RAPID COMMUNICATION

Homzygous Deletions of p16/MTSl Gene Are Frequent But Mutations Are Infrequent in Childhood T-Cell Acute Lymphoblastic Leukemia

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Fifty-six primary childhood T-cell acute lymphoblastic leukemia (T-ALL) samples and 17 T-ALL cell lines were examined for mutations and homozygous deletions of the p16/MTSl gene using polymerase chain reaction single-strand conformation polymorphism and Southern blot analysis. Homozygous deletions were found in 22 primary samples (39%) and in 10 cell lines (59%). In contrast, mutations including small deletions and/or insertions were identified in only 4 primary samples (7%) and in 2 cell lines (12%). Mutations included one nonsense mutation at codon 72, one missense mutation at codon 58, one deletion (29 bp from codon 52-61), one insertion (7 bp into codon 50), and two deletion/insertions (codon 63 and intron 1). Four of the six mutations caused subsequent stop codon and presumably produced truncated p16 protein. Our results suggest that p16 gene alterations are involved in the development of T-ALLs and that the inactivation of the p16 gene occurs mainly through homozygous deletions rather than mutations.

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MATERIALS AND METHODS

Cell lines. Seventeen human T-ALL cell lines (KCMC-T, L-KAW, SIL, MOLT 16, CCRF-HSB2, KOPT4, p12/Ichikawa, Jurkat, M, MOLT-4F, TALL-1, CCRF-CEM, KOPT-K1, MPB-MLT, DND-41, MOLT14, and PEER) were examined in this study. KCMC-T and L-KAW were generously provided by Dr H. Nishihira (Kanagawa Children’s Hospital, Kanagawa, Japan) and Dr M. Mi-}

KAW, SIL, MOLT 16, CCRF-HSB2, KOPT4, pl2/Ichikawa, Jurkat, 1270 KCMC-T and L-KAW were generously provided by Dr H. Nishihira saki Cell Center (Okayama, Japan). The cells were grown in suspen-

sion culture in RPMI medium supplemented by 10% fetal calf serum.

Patients and preparations of samples. Bone marrow or peripheral blood samples were collected from 56 patients with T-ALL in various institutes after informed consent was obtained. The patients were 41 boys and 15 girls whose ages ranged from 10 months to 19 years. The diagnosis of ALL was based on the criteria of the French-American-British (FAB) classification. T-ALL was defined based on the expression of CD7 plus CD2, CD3, CD4, CD5, CD11c, and CD8 surface antigens on more than 30% of leukemic blasts. In all samples examined, the proportion of leukemic cells exceeded 80%. Mononu-

clear cells were separated from the samples on Ficoll-Hypaque (Lymphoprep) density gradient, suspended in RPMI medium con-

taining 10% of dimethylsulfoxide. and kept at -80°C. High molecu-

lar weight DNA from primary samples and cell lines was prepared by proteinase K-phenol-chloroform extraction, as previously de-

scribed.27,28

PCR method. PCR of genomic p16 gene was performed as previously described,29 with some modifications. Exon 1 was amplified as one fragment, whereas exon 2 was split into two fragments (2a and 2b) for amplification. The PCR reaction mix (10 μL) consisted of 1× Taq Extender Reaction buffer (Stratagene, La Jolla, CA), 50 μmol/L dNTPs, 1 U Taq polymerase, 1 U Taq extender (Stratagene), 6% dimethylsulfoxide, 1 μmol/L primers, and 200 ng template. A total of 2 μCi [α-32P]dCTP (3,000 Ci/mmol; Amersham, Bucking-

hamshire, UK) per tube was added to the mixture when SSCP was performed. The sequences of primers used for PCR were as follows: 5'-TCTGGCGAGGAGGGAGACGACA-3' (PQ1S) and 5’-GCCAATCTGTTCATCCTTCAGC-3' (PQ1A) for exon 1; 5'-A-

CAAGCTTTCCCTCCCCGATCTGACG3'-3' (PQ2AS) and 5'-CCA-

GGATCTGCAGCAGCAGACTCC-3' (PQ2AA) for fragment 2a; and 5'-TTCTGAGCAGCGCTGTGGT-3' (PQ2BS) and 5'-TCTGAG-

CTTGGGAAGCTTCAG-3' (PQ2BA) for fragment 2b. PCR parame-

ters were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 50 seconds for 35 cycles.

SSCP analysis. The 32P-labeled PCR products were heated for 5 minutes at 85°C with 40 μL of formamide denaturing dye mixture (95% formamide, 20 mmol/L EDTA, 0.05% xylene cyanol, 0.05% bromphenol blue) and then applied to a 3.3% agarose gel (pH 8.3) and 4 mmol/L EDTA with and without 10% glycerol. Electroforesis was performed at 40 W for 2 to 3 hours with a water cooling system. The gel was dried on filter paper and exposed to x-ray film for 1 to 2 days. Samples exhibiting band shifts were reassayed using separate PCR products to verify that the shifts were not due to Taq-induced error.

Direct sequencing. Direct sequencing was performed as pre-

viously described,29,30 with some modifications. A small piece of the gel corresponding to the abnormal band detected by SSCP analysis was cut out, immersed in 20 μL of water, and heated at 80°C for 15 minutes. The centrifuged water was extracted (4 μL), subjected to 35 cycles of PCR, and purified with Centricon 30 (Amicon, Bev-

more, MA). The purified DNA fragments were sequenced by deoxy-

chain termination method using 5' 32P-labeled primers and Taq DNA polymerase (dsDNA Cycle Sequencing System; Gibco, Gaithers-

burg, MD) for symmetric PCR products. The same 5' and 3' side primers as used for PCR-SSCP were available. The products were applied to 5% polyacrylamide gel containing 7 mol/L urea. All mutations and base changes were verified by sequencing two differ-

ent PCR products in separate experiments.

Southern blot analysis. The 0.5-kb EcoRI fragment containing the coding sequence of p16 cDNA21 was labeled with [α-32P]dCTP by the random priming method and used as the probe for Southern blot analysis. A human c-sis exon 6 probe obtained from Oncogene Science, Inc (Mathasset, NY) was cohybridized to confirm the integ-

rity of the sample DNA. Five micrograms of DNAs extracted from cell lines and patients’ samples was digested with BamHI, electro-

phoresed in TAE buffer (40 mmol/L Tris, 20 mmol/L sodium acetate, 2 mmol/L EDTA, pH 7.2) through 1% agarose gel at 28°C overnight, transferred to a nylon filter, and fixed to the filter by UV light. The filters were hybridized with 32P-labeled probes overnight in a solution with 50% formamide, 5× SSC, 5× Denhardt’s solution, and 0.5% sodium dodecyl sulfate (SDS) at 42°C. After high stringency washing with 0.2× SSC containing 1% SDS at 55°C for 20 minutes three times, filters were exposed to x-ray film with an intensifying screen at -80°C for 2 to 4 days.

RESULTS

Homozygous deletion of the p16 gene. We examined 17 T-ALL cell lines and 56 T-ALL patients’ samples by Southern blot analysis. Hybridization of 32P-labeled p16 cDNA probe to normal genomic DNA digested by BamHI showed a 21-kb band containing exons 1 and 2 of the p16/MTSI}

kb 0 1 2 3 4 5 6 7 8

23

9.4

6.6

4.4

2.3

2.0

29.1

67.8

1270

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Fig 1. Southern blot analysis of p16 gene deletions in primary T-ALLs. DNA of leukemic cells from T-ALL patients were digested with BamHI and were hybridized by p16 cDNA probe. A 21-kb band and a 5.8-kb band show the genomic DNA fragment containing exons 7 and 2 of the p16 gene and exon 2 of the p15 gene, respectively. A human c-sis probe was cohybridized as an internal control and shown as a band at 1.8 kb. Lane 1, a normal peripheral blood sample used as a positive control. Lanes 2 through 8, T-ALL patients. p16 gene is homozygously deleted in UT 349 (lane 2) and UT 685 (lane 4).
gene and a 5.8-kb band containing exon 2 of the p15/MTS2 gene, which had a great extent of homology and cross-hybridized to p16 (Fig 1). We judged the cases with no bands or a faintly visible 21-kb band compared with the control c-sis band as having homozygous deletion of p16. Homozygous deletions of p16 were identified in 10 (59%) cell lines and in 22 (39%) primary leukemia samples (Table 1 and Fig 1). Among these cases, only 2 retained the p15 band; the remaining 30 cases showed simultaneous p15 deletion. We found no case that had an intact p16 band with a deleted p15 band.

**SSCP analysis and direct sequencing.** We screened 17 cell lines and 56 patients’ samples by SSCP for the detection of the p16 gene mutation. In the two cell lines (KOPT-K1 and DND-41), abnormal band migrations were detected (Fig 2). Direct sequencing analysis showed a point mutation from CGA(Arg) to TGA(stop codon) at codon 72 in KOPT-K1 (Fig 3). This nucleotide change is assumed to cause abnormal termination of translation, leading to truncated p16 protein. In DND-41, 29 bp from nucleotide number 221 to 249 were deleted (Table 2). This deletion causes a frameshift and a subsequent stop codon. Patients UT289 and UT48 showed only an abnormally migrated band in fragment 2a; a normal band was not detected in these patients. Direct sequencing showed a 1-bp deletion and a 5-bp insertion at codon 63 in patient UT 289 and a 5-bp deletion and 9-bp insertion in intron 1, 16 bp upstream of exon 2, in patient UT48. In this study, we did not identify polymorphisms at codons 98 and 140, as were commonly found in Caucasians in previous reports.

**DISCUSSION**

Screening of 56 T-ALL patients’ samples by Southern blot analysis showed homozygous deletion of p16 gene in 22 cases (39%). This rate is higher than the previously reported rate of cytogenetic 9p loss in T-ALL (approximately 10%), implying that p16 gene alterations are not restricted to patients with cytogenetically detectable 9p deletion. Herbert et al have reported higher frequency (83%) of p16 homozygous deletion in both childhood and adult T-ALLs. The lower frequency in our study may be due to racial differences in genetic background or to different leukemogenic factors in childhood and adult T-ALL. In addition, it is possible that the contamination of normal leukocytes created a false-positive band in some patients with homozygous loss and resulted in the lower rate of homozygous deletion. However, even in cell lines without normal cell contamination, we found homozygous deletions in only 59%. Diaz et al have reported that LOH of interferon α and/or β gene on 9p21 was found in 18 of 62 (29%) ALL patients and that phenotypic difference was not significant. More recently,
Quesnel et al. have reported a lower rate (25%) of p16 homozygous deletions in a smaller series of primary T-ALL than that of our result. If we take these findings into consideration, the frequency of p16 homozygous deletions in primary T-ALLs in our study is not too low. In the present study we did not determine the incidence of p16 gene hemizygous deletions because it is difficult to show the loss of one allele in leukemic cells contaminated with up to 20% of normal cells.

In contrast, screening of p16 gene mutation in 56 T-ALL patients by PCR-SSCP analysis showed a point mutation in only 4 cases (7%). The sensitivity to mutation detection by SSCP analysis is assumed to be 90% or more. We performed direct sequencing of exons 1 and 2 in 10 samples from patients with normal SSCP band patterns, but all samples showed the sequence of wild-type p16 gene. SSCP analysis has been used successfully in detecting single base changes in our previous studies, and we readily detected three single-base mutations in the present study. Accordingly, we conclude that a low detection rate of mutations in p16 gene reflects a real low frequency of p16 gene point mutation in primary T-ALL samples rather than false-negative results by SSCP analysis.

Lower frequency of point mutations of p16 gene than of homozygous deletion in T-ALLs is different from the previous results for p53 gene, another tumor-suppressor gene involved in diverse tumors. In p53 gene, most of the alterations are single-base missense mutations; homozygous deletion is reported to be extremely rare in various types of tumors. Our results suggest that p16 gene inactivation may be caused mainly by homozygous deletions. The low frequency of mutation of p16 raises the possibility that another tumor-suppressor gene(s) on chromosome 9p21 closely linked to p16 gene may be associated with the development of T-ALL patients with p16 gene homozygous deletions. p15/MTS2 gene, juxtaposed to p16 on 9p21, has been cloned as a highly homologous gene with p16 and is assumed to be one of the candidate tumor-suppressor genes on 9p21. In the present study, we found 2 cases (4%) that have an intact 5.8-kb band (probably representing p15) but have no 21-kb band (p16 band) in Southern blot by BamHI digestion. In contrast, no case showed an intact p16 band along with a deleted p15 band. These results are consistent with those of the previous report and suggest that p16 is more likely to be the target gene for 9p21 deletion than p15. However, it would be premature to conclude it because we did not perform SSCP of p15 gene or Southern blot analysis using true p15 probe. Another possibility is that exon 3 or the promoter region of p16 gene, which were not investigated in the present study, may have mutations in some patients.

Table 2. Clinical Data on 4 T-ALL Patients With p16 Gene Mutation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>WBC (×10^9/L)</th>
<th>Positivity of T-Cell Phenotype</th>
<th>Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT 48</td>
<td>8/F</td>
<td>350.0</td>
<td>CD 2, 3, 4, 5, 7, 8</td>
<td>15</td>
</tr>
<tr>
<td>UT 233</td>
<td>10/M</td>
<td>45.0</td>
<td>CD 5, 7</td>
<td>17+*</td>
</tr>
<tr>
<td>UT 289</td>
<td>7/F</td>
<td>25.9</td>
<td>CD 2, 4, 7</td>
<td>58+*</td>
</tr>
<tr>
<td>UT 373</td>
<td>4/M</td>
<td>58.0</td>
<td>CD 2, 3, 5, 7, 8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Alive.
Of 17 T-ALL cell lines, 12 (71%) showed p16 gene alterations, which is a higher rate than that for primary T-ALL samples (26 of 56 [46%]). Homozygous deletions were found in 10 (59%) cell lines, which is consistent with the results of previous reports. The higher frequency of p16 gene alterations in cell lines than in patients’ samples has been reported by several investigators, and the results are similar to those with other tumor-suppressor genes such as p53 gene.  

The nonsense mutation at codon 72 (CGA to TGA) found in 2 cell lines, which is consistent with the predicted mechanism of p53 gene inactivation. Another possible explanation is that normal leukocytes are contaminated in this sample and we could not detect hemizygous deletion of one allele. Patient UT48 had a deletion/insertion in intron 1. Although we did not examine whether this nucleotide change causes abnormal splicing of exon 2 or not, the loss of the other allele suggested that this mutation may result in inactivation of the p16 gene in this patient. Four of the six mutations found in our study created stop codon as a result of point mutation or frameshift. Nonsense mutations, frameshifts, or large deletions, which lead to grossly changed or no p16 protein, may be required to inactivate p16 gene and contribute to the development in T-ALLs.

In conclusion, we found frequent p16 homozygous deletions but lower frequency of p16 gene mutations in T-ALL cell lines and patients’ samples. Our results confirm that p16 gene aberrations are involved in the pathogenesis of T-ALL. Further studies are needed to determine whether p16 aberration contributes to the early development or progression of T-ALL.

ACKNOWLEDGMENT
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REFERENCES

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**Table 3. p16 Gene Mutations in T-ALL Cell Lines and Patients’ Samples**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOPT-K</td>
<td>2</td>
<td>72</td>
<td>CGA to TGA</td>
<td>Arg to stop codon</td>
</tr>
<tr>
<td>DND-41</td>
<td>2</td>
<td>52-61</td>
<td>29-bp deletions*</td>
<td>Frameshift</td>
</tr>
<tr>
<td>UT 48</td>
<td>Intron 1</td>
<td>t</td>
<td>CGTGT to TGAGGCC</td>
<td>Mutant</td>
</tr>
<tr>
<td>UT 233</td>
<td>2</td>
<td>58</td>
<td>CAC to CGC</td>
<td>His to Arg</td>
</tr>
<tr>
<td>UT 289</td>
<td>2</td>
<td>127</td>
<td>GGG to GGA*</td>
<td>None</td>
</tr>
<tr>
<td>UT 373</td>
<td>2</td>
<td>63</td>
<td>AAC to AAGTCG</td>
<td>Frameshift</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50</td>
<td>CGA to CCGAGGGGA</td>
<td>Frameshift</td>
</tr>
</tbody>
</table>

*CGGAGCCTGCTGCTGCTCAAGGCAGGCAGGAG.
† Sixteen base pairs upstream of exon 2.
‡ Polymorphism.


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