Mutations of the p53 Gene in Lymphoid Leukemia

By Koichi Sugimoto, Hideo Toyoshima, Ryuichi Sakai, Kiyoshi Miyagawa, Koichii Hagiwara, Hisamaru Hirai, Fuyuki Ishikawa, and Fumimaro Takaku

p53 is currently considered to be a tumor suppressor gene product, and its alterations are suggested to be involved in several human malignancies. Here we show evidence of the possible involvement of p53 gene mutations in lymphoid leukemias studied by reverse transcriptase-polymerase chain reaction, single strand conformation polymorphism analysis, and nucleotide sequencing. Fourteen patients with various leukemias were examined and two with acute lymphoblastic leukemia and one with Waldenström's macroglobulinemia were identified to have mutations in the coding region of the p53 gene. These mutations included point mutation, triplet deletion, and single nucleotide insertion. Furthermore, expression of the wild-type p53 mRNA was not detected in the samples from these three patients. In one of them, chromosome 17p was deleted, suggesting the absence of the nonmutated p53 gene, whereas in the other two patients, chromosome 17p seemed to be intact by cytogenetic analysis. Our results suggest that alterations of the p53 gene may have a role in the genesis of some leukemias.

Rapid Communication

MATERIALS AND METHODS

Patients. Bone marrow samples from 14 patients, including six patients with acute myelogenous leukemia (AML), four with Ph'-negative ALL, three with Ph'-positive ALL, and one with Waldenström's macroglobulinemia, were collected after informed consent. The percentages of leukemic cells in the bone marrow samples were more than 80% in seven cases, 50% to 80% in two cases, and 20% to 50% in five cases.

RT-PCR method. Primers used in this study were prepared by the 381A DNA synthesizer (Applied Biosystems, Foster City, CA). Using the nucleotide numbers of the sequence published by Zakut-Houri et al,4 the sense primers were: ST1, nucleotide (nt) 361 to 380; SN2, nt 373 to 392; and SC3, nt 603 to 622. The antisense primers were: AST1, nt 1,000 to 981; ASN2, nt 777 to 758; and ASC3, nt 980 to 961. The RT-PCR was performed as follows. Complementary DNA was synthesized from 1 μg of total cellular RNA from bone marrow mononuclear cells using 100 ng of 3'-primer AST1 and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in a 25 μL solution containing 200 μmol/L each of all four dNTPs, 80 U of RNase inhibitor, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 10 mmol/L dithiothreitol (DTT), 3 mmol/L MgCl2. The reaction was allowed to proceed for 60 minutes at 37°C and used as substrate for the PCR. To the RT reaction solution, 25 μL of a solution containing 250 μmol/L each
of all four dNTPs, 100 ng of 5'-primer ST1, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 3 U of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) was added. PCR was performed for 25 cycles of 94°C (1 minute), 50°C (1 minute) and 72°C (2 minutes).

**SSCP analysis.** For the SSCP analysis, 5' and 3'-side p53 cDNA fragments were generated using 1 μL (one-fiftieth) of RT-PCR reaction solution by second PCR of 30 cycles (94°C 1 minute, 50°C 1 minute, and 72°C 2 minutes). The 5' ends of the primers used in the second PCR were labeled with [γ-32P] adenosine triphosphate (ATP) and T4 polynucleotide kinase (Takara, Kyoto, Japan). Primers SN2 (100 ng) and ASN2 (100 ng) were used to amplify the 5'-side fragment, and primers SC3 (100 ng) and ASC3 (100 ng) were for the 3'-side fragment. The reaction solution heated at 94°C for 3 minutes. The mixture was quickly chilled on ice and 2 μL was loaded onto 5% polyacrylamide gels containing 90 mmol/L Tris-borate pH 8.3, and 4 mmol/L EDTA with or without 10% glycerol. Electrophoresis was performed at room temperature and at 4°C.

**Sequencing.** For direct sequencing, 1 μL of RT-PCR reaction solution was used in 50-cycle second PCR with a 10- to 20-fold reduction of one of the primers. The resulting single-stranded DNA was purified and sequenced by the dideoxy chain termination method. Sequencing primers were SS4 for nt 622 to 641 (sense) and ASS4 for nt 962 to 943 (antisense). For conventional sequence analysis, the 5' and 3'-side p53 cDNA fragments were cloned and sequenced by the dideoxy chain termination method.

## RESULTS

**RT-PCR/SSCP analysis.** The positions of p53 gene mutations previously reported are clustered in four regions spanning about 650 bp of the p53 coding sequence that are highly conserved among wide species (regions A, B, C, and D in Fig 1). Therefore, the RT-PCR method was used to amplify the 650-bp coding sequence. Because SSCP analysis can detect mutations effectively in DNA fragments less than 400 bp, the 650-bp RT-PCR product was analyzed in two fragments (5'- and 3'-side fragments generated by the second PCR). The DNA fragment was denatured into separate single strands and each strand folded back on itself in a unique conformation during electrophoresis under nondenaturing conditions. Even a single nucleotide substitution can usually be detected, because it alters the three-dimensional conformation of the single-stranded DNA fragment and, therefore, its electrophoretic mobility.15

Figure 2 shows the SSCP analysis of 3'- and 5'-side p53 cDNA fragments performed at room temperature in the presence of 10% glycerol. Positive and negative control fragments are also included (P and N, respectively). One patient for the 3'-side (patient 1 in Fig 2A) and two patients for the 5'-side SSCP analysis (patients 2 and 3 in Fig 2B) can be identified to have aberrantly migrating fragments. All three samples lacked normally migrating fragments, suggesting that the leukemic cells of these patients did not express wild-type p53 mRNA. The SSCP analysis under all of the four conditions (at room temperature or at 4°C in the presence or absence of 10% glycerol) detected aberrantly migrating fragments in the three patients. We also performed the 5'- and 3'-side SSCP analysis on 32 healthy volunteers at room temperature in the presence of 10% glycerol and found no aberrantly migrating fragments (data not shown).

**Sequencing analysis.** The 3'-side p53 cDNA fragment of patient 1 was directly sequenced to determine the nucleotide change responsible for the mobility shift in SSCP analysis. If both wild-type and mutant p53 mRNAs are expressed, we can obtain sequence ladders of both wild-type and mutant p53 mRNA with different intensities depending on their relative amount. The analyzed sequence exactly matched the published one,16,17 except for a G to C transition at nt 826 (Fig 3, panel 2), which results in a change of the encoded amino acid from alanine to proline at codon 276. A faint band at nt 826 of the G lane in this panel may reflect the wild-type p53 mRNA from the residual normal cells, which accounted for 20% of the marrow nuclear cells. For patients 2 and 3, we cloned the 5'-side p53 cDNA fragment into a M13-derived vectors and sequenced four independent clones for each fragment to avoid random errors generated by the PCR. Patient 2 was shown to have a triplet deletion (CCT), which removes
proline at codon 190 (data not shown). In patient 3, a single nucleotide (T) insertion was identified between nucleotides 714 and 715, and the inserted T with the normal mononuclear cells of a volunteer (panel 1). Nucleotide sequencing of the p53 cDNA from patient 3 showed that a T nucleotide was inserted between nucleotides 714 and 715 (panel 4) compared with the corresponding region of the normal control p53 cDNA (panel 3).

**DISCUSSION**

We have identified three mutations of the p53 gene in two cases of Ph'-negative ALL (pre-B cell and null cell types) and one case of Waldenström's macroglobulinemia by RT-PCR/SSCP analysis and sequencing (Table 1). Furthermore, our results suggested the possibility of the absence of wild-type p53 mRNA expression in the leukemia cells of the three patients.

Because the p53 gene is now considered to be a tumor suppressor gene, it is important to show inactivation of the gene in both alleles to demonstrate that the p53 gene has a role in leukemogenesis. RT-PCR/SSCP analysis is useful for this purpose because it can separate mRNAs from both alleles unless they are identical. However, if there is far less mutant p53 mRNA than that of wild type in the bone marrow specimen, the SSCP analysis cannot detect the aberrantly migrating fragments. We chose 14 cases containing leukemic cells more than 20% of bone marrow cells, because PCR-SSCP method was reported to detect point mutations of about 10% of total DNA.\(^{18}\) To optimize the detection of the p53 gene mutation, we performed electrophoresis at room temperature or at 4° C in the presence or absence of 10% glycerol. The SSCP analysis detected aberrantly migrating fragments in the three patients under all four conditions. Mobility shifts of mutant p53 cDNA fragments from the patients and positive controls were best resolved when electrophoresis was performed at room temperature in the presence of 10% glycerol.

Because the effect of sequence alterations on electrophoretic mobility is unpredictable, it is true that some of the sequence mutations may not appreciably affect the mobility. However, Orita et al reported that single base changes can be detected as mobility shift with SSCP analysis in all 12 arbitrary chosen tumor cell lines that are known to contain mutated H-ras, K-ras, or N-ras.\(^{15}\) We observed normal migration of the p53 gene in the SSCP analysis on the 32 normal samples. Moreover, we sequenced 10 normally migrating fragments and confirmed that they contain no mutations. These findings suggest that the SSCP analysis has relatively high sensitivity and, therefore, usefulness for detecting mutations.

The mutations of patients 1 and 3 are mapped in the four regions highly conserved among five species from *Xenopus laevis* to human (regions D and C, respectively, in Fig 1). Although the triplet deletion at codon 190 of patient 2 is out of these regions, the amino acid sequences between codons 189 and 200 are also highly conserved.\(^{17}\) Therefore, the mutations of all the three cases may affect the normal function of the p53 protein.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Blast (%)</th>
<th>Karyotype</th>
<th>Codon</th>
<th>Nucleotide</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre-B cell ALL</td>
<td>80</td>
<td>Normal</td>
<td>276</td>
<td>GCC → CCC</td>
<td>Ala → Pro</td>
</tr>
<tr>
<td>2</td>
<td>Null-cell ALL</td>
<td>99</td>
<td>[46xy, 6q−, 17p−]</td>
<td>190</td>
<td>Deleted CCT</td>
<td>Deleted Pro</td>
</tr>
<tr>
<td>3</td>
<td>Waldenström's macroglobulinemia</td>
<td>90</td>
<td>[46xy, −1, −6, −16, 3p−]</td>
<td>239</td>
<td>Inserted a T</td>
<td>Asn → Stop</td>
</tr>
</tbody>
</table>
the p53 gene expression. In any case, our results suggest that leukemic cells of the three patients lacked the wild-type p53 mRNA expression.

Both activation of oncogene(s) and inactivation of tumor suppressor gene(s) are recently considered to be necessary for tumorigenesis in some human malignancies. Alterations of the p53 gene seem to act in concert with the activated abl protein, a bcr-abl fusion product, in blast crisis of CML. In the case of patient 2, we detected K-ras gene activation at codon 12 (unpublished result), which may suggest that the inactivated p53 gene may cooperate with the activated K-ras gene in the leukemogenesis of this patient.

If both of the p53 alleles are transcriptionally inactivated in leukemic cells, then the RT-PCR reaction may amplify wild-type p53 mRNA from the residual normal cells in the bone marrow samples. Some of the cases showing the normally migrating fragments in the SSCP analysis may, therefore, have had leukemic cells that entirely lacked the p53 gene expression. Our results suggest that loss of the normal p53 function may be important in the genesis of some human leukemias. More extended study of the p53 gene mutation will give an important insight into the mechanism of human leukemogenesis.

ACKNOWLEDGMENT

We thank Dr A. Aoyagi of Yokosuka Mutual Aid Hospital, Dr Y. Miura of the Department of Hematology, Jichi Medical School, and Dr A. Fujita of Showa Hospital for providing samples from patients, and Dr K. Hayashi of National Cancer Center Research Institute for his kind advice.

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