RAPID COMMUNICATION

P53 Gene Mutations in Acute Myeloid Leukemia With 17p Monosomy

By Pierre Fenaux, Philippe Jonveaux, Isabelle Quiquandon, Jean Luc Laf, Jean Michel Pignon, Marie Henriette Louiseaux-Lefebvre, Francis Bauters, Roland Berger, and Jean Pierre Kerckaert

We looked for mutations of exons 5 to 8 of the P53 gene in 10 patients with acute myeloid leukemia (AML) and 17p monosomy, and 36 patients with AML and no cytogenetic abnormalities of 17p. DNA was analyzed by polymerase chain reaction, single-strand conformation polymorphism analysis, and nucleotide sequencing. Four of the 10 patients with 17p monosomy showed point mutation, single-nucleotide deletion, or insertion in exons 7 or 8. By contrast, only 1 of the 36 patients with AML and no cytogenetic abnormalities of 17p showed a mutation of the P53 gene in exons 5 to 8 (P < .01). These results suggest that alterations of the P53 gene may have a role in leukemogenesis in some cases of AML. The fact that P53 gene mutations occurred more often in patients with 17p monosomy seems to support the "recessive" model of tumor suppressive activity of the P53 gene rather than the "dominant" model, in which alteration of only one allele is sufficient for the development of malignancy.

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MATERIALS AND METHODS

Patients. Bone marrow samples of AML patients were collected at diagnosis after informed consent. Cases were classified according to French-American-British (FAB) criteria.

Cytogenetic analysis. Cytogenetic analysis was performed at diagnosis on bone marrow cells (except in patients 178 and 636, where it was performed on blood leukocytes) after a 24-hour culture without stimulation. Chromosomes were identified by RHG and GTH banding, and classified according to the International System for Cytogenetic Nomenclature.

Ten patients with 17p monosomy, along with 36 patients without cytogenetic abnormalities of 17p, were subjected to polymerase chain reaction (PCR)-SSCP analysis. The karyotype of one patient with 17p monosomy was not available.

PCR-SSCP analysis. Oligonucleotide primers were purchased from Genset (Paris, France). The names and nucleotide sequences of the primers used in this work are listed in Table 1. Two genomic regions were amplified: region 1, encompassing exons 5 and 6 and intron 5, and measuring 408 bp; region 2, encompassing exons 7 and 8 and intron 7, and measuring 610 bp. Because SSCP analysis seems to require fragments of less than 400 bp, region 2 was digested, after amplification and before SSCP analysis, by DraI enzyme, as a DraI restriction site is present in intron 7. This led to two fragments, region 2a and region 2b, each encompassing the corresponding exon, and measuring 392 and 218 bp, respectively.

Genomic DNAs (0.1 µg) were subjected to PCR in a 10-µL solution containing 200 µmol/L each of dATP, dGTP, dTTP, 0.1
was first digested by Tris-borate pH solution. Then mmol/L EDTA solution. For region 2, pL of the reaction mixture after amplification, 1 pL of the reaction mixture for region 1 was mixed with 19 pL of 0.1% sodium dodecyl sulfate (SDS) 20 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 U of Taq polymerase (Boehringer, Mannheim, Germany) in a thermocycler (Techne, Princeton, NJ). PCR was performed as follows: 2 minutes, followed by final elongation at 72°C for 7 minutes.

polymerase (Boehringer, Mannheim, Germany) in a thermocycler (Techne, Princeton, NJ). PCR was performed as follows: 94°C; 1128 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, followed by final elongation at 72°C for 7 minutes. After amplification, 1 μL of the reaction mixture for region 1 was mixed with 19 μL of 0.1% sodium dodecyl sulfate (SDS) 20 mmol/L EDTA, 0.05% xylene cyanol, heated at 80°C and applied (2 pL/lane) to a polyacrylamide gel containing 90 mmol/L Tris-borate pH 8.3, 4 mmol/L EDTA, and 10% glycerol. Electrophoresis was performed at 35 W for 5 to 6 hours at room temperature, with cooling using a fan.

Sequencing. For direct sequencing, asymmetric PCR using a 50 to 100 reduction of one of the primers was performed. The resulting single-stranded DNA was purified and sequenced by the dideoxychain termination method, using the sequenase kit (USB, Cleveland, OH) and analyzed on a polyacrylamide gel containing 7 mol/L urea.

Southern analysis. DNA samples from the 10 AML patients with 17p monosomy were also subjected to Southern analysis with conventional methods, after digestion with the four following restriction enzymes: EcoRI, BamHI, HindIII, BgII. The probe specific for the P53 gene, kindly provided by M. Oren, MD (Tel Aviv, Israel), was an XbaI XbaI 1.9-kb cDNA probe encompassing exons 2 to 11 and part of exon 1 of the P53 gene.

RESULTS

Patients with 17p monosomy. Hematologic, cytogenetic, PCR-SSCP, and sequence findings in the 10 patients with 17p monosomy are shown in Table 2.

Cytogenetic and hematologic findings at diagnosis. Six patients had loss of a whole chromosome 17, whereas the other four patients (nos. 178, 1209, 636, 1068) had loss of the short arm of chromosome 17 (Table 2). A cell population with two normal chromosomes 17 coexisted with the

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/ Age</th>
<th>Diagnosis</th>
<th>Karyotype</th>
<th>SSCP Analysis</th>
<th>Mutation in Exons 5 to 8</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>F/31</td>
<td>MAML</td>
<td>44,XX, -2, -6, -7, -8, -13, -16, -17, -18, +6mar</td>
<td>Region 2a abnormal</td>
<td>8 272 GTG → ATG</td>
<td>Val → Met</td>
</tr>
<tr>
<td>178</td>
<td>F/60</td>
<td>MAML</td>
<td>43,XX, -4, -5, -7, -17,t(12;7)</td>
<td>Region 2a abnormal</td>
<td>8 282 CGG → TGG</td>
<td>Arg → Trp</td>
</tr>
<tr>
<td>452</td>
<td>M/79</td>
<td>MAML</td>
<td>45,XX, Y, 11q+ (6 cells)</td>
<td>Region 2a abnormal</td>
<td>7 256 Inserted A between codons 255 and 256</td>
<td>Frameshift</td>
</tr>
<tr>
<td>1209</td>
<td>F/52</td>
<td>MAML</td>
<td>45,XX, -1, -5, -6, -12, -17, -18, +5mar, t(15;7)(q23;7), der(17)(t5;17)</td>
<td>Region 2a abnormal</td>
<td>8 264 Deleted C (CTA → TA)</td>
<td>Frameshift</td>
</tr>
<tr>
<td>614</td>
<td>M/6</td>
<td>MAML</td>
<td>49,XY, -5, -7, -18, -18, t(7q;7)(q35;7), del(11)(q14), t(16;7)</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td>636</td>
<td>M/69</td>
<td>MAML</td>
<td>45,XY, -3, -4, -5, -7, -12, -17, +11, +3mar, der(17)(t7;7)</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
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<tr>
<td>722</td>
<td>M/29</td>
<td>MAML</td>
<td>39,XY, -2, -4, -5, -7, -8, -12, -14, -15, -16, -17, +4mar, +r</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td>1060</td>
<td>F/67</td>
<td>MAML</td>
<td>46XX(8 cells)/37XX, -X, -3, -4, -5, -7, -9, -10, -12, -16, -17, -18, +20, der3, +4mar</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td>1068</td>
<td>M/68</td>
<td>MAML</td>
<td>46XX, t(17q)(15 cells)</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td>1128</td>
<td>M/61</td>
<td>MAML</td>
<td>43,XY, -7, -10, -14, -15, -16, -17, -19, -20, -20, -21, -22, +4del(4)(p11); del(8q), t12(7p)</td>
<td>Normal</td>
<td>None detected</td>
<td></td>
</tr>
</tbody>
</table>
 clone having chromosome 17 change in 2 patients (nos. 452, 1060).

Nine patients had de novo AML and one patient (no. 452) had AML secondary to prolonged chemotherapy. Except for patient 452, all patients had partial bone marrow blastic infiltration (32% to 57%), and dysplastic features involving all three myeloid series, suggesting possible evolution from a preceding phase of myelodysplastic syndrome and multilineage involvement. Eight of the patients were treated with intensive chemotherapy and two with low-dose cytosine arabinoside, but only two achieved complete remission, of 3 and 5 months duration.

**PCR-SSCP analysis.** Of the 10 patients, six had a normal PCR-SSCP pattern for regions 1, 2a, and 2b. The four other patients had an abnormal pattern, with aberrantly migrating fragment(s) for region 2a (patient 452), and region 2b (patients 20, 178, 1209). No patient had abnormal migrating fragments for region 1. Examples of normal and abnormal SSCP profiles are shown in Fig 1. In patients 20 and 178, the normally migrating fragment(s) were apparently still present, while they were not found in patient 1209. In patient 452, the normal fragment for region 2a was apparently absent after amplification of region 2 with primers IIa and IIas, and digestion of the amplified product with Dra I (Fig 1B). In this patient we also performed SSCP analysis of a shorter DNA fragment of 134 bp encompassing exon 7, amplified with primer II and the followed antisense primer (7as: 5'TGCAAGGGTGGAAGTGACTC3') situated immediately 3' of exon 7. SSCP analysis of this shorter fragment showed persistence of a faint normal band in addition to the abnormal bands (Fig 1C).

**Sequencing analysis.** Direct sequencing of the four samples with aberrantly migrating fragments showed point mutations in all cases, involving exons corresponding to the abnormally migrating region in SSCP analysis (Table 2, Fig 2). One mutation involved exon 7, and three involved exon 8. In patients 20 and 178, one base was substituted for another, resulting in a change of the encoded amino acid. In both patients, a faint band corresponding to the normal nucleotide was still present. In patient 1209, deletion of a nucleotide at codon 264 was seen. In patient 452, the sequence was as expected until the position of codon 256,
Fig 2. Examples of sequencing reactions showing P53 gene mutations. (A) Mutation at codon 282 in patient 178 (TGG, instead of the wild-type sequence CGG). (B) Insertion of an A nucleotide between codon 255 and 256 in patient 452, with every band 5' of the inserted nucleotide appearing as a doublet. (C) Deletion of a C nucleotide at codon 264 in patient 1209.

where the sequence ladder became unreadable due to every band appearing as a doublet. This result was compatible with the insertion of a single base pair between codons 255 and 256 (Fig 2). Therefore, in patients 452 and 1209 the mutation resulted in a frameshift in the reading sequence.

In the six patients with normal migrating fragments for regions 1, 2a, and 2b, sequencing of exons 5 to 8 failed to show any abnormalities (data not shown). Finally, Southern blot analysis showed no rearrangements in the 10 patients (data not shown).

Patients with normal 17p. The 36 cases of AML who had no cytogenetic abnormality of 17p consisted of 9 cases of M2, 11 of M4, 5 of M5a, 5 of M5c, 5 of M6, and 1 of M4 AML. Only 1 of the 36 patients had an abnormal SSCP pattern, for region 2b. This patient had M4 AML and the following karyotype: 45,XY,−7,12,+2,del5q,del13q(q24q27). Sequence analysis showed a AG to AT mutation at the splicing acceptor site preceding exon 8. Southern analysis was normal in this patient (data not shown). Therefore, P53 gene mutations were significantly more frequent in patients with 17p monosomy (4 of 10) than in patients with cytogenetically normal 17p regions (1 of 36) (corrected chi square: 7.68, P < .01).

DISCUSSION

In 10 AML patients, cytogenetic analysis showed loss of the short arm of one chromosome 17, due to complete loss
of the whole chromosome, isochromosome 17q formation, or unbalanced t(5;17) and t(7;17) translocation. From cytogenetic data, three patients clearly had 17p monosomy and therefore 17p13 monosomy. The remaining patients also very probably had 17p monosomy, although they had marker chromosomes, so that cytogenetic analysis could not completely rule out the presence of 17p chromosome material in those markers. Four of the 10 patients showed mutations in exons 7 or 8 of the remaining P53 gene. Mutations consisted of nucleotide substitution in two cases, leading to a change in the encoded amino acid. The remaining two patients had deletion (no. 1209) or insertion (no. 452) of one nucleotide, leading to a frameshift in the reading sequence. All four mutations would lead to an abnormal p53 protein. Although P53 protein was not studied, it is therefore probable that no normal P53 protein was synthetized by the abnormal clone in those patients.

Five highly conserved regions among species from *Xenopus laevis* to humans have been described in the P53 gene. Four of them are situated in exons 5, 7, and 8 of the gene, and are mutation "hotspots," where most reported mutations of p53 genes in solid tumors have been reported. All four mutations, in the present patients with 17p monosomy, were found in the two last conserved regions, situated in exons 7 and 8, respectively. We found no mutations in exon 5, where two mutations hotspots are situated, or in exon 6, where fewer mutations have been reported. Mutations in exons 1 to 4 and 9 to 11 of the P53 gene are rare. Therefore, although we cannot rule out the presence of P53 gene mutations in those exons, they are relatively improbable in the six patients without mutations in exons 5 to 8.

In patients 20 and 178, with nucleotide substitution, the normal nucleotide band was still present on the sequencing gel and SSCP analysis, although both patients had lost the other P53 allele. This result could probably be explained by the persistence of nonleukemic cells in the sample analyzed. In patient 452, who had a nucleotide insertion between codon 255 and 256, SSCP and sequence analysis suggested the persistence of a normal P53 allele in addition to the mutated one. This finding was in agreement with cytogenetic findings, which found mitoses with normal chromosomes 17 in addition to the mitoses with loss of one chromosome 17. Likewise, disappearance of the normal SSCP bands, in patient 1209, was consistent with cytogenetic findings showing 17p monosomy in all mitoses.

Orita et al. first showed that SSCP was able to detect most if not all single-nucleotide substitutions in RAS genes. Our preliminary findings had shown us the validity of SSCP analysis in detecting point mutations in exons 5 to 8 of the P53 gene: four known P53 mutations in those exons in three tumor cell lines (namely HOS, HUT 78, and CEM, the latter cell line having two mutations, one in exon 5 and one in exon 7) all lead to mobility shift variants in comparison with 30 normal controls. In addition, after sequence analysis, no mutations in exons 5 to 8 were found in 15 cases of hematologic malignancies with normal SSCP findings (including the six patients with 17p monosomy and normal SSCP presented here). By contrast, a single point mutation was found in each of the 13 cases with abnormal SSCP (including the five patients with P53 mutation reported in this study and in ref 26). Sugimoto et al. also recently applied SSCP to the P53 gene, although with a different approach, using SSCP on P53 cDNA, amplified after reverse transcription. The investigators could detect, with SSCP, P53 mutations in 3 of 14 patients with hematologic malignancies. Mutations in those three patients and the absence of mutations in other patients were confirmed by sequence analysis.

The high incidence of mutations of the P53 gene in the 10 AML patients with 17p monosomy contrasted with the low incidence in AML with normal 17p (1 of 36). SSCP analysis in those 36 patients detected only one case of mutation in exon 5 to 8, which was confirmed by sequence analysis. Sugimoto et al. found no mutation of the P53 gene in six AML patients and Slingerland et al. found point mutations of both P53 alleles in one of the five cases of AML they analyzed. This patient had no chromosome 17 abnormalities, whereas cytogenetic findings in the other four patients and in the six patients of Sugimoto et al. were not reported.

The P53 gene is currently believed to be a tumor suppressor gene, and deletion of both normal alleles or deletion of only one allele and mutation of the other allele are considered to play a role in the oncogenic process. However, the P53 gene abnormalities detected here were always associated with several chromosome changes, prominent myelodysplastic features, and poor response to treatment, suggesting advanced disease, probably resulting from a multistep process and the occurrence of several genetic events. In this setting, mutations of the P53 gene in malignant cells could have been one of the important genetic events leading to tumor progression. Alternatively, those tumors with markedly abnormal karyotypes could simply be more genetically unstable, and contain a high frequency of point mutations in some genes possibly not related to the tumor phenotype. From this viewpoint, P53 mutations could simply represent epiphenomena. Still, in this report, P53 mutations were found more frequently in patients with 17p monosomy than in patients with two apparently normal chromosome 17. This finding seems to fit with the "recessive" model of tumor suppressive activity of the P53 gene and may suggest a role for those mutations in the malignant process in those patients.

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REFERENCES


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