Mutations of the p53 Gene in Myelodysplastic Syndrome (MDS) and MDS-Derived Leukemia

By Koichi Sugimoto, Naoto Hirano, Hideo Toyoshima, Shigeru Chiba, Hiroyuki Mano, Fumimaro Takaku, Yoshio Yazaki, and Hisamaru Hirai

The p53 gene is currently thought to be a tumor suppressor gene, and its alterations have been suggested to be involved in the pathogenesis of several human malignancies, including some leukemias and lymphomas. We present here evidence for the possible involvement of p53 gene mutations in the myelodysplastic syndrome (MDS), although the incidence is relatively low. Forty-four patients with MDS and six patients with overt leukemias that developed from MDS were studied for p53 gene alterations using reverse transcriptase-polymerase chain reaction, single-strand conformation polymorphism analysis, and nucleotide sequencing. Three patients with MDS (2 RAEB and 1 RAEB-in-T) had missense point mutations in the conserved regions of the p53 coding sequence. Furthermore, expression of the wild-type p53 mRNA was not detected in these three patients. The probable absence of normal p53 function in these three cases studied here suggests that alterations in the p53 gene may occasionally play a role in MDS. These three MDS patients with p53 gene mutations and an MDS-derived erythroleukemia cell line that we had previously reported to carry a p53 gene mutation showed no N-ras gene mutations, suggesting heterogeneity in the oncogenic mechanism of MDS.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Patients. Bone marrow samples from 50 patients, 13 with refractory anemia (RA), 2 with RA with ringed sideroblasts (RARS), 13 with RA with excess blasts (RAEB), 9 with RA with excess blasts in transformation (RAEB in T), 7 with chronic myelomonocytic leukemia (CMMoL), and 6 with overt leukemia derived from MDS, were collected. Approval was obtained from the Institutional Review Board for these studies. Patients and volunteers were informed that their blood or bone marrow samples were obtained for research purposes, and that their privacy would be protected. Bone marrow mononuclear cells were isolated with density sedimentation. One MDS-derived human erythroleukemia cell line, TF-I, for which we have previously reported the results of RT-PCR/SSCP analysis and sequencing of the p53 gene, was also analyzed for N-ras gene activation.

RT-PCR/SSCP analysis of the p53 gene. The RT-PCR/SSCP analysis was performed basically as previously described. Four p53 cDNA fragments covering the whole p53 gene-coding region were amplified by nested PCRs (first RT-PCR and second PCR). The primers used in the reactions are listed in Table 1. Using the nucleotide number of the p53 sequence published by Zakut-Houri et al., the sense primers were: EN5-Out, nucleotide (nt) -49 to -30; EN5-In, nt -33 to -14; ST1, nt 361 to 380; SN2, nt 373 to 392; SC3, nt 603 to 622; EC5-Out, nt 856 to 875; and EC5-In, nt 926 to 945. The antisense primers were: EN3-Out, nt 497 to 478; EN3-In, nt 450 to 431; AST1, nt 1000 to 981; ASN2, nt 777 to 758; ASC3, nt 980 to 961; EC3-Out, nt 1276 to 1257; and EC3-In, nt 1202 to 1183. Total RNA was isolated by the single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction from bone marrow mononuclear cells. Complementary DNA was then synthesized from 1 μg of the total RNA using 100 ng of the antisense first RT-PCR primer 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), and 3 mmol/L MgCl₂. The reaction was allowed to proceed for 60 minutes at 37°C. To the RT reaction, 25 µL of a solution containing 250 pmol/L each of all four dNTPs, 3 mmol/L KCl, 50 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 3 mmol/L MgCl₂ was performed for 25 cycles of 94°C (1 minute), 50°C (1 minute), and 72°C (2 minutes). For the SSCP analysis, the 5'-ends of the second PCR primers were labeled with [γ-³²P]ATP and T4 polynucleotide kinase (Takara, Kyoto, Japan). The 5'-ends of the second PCR primers were labeled with [γ-³²P]ATP and T4 polynucleotide kinase (Takara, Kyoto, Japan). The second PCR of 30 cycles (94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes) was performed using 100 ng each of the labeled primers and the p53 cDNA fragment generated by the first RT-PCR (1 µL of the first RT-PCR solution). The second PCR 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 a glycerol-containing nondenaturing gel at room temperature and on glycerol-free nondenaturing gel at 4°C.

Sequence analysis of the p53 gene. The p53 cDNA fragments showing aberrant electrophoretic mobility in the SSCP analysis were cloned and sequenced by the dideoxy chain termination method.

RT-PCR/direct sequencing of N-ras gene. The RT-PCR was performed as described above for the p53 cDNA fragments, except for the primers. Using the nucleotide numbers of the N-ras cDNA sequence published by Hall and Brown, the sense primers were: RO1, nt -34 to -15; and R12, nt -14 to 6. The antisense primers were: AR1, nt 253 to 244; and AR12, nt 243 to 224. N-ras complementary DNA was synthesized from 1 µg of total RNA using 100 ng of 3' primer AR01 and 200 U of M-MLV reverse transcriptase. The RT reaction solution with 100 ng of 5'-primer RO1 and 3 U of Taq polymerase was subjected to 25 cycles of PCR. For direct sequencing, a 500-fold dilution of RT-PCR reaction solution was used in a second 25-cycle PCR with 10 ng of 5'-primer R12 and 100 ng of 3'-primer AR12. The resulting single-stranded DNA fragment was purified and sequenced by the dideoxy chain termination method. The end-labeled 5'-primer R12 was used as the sequencing primer.

RESULTS

RT-PCR/SSCP analysis of the p53 gene. In this study, we performed RT-PCR/SSCP analysis on four p53 cDNA fragments (Extra 5'-, 3'-, 3'-side, and Extra 3'-side) that cover the entire p53 coding sequence. To make sure of the specificity of these PCR fragments, they were generated by nested PCRs (first RT-PCR and second PCR). The resultant DNA fragments were denatured into separate single strands, and each strand assumed 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.

Table 1. Primers Used for RT-PCRs

<table>
<thead>
<tr>
<th>Amplified Fragment</th>
<th>First RT-PCR</th>
<th>Second PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primer</td>
<td>Primer</td>
</tr>
<tr>
<td>Extra 5'-side p53 cDNA</td>
<td>EN5-Out</td>
<td>EN5-In</td>
</tr>
<tr>
<td>5'-side p53 cDNA</td>
<td>EN3-Out</td>
<td>EN3-In</td>
</tr>
<tr>
<td>3'-side p53 cDNA</td>
<td>ST1</td>
<td>AST1</td>
</tr>
<tr>
<td>Extra 3'-side p53 cDNA</td>
<td>EC5-Out</td>
<td>EC5-In</td>
</tr>
</tbody>
</table>

Three of the 50 patients were identified to have aberrantly migrating fragments in the 5'-side SSCP analysis performed at room temperature in the presence of 10% glycerol (lanes 1, 2, and 3 in Fig 1). These three patients were designated patients 1, 2, and 3, respectively. The lanes of these three patients lacked normally migrating fragments. In lanes 1 and 2, three fragments could be detected. Because more than one metastable conformation of a single strand is sometimes allowed in the SSCP analysis, we suppose that two of the three bands are different conformers of the same sequence. Only these three patients had aberrantly migrating 5'-side SSCP fragments also at 4°C in the absence of 10% glycerol. None of the Extra 5', 3', and Extra 3'-side p53 cDNA fragments from the 50 patients showed abnormal electrophoretic mobility either at room temperature in the presence of 10%
Fig 3. Example of mutant p53 cDNA sequencing. The p53 coding sequence of patient 1 contains a point mutation at codon 175 (CAC, panel 2) instead of the wild-type sequence (CGC) found in the p53 cDNA of a normal control (panel 1). Nucleotide sequencing of the p53 cDNA from patient 3 shows a point mutation at codon 190 (CTT, panel 4) instead of the corresponding sequence (CCT) of the wild-type p53 cDNA (panel 3).

Glycerol or at 4°C in the absence of 10% glycerol (Fig 2). We furthermore performed the 5'- and 3'-side SSCP analysis on 40 healthy volunteers at room temperature in the presence of 10% glycerol and found no aberrantly migrating fragments (data not shown).

Sequence analysis of the p53 gene. For patients 1, 2, and 3, we cloned the 5'-side p53 cDNA fragments into M13-derived vectors and sequenced four independent clones for each fragment to avoid random errors generated by the PCR. Patient 1 was found to have a missense point mutation at nt 524 (G to A), which results in a change of the encoded amino acid at codon 175 from arginine to histidine (Fig 3, panel 2). In patient 2, a missense point mutation at nt 410 (T to C) was identified, which generates an amino acid substitution at codon 137 from leucine to proline. Patient 3 had a missense point mutation at nt 569 (C to T) with a predicted amino acid substitution at codon 190 from leucine to proline (Fig 3, panel 4). The 5'-side p53 cDNA fragments of the three patients were amplified in two additional independent RT-PCRs and a total of 12 clones of the fragment was sequenced for each patient. In patients 1, 2, and 3, all of the 12 clones had the same mutation as mentioned above. We sequenced normally migrating Extra 5', 5', 3', and Extra 3'-side SSCP fragments from eight other patients (2 with RA, 2 with RAEB, 2 with RAEB in T, 1 with CMMoL, and 1 with overt leukemia from MDS) and confirmed that they contain no p53 mutations (data not shown).

RT-PCR/direct sequencing of N-ras gene. The three patients and the TF-1 cell line with a p53 gene mutation were studied for alterations of the N-ras gene using RT-PCR and direct sequencing. Direct sequence analysis showed the wild-type N-ras cDNA sequence in all four cases (data not shown).

DISCUSSION

Using RT-PCR/SSCP analysis and sequencing, we have identified mutations of the p53 gene in two patients with RAEB and one patient with RAEB in T. Taking into account that we analyzed 44 patients with MDS and six patients with overt leukemias converted from MDS, the incidence of p53 gene mutations in MDS and MDS-derived leukemias is low. It appears that mutations in the p53 gene occur preferentially in more advanced stages of MDS, although the sample numbers here are limited and the difference is not statistically significant. During this study, patient 1 died of infection and patient 2 developed overt leukemia. Patient 3 died of hemorrhage a few months after diagnosis. Table 2 summarizes the diagnosis, karyotype, and p53 gene mutation in these three patients and one MDS-derived erythroleukemia cell line that we had previously reported to have a p53 gene mutation.18

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 al16 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. We previously reported that 10 normally migrating SSCP fragments had no mutations.7 In this study,
we sequenced the entire p53 coding region from eight MDS patients negative for the SSCP analyses and confirmed that they contained no mutations. Moreover, we observed normal migration of the 5'- and 3'-side SSCP fragments on healthy volunteers. These results suggest that the SSCP analysis has relatively high sensitivity and, therefore, usefulness for detecting mutations. When both of the p53 alleles are transcriptionally inactivated in abnormal cells, the RT-PCR will amplify wild-type p53 mRNA from the residual normal cells in the bone marrow samples and the RT-PCR/SSCP analysis cannot detect the p533 gene alteration. Furthermore, if there is far less mutant p53 mRNA than wild-type mRNA in the bone marrow specimen, the SSCP analysis cannot detect the aberrantly migrating fragments. PCR-SSCP analysis was reported to detect a point mutation if more than 10% of the amplified sequence contained the mutation.23 MDS is considered to be a stem cell disorder involving several cell lineages, and morphologic evaluation suggests that not only blast cells but also a considerable part of the other cells in the patient's bone marrow may be of abnormal clone origin.24 We think, therefore, that most cases analyzed in the present study were free from this problem of normal cell contamination.

Although we performed RT-PCR/SSCP analysis on the entire p53 coding sequence in this study, the detected mutations were all missense point mutations located within or near the four regions highly conserved during evolution.6,25 The G to A transition at codon 175 presented in patient 1 is one of the most frequent mutations of the p53 gene.25 This transition, which occurs at a CpG dinucleotide, a well-known hot spot for spontaneous mutations, was previously found in many cases of colon, breast, and brain tumors.6,25 The same mutation was also detected in human leukemia and lymphoma cell lines, KY821,18 CEM,26 and CW678.8 To our knowledge, the transition at codon 137 in patient 2 is a novel mutation of the p53 gene. A different mutation at the codon was reported in a case of sarcoma.25 Although a missense mutation at codon 190 in patient 3 is novel, we have previously reported a three-base deletion at this codon.7 The MDS-derived leukemia cell line had a deletion of a T nucleotide, causing a frame shift in the p53 coding sequence.18 Therefore, these four mutations may affect the normal function of the p53 protein product. In these patients and the cell line, the RT-PCR/SSCP analysis detected no normally migrating fragments. In each of the three patients, all 12 clones of the 5'-side p53 cDNA fragment contained the identical mutation. These results suggest that only mutant p53 mRNA was expressed in the bone marrow mononuclear cells of the patients and the MDS-derived erythropoietic leukemia cell line. Furthermore, loss of chromosome 17p3 coupled with the p53 gene mutation in patient 3 supports this probability. Although the incidence of p53 gene mutations seems low in MDS patients, the probable inactivation of both p53 alleles in the four examples suggests that alterations in the p53 gene may play a role in a small fraction of cases of MDS.

All the p53 gene mutations detected here were in association with several chromosomal abnormalities. The five MDS patients with mutations in the p53 gene reported by Jonveaux et al21 also showed variable cytogenetic abnormalities. Recently emerging evidence suggests that normal p53 monitors the integrity of the genome and, if DNA is damaged, switches off replication.28,29 On the contrary, tumor cells without normal p53 cannot carry out this arrest. Therefore, they are genetically less stable and accumulate chromosomal abnormality. This new view suggests that the mutations in the p53 gene may not be a mere result of multiple chromosomal changes, but instead are involved in the genomic instability and progression of some part of MDS.

Both activation of oncogene(s) and inactivation of tumor suppressor gene(s) are considered to be necessary for tumorigenesis in some human malignancies. In a genetic model for colorectal cancer, inactivation of the p53 gene is thought to be one of multiple steps to the fully tumorigenic phenotype.30 Although MDS is a syndrome that comprises heterogeneous disorders, at least some cases of MDS are considered to be clonal diseases caused by genetic changes. We previously reported the significance of mutations in the N-ras gene in the progression from MDS to overt leukemias,4,3 and other groups showed fms gene mutations in MDS.31 We detected no N-ras gene activation in the three MDS patients and the MDS-derived leukemia cell line with p53 gene mutations in this study. The results suggest that MDS is heterogeneous not only in its phenotype but also in the molecular mechanism of its genesis and progression. More extended and detailed study is necessary to understand the molecular basis of MDS development.

ACKNOWLEDGMENT

We thank Dr Y. Miura (Department of Hematology, Jichi Medical School, Tochigi, Japan) for providing samples from patients and Dr K. Takeshita for fruitful discussion and proofreading of the text.

REFERENCES


Mutations of the p53 gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia

K Sugimoto, N Hirano, H Toyoshima, S Chiba, H Mano, F Takaku, Y Yazaki and H Hirai