Frequent Mutations in the p53 Gene in Human Myeloid Leukemia Cell Lines

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

The p53 gene is currently considered to function as a tumor-suppressor gene in various human malignancies. In hematologic malignancies, alterations in the p53 gene have been shown in some human leukemias and lymphomas. Although mutations in the p53 gene are infrequent in acute myelogenous leukemia (AML) patients, we show in this report that alterations in the p53 gene are frequent in myeloid leukemia cell lines. We studied alterations of the p53 gene in nine human myeloid leukemia cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR), single-strand conformation polymorphism (SSCP) analysis, and direct sequencing. Expression of the p53 gene was not detected at all by RT-PCR in two of the nine cell lines. In these two cell lines, Southern blot analysis showed gross rearrangements and deletions in both of the p53 alleles. Six of the nine cell lines were found to express only mutant p53 mRNA by RT-PCR/SSCP analysis and direct sequencing, and wild-type p53 mRNA was not detected. Two of the mutant p53 mRNAs were shown to be products of abnormal splicing events induced by intronic point mutations. Taken together, eight of nine human myeloid leukemia cell lines expressed no or an undetectable amount of wild-type p53 mRNA. Three of the eight cell lines were growth factor-dependent. Our results suggest that inactivation of the p53 gene may be a common feature in myeloid leukemia cell lines and may play an important role in the establishment of these cell lines.

S EVERAL LINES of research have shown that the p53 gene is a tumor-suppressor gene. Transfection of wild-type p53 cDNA was found to inhibit the ability of an adenovirus E1A or mutant p53 gene plus an activated ras oncogene to transform primary rat embryo fibroblasts. Inactivation of both alleles of the p53 gene through deletion, insertion, or point mutation was shown in various types of human malignancies. As for hematologic malignancies, several groups have observed alterations in the structure and expression of the p53 gene in blast crisis of chronic myelogenous leukemia (CML). Mutations of the p53 gene were shown to be associated with Burkitt’s lymphoma and B-cell chronic lymphocytic leukemia. We reported two cases of acute lymphoblastic leukemia (ALL) and one case of Waldenström’s macroglobulinemia that had mutations in the coding region of the p53 gene. Expression of the wild-type p53 mRNA was not detected in these three cases. Slingerland et al showed mutations of both p53 alleles in one of five AML patients. Fenaux et al found mutations of the p53 gene in four of 10 patients with AML and 17p monosomy, and only one of 36 AML patients without cytogenetic abnormalities of 17p. Furthermore, five of 10 human T-cell leukemia cell lines and 10 of 12 Burkitt’s lymphoma cell lines were found to possess mutations in the coding region of the p53 gene. Koeffler et al found negligible amounts of p53 mRNA in a variety of human myeloid leukemia cell lines. Although gross alteration in the p53 gene was not detected by Southern blot analysis in these cell lines except for HL-60, this observation suggested that small alterations in the p53 gene may exist in human myeloid leukemia cell lines. These findings motivated us to examine for possible mutations of the p53 gene in nine human myeloid leukemia cell lines using the reverse transcriptase-polymerase chain reaction (RT-PCR) method and single-strand conformation polymorphism (SSCP) analysis. The RT-PCR/SSCP analysis has the ability to detect even point mutations in the coding region of the p53 gene. The analysis separates wild-type and mutant p53 cDNA fragments in the gel, and can reveal whether or not the wild-type p53 gene is expressed. Two of the nine human myeloid leukemia cell lines were found to express no p53 mRNA by the RT-PCR method, and six of the nine cell lines were found to express only mutant p53 mRNA by RT-PCR/SSCP analysis.

MATERIALS AND METHODS

Cell lines. Nine human myeloid leukemia cell lines used in this study were CMK (megakaryocyte), ML-1 (myeloid), THP-1 (monocytoid), U937 (monocytoid), UT-7 (megakaryocyte), TF-1 (erythroid), KG-1 (myeloid), KHY821 (myeloid), and PL-21 (myeloid). The cells were grown in suspension culture in RPMI medium supplemented with 10% fetal calf serum, CMK, UT-7, and TF-1 are factor-dependent cell lines and needed addition of 3 µg/mL recombinant human granulocyte-monocyte colony-stimulating factor (rhGM-CSF).

RT-PCR/SSCP analysis. The RT-PCR/SSCP analysis was performed essentially as previously described. Using the nucleotide numbers of the p53 coding sequence published by Zakut-Houri et al., 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 1000 to 981; ASN2, nt 777 to 758; and ASC3, nt 980 to 961. Complementary DNA was synthesized from 1 µg of total RNA from cell lines using 100 ng of 3-primer AST1 and 200 U of M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in a 25-µL solution containing 200 µmol/L each of all four dNTPs, 80 µU 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. The reaction solution, 25 µL of a solution containing 250 µmol/L each of all four dNTPs, 100 ng of

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Fig 1. (A and B) Agarose gel electrophoresis of 5'- and 3'-side p53 cDNA fragments, respectively. In (A) and (B), lanes: N, peripheral mononuclear cells of a volunteer (normal control); 1, CMK; 2, ML-1; 3, THP-1; 4, U937; 5, UT-7; 6, TF-1; 7, KG-1; 8, KY821; 9, PL-21.

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) were added. PCR was performed for 25 cycles of 94°C (1 minute), 50°C (1 minute), and 72°C (2 minutes). The 650 bp of p53 coding sequence generated by the RT-PCR was used as the template in the second PCRs in which 5' and 3' halves of the RT-PCR product were amplified separately. For the SSCP analysis, 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 used for the 3'-side fragment.

Sequencing of genomic DNA. To detect intronic mutations causing abnormal splicing events, the implicated p53 genomic DNA fragments were amplified by PCRs from the cultured cell DNAs. Using the nucleotide numbers of the sequence published by Zakut-Houri et al,27 the sense primers were SGI-5, nt 478 to 497; and SGI-6, nt 594 to 613. The antisense primers were ASG1-5, nt 622 to 603; and ASG1-6, nt 729 to 710. SGI-5 and SGI-6 were used to amplify the implicated p53 genomic DNA from U937, and SGI-6 and ASG1-6 were used for KG-1. The amplified DNA fragments were cloned and sequenced by the dideoxy chain termination method.

Southern blot analysis. DNAs extracted from cultured cells were digested with EcoRI, BamHI or HindIII, separated by electrophoresis on 0.8% agarose gels, and transferred to the nylon filters. The hybridization probe encompassed the entire p53 coding sequence.

RESULTS

RT-PCR and Southern blot analysis. As the previously reported p53 gene mutations are clustered in four regions spanning approximately 650 bp of the p53 coding sequence, we amplified the 650-bp coding sequence by the RT-PCR method. Since SSCP analysis can detect mutations most effectively in DNA fragments less than 400 bp, 5'- and 3'-side p53 cDNA fragments were generated from the 650-bp RT-PCR product by respective second PCR reactions. Before SSCP analysis, the 5'- and 3'-side p53 cDNA fragments were examined by electrophoresis in 2.5% agarose gels. Neither 5'- nor 3'-side p53 cDNA fragment was detected in CMK and ML-1 (Fig 1). We then performed RT-PCR with ST1 and ASN2 for the 5'-side fragment and with SC3 and AST1 for the 3'-side fragment. The second PCR reaction solution was mixed with 20 μL of 95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, and heated at 94°C for 3 minutes. The mixture was quickly chilled on ice and 2 μL was electrophoresed on glycerol containing nondenaturating gel at room temperature and on glycerol-free nondenaturating gel at 4°C.

Direct 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 SN2 and ASN2 for the 5'-side p53 cDNA fragments and SC3 and ASC3 for the 3'-side p53 cDNA fragments.

DNAs extracted from cultured cells were digested with EcoRI, BamHI or HindIII, separated by electrophoresis on 0.8% agarose gels, and transferred to the nylon filters. The hybridization probe encompassed the entire p53 coding sequence.
Southern blot analysis was performed on CMK and ML-1 to detect genetic alterations that might be responsible for the total loss of p53 mRNA expression. Southern blots of EcoRI-, BamHI-, and HindIII-digested DNA hybridized with a probe encompassing the entire p53 coding sequence are shown in Fig 2 A, B, and C, respectively. The normal bands were lacking and abnormal bands were detected in the cell lines with these three restriction enzymes, suggesting gross structural alterations and loss of both normal alleles of the p53 gene in these cell lines.

**SSCP analysis.** RT-PCR/SSCP analysis was performed on the seven cell lines from which detectable 5'- and 3'-side p53 cDNA fragments were generated by the second PCRs. In the SSCP analysis, the DNA fragment is denatured into separate single strands and each strand folded back on itself in a unique conformation during electrophoresis under nondenaturing conditions. Under these 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. \(^\text{17}\) Figures 3A and B show the 5'- and 3'-side SSCP analysis, respectively, on the seven cell lines at room temperature in the presence of 10% glycerol. The 5'-side p53 cDNA fragments from THP-1, U937, KG-1, and KY821 showed mobility shift in the analysis. Although mobility shift of the 5'-side p53 cDNA fragments from TF-1 was indistinct in the SSCP analysis at room temperature in the presence of glycerol, the mobility shift could be clearly detected in the SSCP analysis at 4°C in the absence of glycerol (data not shown). UT-7, TF-1, and KG-1 were found to have aberrantly migrating fragments in the 3'-side SSCP analysis. Mobility shift of the 3'-side p53 cDNA fragment from UT-7 was so large that the fragment could not be included in Figure 3B. Normally migrating fragments were not detected by the SSCP analysis in all the cases having aberrantly migrating fragments. As a whole, six of the seven cell lines were positive for the 5'- or 3'-side SSCP analysis of the p53 cDNA (Table 1), and these cell lines lacked wild-type p53 cDNA.

**Direct sequencing.** The p53 cDNA fragments positive in the SSCP analysis were directly sequenced to determine the nucleotide(s) change responsible for the mobility shift. If both wild-type and mutant p53 mRNAs are expressed, we can obtain sequence ladders of both wild-type and mutant p53 cDNAs with different intensities depending on their relative amounts. Figure 3 shows examples of the direct sequencing. THP-1 has a 26-base deletion from the first letter of codon 174 of the p53 coding sequence, and sequence ladder of the wild-type p53 cDNA cannot be detected (Fig 4, panel 2). Sequence ladder from KG-1 shows a 5-base insertion between codons 224 and 225 of the p53 coding sequence with no wild-type p53 cDNA sequence detected (Fig 4, panel 4). As a whole, direct sequencing identified four deletions, one insertion, and one point mutation of the p53 cDNA in six myeloid leukemia cell lines (Table 1), and wild-type p53 coding sequence was not detected in any of the six cell lines. These findings were confirmed by two additional independent RT-PCR analyses followed by direct sequencing.

**Sequencing of genomic DNA.** The mutant p53 mRNA of U937 had a 46-base deletion ending exactly at the 3' end of exon 5. A 5-base insertion in the coding sequence of KG-1 was identified between exon 6 and exon 7. Since these p53 mRNA mutants are highly suggestive of abnormal splicing events, we amplified and sequenced the implicated p53 genomic DNA in U937 and KG-1. Sequencing of the implicated p53 genomic DNA in U937 showed a point

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RT-PCR Product 5'-side</th>
<th>RT-PCR Product 3'-side</th>
<th>SSCP Analysis of p53 cDNA 5'-side</th>
<th>SSCP Analysis of p53 cDNA 3'-side</th>
<th>Alteration of p53 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMK</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Not examined</td>
<td>Not examined</td>
<td>No expression of p53 mRNA</td>
</tr>
<tr>
<td>ML-1</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Not examined</td>
<td>Not examined</td>
<td>No expression of p53 mRNA</td>
</tr>
<tr>
<td>THP-1</td>
<td>Smaller size</td>
<td>Normal</td>
<td>Positive</td>
<td>Negative</td>
<td>Deleted 26 bases from the 1st letter of codon 174</td>
</tr>
<tr>
<td>U937</td>
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<td>Positive</td>
<td>Negative</td>
<td>Deleted 46 bases from the 1st letter of codon 172</td>
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<tr>
<td>UT7</td>
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<td>Positive</td>
<td>Deleted 137 bases from the 3rd letter of codon 261</td>
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<tr>
<td>TF-1</td>
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<td>Positive</td>
<td>Positive</td>
<td>Deleted a T at codon 251</td>
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<tr>
<td>KG-1</td>
<td>Normal</td>
<td>Normal</td>
<td>Positive</td>
<td>Positive</td>
<td>Inserted 5 bases (ATCTG) between codons 224 and 225</td>
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<tr>
<td>KY821</td>
<td>Normal</td>
<td>Normal</td>
<td>Positive</td>
<td>Negative</td>
<td>CGC[Arg] (\rightarrow) CAC[His]</td>
</tr>
<tr>
<td>PL-21</td>
<td>Normal</td>
<td>Normal</td>
<td>Negative</td>
<td>Negative</td>
<td>None detected</td>
</tr>
</tbody>
</table>

Table 1. Alterations of p53 mRNA in Human Myeloid Leukemia Cell Lines
p53 GENE IN MYELOID LEUKEMIA CELL LINES

ACCT

a-

-5

'1

1 2

3

4

Fig 4. Direct sequencing of mutant p53 cDNAs. Panel 1 shows the p53 cDNA sequence of a normal control, with the square bracket indicating the 26 bases that are deleted starting at the first letter of codon 174 in THP-1 (arrowhead, panel 2). Panel 3 shows the p53 cDNA sequence of a normal control of the region corresponding to panel 4, which shows a 5-base insertion between codons 224 and 225 (square bracket with a bold side, panel 4).

mutation that converts G into A in the splice donor site at the first base of intron 5 (Fig 5A). The point mutation found in KG-1 converts G into A in the splice donor site at the first base of the intron 6 (Fig 5B). No normal sequence was detected in the relevant regions of the p53 gene in U937 and KG-1, indicating that the mutations are hemizygous or homozygous in these cell lines.

DISCUSSION

We have analyzed nine human myeloid leukemia cell lines for alterations in structure and expression of p53 mRNA by the RT-PCR/SSCP analysis. No p53 mRNA could be detected by the RT-PCR method in two cell lines, CMK and ML-1. Southern blot analysis showed gross rearrangements and loss of normal alleles of the p53 gene in the two cell lines. As previously reported for HL-60, the loss of p53 expression in these cell lines seems to be induced by major deletions and rearrangements in the p53 genomic structure. Six cell lines were found to express aberrant p53 transcripts having deletions, insertion, or point mutation. Wild-type p53 mRNA expression could not be detected in these six cell lines by either RT-PCR/SSCP analysis or direct sequencing. The p53 mRNA from THP-1, U937, and UT-7 had partial deletions of 26, 46, and 136 bases, respectively. The structural alterations of p53 mRNA in the other three lines were a point mutation, 5-base insertion, and 1-base deletion. Except for the point mutation, which results in an amino acid substitution in the highly conserved region, the other five mutations cause frame shift of the p53 coding sequence. These six mutations of the p53 coding sequence may affect the normal function of p53 protein.

Deletions and an insertion in the mutant p53 mRNAs in U937, UT-7, and KG-1 are highly suggestive of abnormal splicing events. To confirm this expectation experimentally, we sequenced the implicated regions of the p53 genomic DNA in U937 and KG-1. The p53 genomic DNAs in U937 and KG-1 had point mutations that convert G into A at the first base of intron 5 and 6, respectively. A CML blast crisis cell line, JOSK-M, was reported to have the same splice junction mutation in the p53 gene as U937. These intronic point mutations appear to inactivate the normal splicing junction and alter the p53 mRNA processing. Takahashi et al reported that two lung cancer cell lines had intronic point mutations in the splicing donor or acceptor site of the p53 gene. Furthermore, they found three other splicing mutations among 36 completely characterized p53 mutations in lung cancer (5/36, 14%). Although previously reported p53 gene mutations are clustered in the four highly conserved regions of the p53 coding sequence, our results and those of Takahashi et al suggest that splicing mutation may also be an important mechanism to inactivate normal p53 gene expression.

Including HL-60, our result suggests that nine of 10 myeloid leukemia cell lines lack the normal p53 transcript. Our previous study on six patients with AML detected no p53 gene mutations. Slingerland et al showed mutations of both p53 alleles in one of five AML patients. Although Fenaux et al found mutations of the p53 gene in four of 10 patients with AML and 17p monosomy, only one of 36 AML patients without cytogenetic abnormalities of 17p was shown to have a mutation in the p53 gene. These observations suggest that alterations of the p53 gene are more frequent in myeloid leukemia cell lines than in myeloid leukemia samples from patients. It remains to be known whether alterations of the p53 gene occurred during in vitro culture or were present in original myeloid leukemia cells, which might have the advantage in establishment of cell lines.

The cell lines analyzed in this study have several chromo-

Fig 5. Schematic diagrams explain the mechanisms of the partial deletion in the p53 mRNA of U937 (A) and the 5-base insertion in the p53 mRNA of KG-1 (B). Intronic point mutations that convert G into A at the first base of intron 5 and 6 in the respective p53 genomic DNAs of U937 and KG-1 (shown by the star marks) cause abnormal splicing events.
osomal changes and are expected to contain a high frequency of genetic mutations, some of which may not be related to the malignant process or establishment of the cell lines. We must take account of the possibility that the mutations in the p53 gene may be one of these nonessential changes caused by genetic instability. However, our results and the previous report on HL-60 suggest that nine of the ten myeloid leukemia cell lines have both p53 alleles inactivated through deletion, rearrangement, point mutation, and so on. We also analyzed 15 human lymphoid leukemia cell lines for mutations of the p53 gene by RT-PCR/SSCP analysis. Nine of 15 cell lines were positive for the SSCP analysis and all of the nine cell lines lacked normal p53 transcript (unpublished result). The high incidence of loss of the normal p53 expression in leukemia cell lines, which contrasts with the relatively low incidence in samples from leukemia patients, suggests that inactivation of the normal p53 function cannot render myeloid leukemia cells factor independent. Recently, Yonish-Rouach et al. reported that introduction of wild-type p53 into a murine leukemia cell line lacking p53 resulted in rapid loss of cell viability in a way characteristic of apoptosis (programmed cell death). The bcl-2 oncogene product, which has been shown to block the apoptosis independent of stimulating cell division, promotes the eventual immortalization of B-lineage cells constitutively expressing c-myc. If wild-type p53 is indeed involved in this apoptosis, then the loss of normal p53 function could cooperate with other genetic events to immortalize the affected clone in a manner similar to activation of bcl-2 oncogene. More extended study of the normal p53 function will give insight into the mechanism of establishment of human myeloid leukemia cell lines.

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