Mutations of the p53 and ras Genes in Childhood t(1;19)-Acute Lymphoblastic Leukemia

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We have investigated the alterations of p53 and ras genes including H- and K-, and N-ras genes in 22 acute lymphoblastic leukemia (ALL) cases and five cell lines carrying t(1;19) by use of polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP) analysis and direct sequencing. The mutations of the p53 gene were found in 2 of 20 t(1;19)-ALL cases at diagnosis (10%), all of 4 cases at relapse (100%), and 4 of the 5 cell lines (80%). Four of the five patients who died had missense mutations at codons 49, 177, 179, and 248. In cases examined sequentially, one had the same point mutation at codon 179 at both diagnosis and relapse, and another had the same p53 gene mutation at codon 240 both in leukemic cells at relapse and in a cell line derived at that time. The other case had no mutation at diagnosis but had the mutation at codon 177 at relapse and cell lines derived from blast cells at diagnosis, suggesting that a small number of leukemic cells with the p53 gene mutation at diagnosis might have escaped PCR-SSCP analysis. In cell lines, SCMC-L9 had three point mutations in the p53 gene at codons 175, 248, and 358, whereas SCMC-L10 had frame shift at codons 209-211. One case had a rare polymorphism at codon 11. We found only one mutation of the N-ras gene that was a 2-bp substitution of GGT(Gly) to GTC(Val) at codon 13 among 22 t(1;19)-ALL cases and five cell lines. This case showed no mutation of the p53 gene and had a good course. These results suggest that in t(1;19)-ALL, mutations of the p53 and ras genes are frequent at diagnosis and that p53 gene alterations may be associated with relapse phase or progression of t(1;19)-ALL.

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CHROMOSOMAL TRANSLOCATIONS are important events in the pathogenesis of leukemias and lymphomas. One of the most frequently reported cytogenetic changes in acute lymphoblastic leukemia (ALL) is the t(1;19)(q23;p13).1-4 Observed in up to 6% of pediatric ALL and in approximately 25% of ALL with a pre-B cell phenotype,1,5,6 which express cytoplasmic heavy chain Ig (cIg), but not surface Ig (sIg), t(1;19)-ALL cases have been reported to have a poor clinical outcome or poor prognostic features.7

The E2A gene, which encodes proteins that bind to enhancer of the ke2, has been isolated,7 mapped to 19q13, and shown to be rearranged in t(1;19)-ALL.8 Similarly, the breakpoint in chromosome 1q23 interrupts a homeobox gene known as PBX1.9,10 Consequently, the production of a chimeric E2A-PBX1 protein may contribute to the development of t(1;19)-ALL.9,10

It has recently been shown that the p53 gene is a tumor suppressor gene located on chromosome 17p13. Alterations of the p53 gene are involved in various types of human cancers.11 Loss of the normal growth-inhibitor activity of p53 protein in most of these tumors occurs as a result of point mutations of the other p53 allele. In myeloid malignancies, p53 gene mutations have been reported in myelodysplastic syndrome,12 blast crisis of chronic myelogenous leukemia,13 and acute myelogenous leukemia (AML),14 at a low frequency. In lymphoid malignancies, Burkitt's lymphoma,15,16 B-cell lymphoma,17 B-cell ALL,18 multiple myeloma,19 and T-cell-ALL cell line20 had the p53 mutations at high frequency whereas early pre-B ALL had low frequent mutations of the p53 gene.21 However, there have been few reports of this gene aberration in t(1;19)-ALL with pre-B phenotype.21

Altered ras genes have been detected with varying frequencies in a number of human malignancies.22 N-ras mutations are found in between 25% and 40% cases of AML23,24 whereas they were found at lower frequency in ALL.25 Here we analyze the rearrangement of the E2A gene and mutations of the p53 and ras genes to investigate the frequency and spectrum of these mutations in a series of t(1;19)-ALL. We find that mutations of the p53 and ras genes were infrequent and that p53 mutations were associated with relapse phase or progression of t(1;19)-ALL.

MATERIALS AND METHODS

Patient samples. From April 1993 to May 1994, we identified 22 ALL cases with the t(1;19) at diverse institutes in Japan. The diagnosis of ALL was based on examination of Wright-stained smears of bone marrow (BM) aspirates classified according to the French-American-British morphologic criteria and negative myeloperoxidase and α-naphthyl butyrate esterase staining. BM cells from 22 t(1;19)-ALL cases including 18 cases at diagnosis, 2 cases at relapse, and 2 cases at both diagnosis and relapse were used in this study (Table 1). In cases 3 and 19, we analyzed peripheral blood (PB) and BM samples at remission, respectively, and in case 10 we analyzed fibroblasts cultured from BM. These included 7 boys and 15 girls whose ages ranged from 2 to 14 years. These cases were mainly treated on the TCCSG ALL protocol.26

Cell lines. Five cell lines (KM0-50,27 SCMC-L9,28 SCMC-L10,25,27 SCMC-L11,28 THP-817) with the t(1;19) were examined. KM090 and SCMC-L11 derived from case 21 at diagnosis and from...
case 10 at relapse, respectively. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

**Cyto genetic studies.** The chromosomes of cell lines and BM or peripheral samples were analyzed by the regular tripsin-Giemsa- or Q-banding method as described previously. DNA and Southern blot analysis. High-molecular-weight DNA of all samples was prepared by proteinase K-phenol-chloroform extraction. DNA was digested with EcoRI, Xba I, BglII, HindIII or BamHI restriction endonuclease, electrophoresed through a 0.8% agarose gel, and transferred to nylon membrane.

**DNA probes and hybridization.** p53 probe was used in the Southern hybridization. The insert was isolated from the vector sequence with HindIII and EcoRI, and was used as a template to prepare a labeled probe with Klenow fragment of DNA polymerase I and [α-32P]dCTP, after priming with random hexanucleotides. Highly stringent condition was used for hybridization and washing. The filters were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) in the presence of intensifying screens. All experiments included control DNA.

**Immunophenotyping.** Cell surface antigens were detected by a standard indirect immunofluorescence assay with the use of monoclonal antibodies to lymphoid-associated antigens, including CD10(15), CD20(15), and CD19(15,24) as well as myeloid antigens including CD13(MY7) and CD33(MY9). Cells were analyzed for fluorescence microscopy of flow cytometry (Coulter EPICSCL, Hialeah, FL). Results were considered positive if 25% or more of the cells expressed a particular antigen. Leukemic cells were also tested for slg and clg, which were considered to be positive if greater than 10% of blast cells had fluorescence.

**Polymerase chain reaction (PCR) method.** Fragments A, B, C, D, E, F, G, H, and I containing the sequences of the p53 gene important for the function of the wild-type protein were analyzed by PCR (Fig 1). Names and sequences of primers for amplification of the fragments have been described previously. Mutations in codon 12, 13, or 61 of one of the three ras genes, H-, K-, and N-ras, convert these genes into active oncogenes. Therefore, amplified fragments from regions carrying exon 1 or 2 of the K-ras gene (K1 or K2), the Ha-ras 1 gene (H1 or H2) and the N-ras gene (N1 or N2) were analyzed by PCR. The sequences of primers used for PCR were the same as reported previously. DNA samples (50 ng) in the mixture (5 µL) with appropriate unlabeled primers and [α-32P]dCTP (20 µCi per tube, 3,000 Ci/mmol; Amersham, Buckinghamshire, UK) as one of the nucleotide substrates were subjected to 30 cycles of the reaction.

**Single-strand conformation polymorphism (SSCP) analysis.** After addition of 45 µL of formamide denaturing dye mixture (95% formamide:20 mMol/L EDTA:0.05% xylene cyanol:0.05% bromophenol blue) PCR mixture was heated at 80°C for 3 minutes, and then 1 µL of the diluted mixture was applied to one lane of 5% polyacrylamide gel containing 45 mMol/L TRIS-borate (pH 8.3) and 4 mMol/L EDTA. The gel contained 10% glycerol when it was specified. Electrophoresis was performed at 40 W for 1 to 3 hours with cooling by fan. The gel was dried on filter paper and exposed to x-ray film.

**Direct sequencing of PCR-amplified fragments.** Direct sequencing was performed as previously described 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, heated at 80°C for 15 minutes, and centrifuged. DNA in extract (1 µL) was subjected to 30 cycles of PCR, and the products were purified with Centricon 30 or Microcon 100 (Amicon, Beverly, MA). The DNA fragments thus obtained were sequenced by dyeoxy chain termination method using 5′-32P-labeled primers and Taq DNA polymerase (dideoxy Cycle Sequencing System; GIBCO BRL, Gaithersburg, MD). Primers for sequencing were the same as those used for PCR.

**Statistical analysis.** Cases were classified into two groups on the basis of the presence or absence of the p53 gene mutations that their leukemias carried. Significance of the different groups with and without p53 gene mutations was examined by χ2 test. The survival curves of each group of cases were estimated by the Kaplan-Meier method, and significant differences were determined by the generalized Wilcoxon test.

**RESULTS**

**Cyto genetic studies.** All cases had t(1;19)(q23;p13) or der(19)(t;1;19) with or without additional chromosome abnormalities. An absence or obvious structural abnormalities of chromosome 17p were not found in any cases.

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| Table 1. Clinical Data on 22 ALL Cases With the t(1;19) Translocation |
|-------------|--------------------|---------------|---------------|---------|
| **Case** | **Age (yrs)** | **WBC (10⁹/L)** | **E2A Re** | **CD10** | **Survival (mos)** |
| 1 | 4 | F | 44.9 | + | + | 75+ |
| 2 | 4 | M | 28.7 | – | + | ND |
| 3 | 11 | M | 6.4 | + | + | 52+ |
| 4 | 8 | M | 33.5 | + | + | ND |
| 5 | 10 | F | 131.1 | + | + | ND |
| 6 | 4 | F | 8.1 | + | + | 55+ |
| 7 | 4 | F | 25.8 | + | + | + | 54+ |
| 8 | 11 | F | 105.9 | ND | + | ND |
| 9 | 3 | F | 7.7 | + | + | 43+ |
| 10 | 6 | F | 107.1 | ND | + | ND |
| 11 | 2 | M | 102.5 | + | + | 41+ |
| 12 | 4 | F | 42.5 | + | + | ND |
| 13 | 8 | F | 112.6 | + | + | ND |
| 14 | 2 | F | 2.2 | ND | + | ND |
| 15 | 12 | F | 10.6 | + | + | 28+ |
| 16 | 14 | M | 9.7 | + | + | ND |
| 17 | 8 | F | 13.5 | ND | + | ND |
| 18 | 15 | M | 23.6 | ND | + | ND |
| 19 | 4 | F | 23.9 | + | + | ND |
| 20 | 13 | F | 8.3 | ND | + | ND |
| 21 | 13 | F | 20.1 | + | + | 10 |
| 22 | 8 | M | 28.3 | + | + | ND |

**Abbreviations:** WBC, white blood cell count; ND, not done; Re, rearrangement.
### Table 2. p53 Gene Mutations in ALL Cases and Cell Lines With t(1;19) Translocation

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide Substitution</th>
<th>Amino Acid Substitution</th>
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</thead>
<tbody>
<tr>
<td>Fresh leukemic cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 10</td>
<td>R</td>
<td>7</td>
<td>240</td>
</tr>
<tr>
<td>Case 19</td>
<td>D</td>
<td>7</td>
<td>248</td>
</tr>
<tr>
<td>Case 20</td>
<td>D</td>
<td>5</td>
<td>179</td>
</tr>
<tr>
<td>Case 21</td>
<td>D</td>
<td>5</td>
<td>179</td>
</tr>
<tr>
<td>Cell lines</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>D*</td>
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<tr>
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<td>SCMC-L11‡</td>
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<td>240</td>
<td>AGT to TGT</td>
</tr>
<tr>
<td>KMO-90§</td>
<td>5</td>
<td>177</td>
<td>CCC to CTC</td>
</tr>
</tbody>
</table>

Abbreviations: D, at diagnosis; R, at relapse.

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bp insertion at codon 209-211 in exon 6; KMO-90 derived from case 21 at diagnosis showed the same missense mutation of CCC to CTC at codon 177 in exon 5 as leukemic cells at relapse, and SCMC-L9 showed the multiple missense mutations of CGC to TGC at codon 175, CGG to CAG at codon 248, and GAG to AAG at codon 358 (Table 2). SCMC-L11, which derived from case 10 at relapse, showed the same mutation at codon 240 as leukemic cells at relapse.

Correlation between the p53 mutations and clinical outcome. Among 20 cases tested at diagnosis, both of the cases with p53 mutations have died while only 1 of 18 cases lacking p53 mutations has died (P < .02 by \( \chi^2 \) test) (Table 3). The Kaplan-Meier analysis of survival times showed a significant difference between the two groups (\( P < .005 \)). All 4 patients who died had p53 aberrations in the course of disease. Among 5 patients with p53 aberrations in the course of disease, 4 patients died and 1 relapsed after BM transplantation, whereas 17 patients without p53 aberrations have been alive for 6 to 75 months without any evidence of recurrence (\( P < .005 \) by \( \chi^2 \) test). Two of 20 cases (cases 2 and 17) had central nervous involvement at diagnosis and had no p53 mutations.

Ras gene mutations. PCR-SSCP analysis of ras gene showed abnormal mobility shift of fragment N1 in case 2. Nucleotide sequence analysis of this case revealed a 2-bp substitution of GGT(Gly) to GTC(Val) at codon 13 of N-ras gene. The presence of the wild type of codon 13 suggested that mutations occurred in a smaller portion of leukemic cells.

DISCUSSION

The E2A gene rearrangements were found in 15 of 16 cases with t(1;19)-ALL tested, and in all five cell lines with t(1;19). This frequency is compatible with that of the previous reports. Case 2 with hyperdiploid chromosome abnormality had no rearrangements of E2A gene and has been alive for 6 years. Similar cases have been reported thus far.

In lymphoid malignancies frequent mutations of p53 gene have been reported in Burkitt’s cell lines36 and fresh tumors,49 as well as T-cell lines.16 However, there have been few reports of p53 gene aberrations in non-T, non-B lymphoid malignancies, especially in t(1;19)-ALL with pre-B phenotype. In this study, alterations of the p53 gene were found in 2 of 20 t(1;19)-ALL cases at diagnosis (10%), 4 of 4 t(1;19)-ALL cases at relapse (100%), and 4 of 5 t(1;19)-ALL cell lines (80%). The frequency of p53 gene mutation was lower at diagnosis than that at relapse and than that of cell lines. These findings suggest that t(1;19)-ALL cases with p53 gene aberrations may be associated with relapse phase or progression of disease as observed in B-cell lymphomas and multiple myelomas, and support the notion that the p53 gene is more frequently mutated in cell lines than in primary tumors.

The occurrence of p53 gene mutations in human cancer is presumably the consequence of several mutagenic factors that may act specially in a particular type of tumor. A notable finding of p53 mutations in human cancer is the fact that transitions at CpG dinucleotides occur in about one third of mammalian cells because of 5-methylcytosine residues. It is important to note that 3 of 8 mutations in this study occurred at CpG dinucleotides in the form of C to T transition (codon 248 in case 19, codons 175 and 248 in SCMC-L9), as was seen in B-ALL, Burkitt’s lymphoma, T-ALL, T-cell lines, and early pre-B ALL. Whether or not this mutation in lymphoid malignancies was induced through exposure to exogenous carcinogens remains to be investigated in the future.

The mutation at codon 248 (CGG to CAG) seen in case 19 has been reported thus far in a case with pre-B phenotype. In respect of codon 248, transversion (CGG to CCG, arginine to proline) has been reported in an infant with pre-B ALL at relapse. Codon 248 of p53 gene is a CpG dinucleotide.
otide site and a hot spot for transitional mutations in other cancers, both sporadic and hereditary. In Li-Fraumeni syndrome, germline mutation commonly found at a codon 248 (CGG to TGG, arginine to triptophan)\(^3\),\(^4\) is different from that seen in ALL. Therefore, the mutations at codon 248 in ALL may be associated with the leukemogenesis of ALL.

Polymorphism at codon 11 found in case 3 has been reported previously.\(^3\) GAT to CAT transversion at codon 49 (case 22) has been reported in a case with CML in the accelerated phase.\(^4\) However, the same base substitution has been reported in DNA from nonneoplastic tissue of patients with sarcoma\(^3\),\(^2\) and a patient with hepatocellular carcinoma.\(^3\)

It was proposed that this polymorphism is a germline mutation causing proneness to cancer because the amino acid of codon 49 is relatively well conserved among species; an allele with a histidine at codon 49 is very rare in normal individuals, and the patient with this mutation had a family history of ovarian and breast cancers. The segregations of this allele in this family remain to be analyzed. Germ line configuration of p53 gene was found in normal cells from 3 of 5 cases with p53 mutations. Moreover, all the 5 cases did not have any family history of cancer. These findings suggest that p53 gene mutations found in t(1;19)-ALL cases are not associated with germ line mutations such as Li-Fraumeni syndrome.

Leukemic cells from case 21 at relapse and cell lines derived from the same case at diagnosis showed the same aberrations of p53 gene, whereas the leukemic cells from the same case at diagnosis had no mutations. This suggested that a small number of leukemic cells with p53 gene mutation at diagnosis might have escaped from PCR-SSCP analysis. Indeed, both the SSCP and direct sequencing technique fail to detect mutations that are present in less than 10% of the cell population.\(^3\) These results of case 21 are compatible with a report that a clonal expansion associated with mutated p53 was observed in clinical sequential studies of brain tumors in which a low percentage of the cells with p53 gene mutation were observed at diagnosis.\(^3\) Alternatively, mutations of the p53 gene in t(1;19)-ALL may have possibly occurred in the later stage of the tumorigenesis step and might have been involved in the tumor progression. The other possible explanation is that the cells with the mutated p53 gene may have dominated over the other cells lacking mutated p53 gene in the process of cell culture.

Cases 19 and 20 with p53 gene mutation at diagnosis did not obtain complete remission with intensive chemotherapy. These results may show that leukemic cells with p53 gene mutations even in the early stage are very resistant to chemotherapy, as are leukemic cells with these mutations at relapse.\(^2\) At the time of diagnosis both of the two patients with p53 alterations died whereas 1 of the 18 without the aberrations died; this is statistically significant (P < .02). Among 5 patients with p53 gene mutations in the course of disease, 4 died and 1 relapsed; 17 survivors lacking p53 aberrations were free from disease (P < .005) (Table 3).

These results may suggest that the presence of the p53 gene mutations is associated with a poor clinical outcome in t(1;19)-ALL. In this regard, p53-dependent apoptotic response has recently been shown to modulate the cytotoxicity of anticancer agents.\(^3\) Mutations of p53 gene could possibly induce drug resistance by interfering with normal apoptotic pathways in leukemic cells.\(^2\)

As ras gene mutations, the N-ras gene was mutated in only 1 of 22 t(1;19)-ALL cases and in 0 of 5 cell lines in this study, suggesting that ras gene mutation is infrequent in lymphoid malignancy, as indicated by previous reports.\(^3\),\(^5\),\(^6\) Case 2 with N-ras mutation, who has been alive for 73 months since diagnosis, had neither p53 gene mutation nor E2A gene rearrangement, and had hyperdiploid chromosome abnormality. Therefore, the N-ras mutation in addition to hyperdiploidy may be involved in the leukemogenesis of this case. Presence of an N-ras mutation in children with ALL has been reported to be associated with leukemic progression\(^5\),\(^3\) and possibly an independent predictor of worse clinical outcome.\(^6\),\(^5\) Our result is incompatible with these reports. Interestingly, ras gene mutations were less frequent than p53 gene mutations in relapsed cases and in cell lines in this study, suggesting that ras gene mutations may not be involved in the development and progression of t(1;19)-ALL. The 2-bp substitution of GGT to GTC at codon 13 identified in case 3 has not been reported so far in ALL.

We conclude that in t(1;19)-ALL mutations of the p53 and ras genes are infrequent at diagnosis and that p53 mutations may be associated with relapse phase or progression of t(1;19)-ALL.

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