<td>8.8</td>
<td>27.0</td>
<td>10.5</td>
<td>83</td>
<td>7</td>
<td>28</td>
<td>352</td>
<td>4.5</td>
<td>79.0</td>
<td>+</td>
<td>One smaller band</td>
</tr>
<tr>
<td>5</td>
<td>12.7</td>
<td>14.0</td>
<td>53.2</td>
<td>82</td>
<td>6</td>
<td>28</td>
<td>183</td>
<td>4.3</td>
<td>82.0</td>
<td>+</td>
<td>One smaller band</td>
</tr>
<tr>
<td>6</td>
<td>12.7</td>
<td>3.0</td>
<td>77.0</td>
<td>88</td>
<td>7</td>
<td>38</td>
<td>877</td>
<td>6.1</td>
<td>71.0</td>
<td>+</td>
<td>One smaller band</td>
</tr>
<tr>
<td>Typical ATL (n = 83)</td>
<td>1.2-101.0</td>
<td>0-60</td>
<td>0.97</td>
<td>30-99</td>
<td>1-20</td>
<td>4-95</td>
<td>252-7,670</td>
<td>4.1-9.8</td>
<td>45.2-106.5</td>
<td>+</td>
<td>Ordinary one clear band</td>
</tr>
</tbody>
</table>

Normal range* | 4.5-7.0 | 25-45 | 0 | 29-53 | 19-33 | 1-5 | 200-450 | 4.5-5.1 | 80-100 | Not detectable |

* HTLV-I-negative Japanese adult subjects.
† Number of patients with it in parentheses.
Our patients with an integration pattern of two bands by \textit{EcoRI} digestion (patients 1, 2, and 3) presented with dyspnea and lymphadenopathy. Severe hypoxemia was always found at presentation in these patients. Patient 1 had been diagnosed as having diffuse panbronchiolitis for 2 years as we previously reported.\(^6\) The morphology of their leukemic T cells was almost the same as that of typical ATL cells, with highly convoluted nuclei. Their clinical subtype was the acute type\(^5\) (mixed type by our proposed classification\(^5\)). Organ involvements were common in their clinical course. Lung, pleura, stomach, and skin involvements were frequent in ATL, but retina and muscle infiltrations by the tumor cells were less frequent.\(^7\)^{13} Severely elevated levels of serum LDH were noted in these patients; hypercalcemia was not found at the first medical examination. Hypercalcemia at presentation was more frequent in patients with typical ATL than in those with two bands.\(^7\) On the other hand, the ATL patients with one smaller band (patients 4, 5, and 6) presented with fatigue but no lymphadenopathy. Their leukemic T cells were relatively small with bilobulated nuclei. Such cells were reported in patients in “an intermediate state” between healthy carrier and smoldering type of ATL.\(^8\) However, patients in an intermediate state are associated with polyclonal integration of HTLV-I proviral DNA, different from the monoclonal integration in the patients presented here. Therefore, abnormal lymphocytes with bilobulated nuclei are not specific for patients in an intermediate state, but are also found in ATL patients with an integration pattern of one smaller band. The clinical subtypes of these patients were smoldering, chronic, and acute by the Lymphoma Study Group,\(^4\) whereas they were all leukemia type by our proposed classification.\(^6\) Our proposed typing seemed to reflect the clinical course of ATL better than those reported by others.\(^3\)^{14} Organ involvements in our patients with one smaller band were less frequent. The serum level of LDH was much lower than that in the patients with two bands. Hypercalcemia was found in one patient, but hypoxemia was not found. The survival was much longer than that of the patients with two bands. Hypercalcemia was found in one patient, but hypoxemia was not found. The survival was much longer than that of the patients with two bands or the other 83 ATL cases with usual integration pattern. Rearrangement of the TCR-\(\beta\) gene was shown in all patients with unusual integration of HTLV-I proviral DNA.

When the cellular DNA of the tumor cells is digested with \textit{Pst I}, which cleaves several sites in the HTLV-I provirus genome, one or more clear bands containing viral-cellular DNA are known to be detected in addition to the three inter-
nal fragments of 2.5, 1.8, and 1.2 kb in typical ATL patients with monoclonal integration of complete HTLV-I. In polyclonal integration, only three internal fragments are usually detected. Patients 1, 2, and 3 in our study showed 5 or more bands including the internal fragments, but patient 2 lacked the internal fragment of 2.5 kb. Patients 4, 5, and 6 showed one or two bands. Patient 4 showed only one band, but no internal fragments. Patient 5 showed one internal fragment of 2.5 kb. Patient 6 showed two bands, one of which is the internal fragment of 1.8 kb. These band patterns were quite different from those of typical monoclonal and polyclonal integration. The band patterns in patients 2, 4, 5, and 6 seemed to indicate defective HTLV-I.

Rearranged bands of the TCR-β gene were detected in all patients with unusual integration of HTLV-I proviral DNA in the present study. This finding supported the clonality of the tumor cells examined. Even the ATL patients with defective HTLV-I showed monoclonal proliferation of CD4+ lymphocytes. This finding indicates that the whole HTLV-I genome is not necessary to initiate or maintain the clonal expansion of the tumor cells in these patients, although their clinical course was not so aggressive. Therefore, the defective HTLV-I may cause different clinical features from those of the complete HTLV-I as shown in the present study.

REFERENCES


Clinical importance of extraordinary integration patterns of human T-cell lymphotropic virus type I proviral DNA in adult T-cell leukemia/lymphoma [see comments]

Y Shimamoto, K Suga, K Shibata, M Matsuzaki, H Yano and M Yamaguchi