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.

© 1992 by The American Society of Hematology.
p53 GENE IN MYELOID LEUKEMIA CELL LINES

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.

RESULTS

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 STl and ASN2 for the 5' side fragment and with SC3 and ASTl for the 3' side fragment. The second PCR reaction solution was mixed with 20 vol 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 non-denaturating gel at room temperature and on glycerol-free non-denaturating 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 dyeoxy 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.

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 ASGI-5, nt 622 to 603; and ASGI-6, nt 729 to 710. SGI-5 and ASGI-5 were used to amplify the implicated p53 genomic DNA from U937, and SGI-6 and ASGI-6 were used for KG-1. The amplified DNA fragments were cloned and sequenced by the dyeoxy 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.

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 using these RT-PCR products generated no detectable fragments in the two cell lines, suggesting the complete lack of p53 gene expression in these lines (data not shown). The 5' and 3' side p53 cDNA fragments could be amplified in the other seven cell lines (Fig 1, A and B). The 5' side p53 cDNA fragments from THP-1 and U937 migrated at a smaller size. The 3' side p53 cDNA fragment of UT-7 moved much faster than the normal control.
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. 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 ladder from KG-1 was indistinct in the SSCP analysis at room temperature in the presence of 10% glycerol. The 3'-side p53 cDNA fragments from TF-1 was indistinct in the SSCP analysis at room temperature in the presence of 10% 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-PCRs 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 1. Alterations of p53 mRNA in Human Myeloid Leukemia Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RT-PCR Product</th>
<th>SSCP Analysis of p53 cDNA</th>
<th>Alteration of p53 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMK</td>
<td>Undetectable</td>
<td>Not examined</td>
<td>No expression of p53 mRNA</td>
</tr>
<tr>
<td>ML-1</td>
<td>Undetectable</td>
<td>Not examined</td>
<td>No expression of p53 mRNA</td>
</tr>
<tr>
<td>THP-1</td>
<td>Smaller size</td>
<td>Positive, Negative</td>
<td>Deleted 26 bases from the 1st letter of codon 174</td>
</tr>
<tr>
<td>U937</td>
<td>Smaller size</td>
<td>Positive, Negative</td>
<td>Deleted 46 bases from the 1st letter of codon 172</td>
</tr>
<tr>
<td>UT7</td>
<td>Normal, Smaller size</td>
<td>Negative, Positive</td>
<td>Deleted 137 bases from the 3rd letter of codon 261</td>
</tr>
<tr>
<td>TF-1</td>
<td>Normal</td>
<td>Positive, Positive</td>
<td>Deleted a T at codon 251</td>
</tr>
<tr>
<td>KG-1</td>
<td>Normal</td>
<td>Positive, Positive</td>
<td>Inserted 5 bases (ATCTG) between codons 224 and 225</td>
</tr>
<tr>
<td>KY821</td>
<td>Normal</td>
<td>Positive, Negative</td>
<td>CGC[Arg] → CAC[His]</td>
</tr>
<tr>
<td>PL-21</td>
<td>Normal</td>
<td>Negative, Negative</td>
<td>None detected</td>
</tr>
</tbody>
</table>
p53 GENE IN MYELOID LEUKEMIA 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 MG1. 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-
somatic 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 may play an important role in the establishment of human myeloid leukemia cell lines.

Three of the myeloid leukemia cell lines shown to lack the normal p53 expression in this study were GM-CSF-dependent. This result 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.

ACKNOWLEDGMENT

We thank Drs Y. Miura and T. Suda of the Department of Hematology, and Drs M. Saito and M. Obta of the Division of Hemopoiesis, Institute of Hematology, Jichi Medical School, for providing some of the leukemia cell lines, and Dr K. Takeshita for proofreading of the text. We also thank Dr N. Komatsu of the Department of Hematology, Jichi Medical School, and Dr T. Sato of the Department of Pediatrics, Chiba University, for providing UT-7 and CMK, respectively. We are grateful to Dr K. Hayashi of the National Cancer Center Research Institute for his kind advice.

REFERENCES

Frequent mutations in the p53 gene in human myeloid leukemia cell lines

K Sugimoto, H Toyoshima, R Sakai, K Miyagawa, K Hagiwara, F Ishikawa, F Takaku, Y Yazaki and H Hirai