Frequent p53 Gene Mutations in Blast Crisis of Chronic Myelogenous Leukemia, Especially in Myeloid Crisis Harboring Loss of a Chromosome 17p

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ABSTRACT

We investigated chromosome alterations and mutations of the p53 gene in 118 samples from 92 patients with chronic myelogenous leukemia in various clinical phases, i.e., chronic phase, accelerated phase, and blast crisis (BC). Single-strand conformation polymorphism analysis and subsequent nucleotide sequencing disclosed no alteration of the p53 gene in chronic phase (no mutation in 80 samples), while five of 31 BC samples showed point mutations: four in myeloid and one in lymphoid crisis. One of seven accelerated phase samples also showed a p53 gene mutation. Ten of 31 BC samples showed loss of one of the short arms of chromosome 17 (17p) through the formation of iso(17q), i(17q), or unbalanced translocations. Loss of heterozygosity at the p53 locus in the accelerated phase and BC was detected only in two cases with i(17q) but not in seven cases with normal chromosome 17 homologues, suggesting that loss of one p53 allele is rare without cytogenetically detectable loss of a 17p. Among those six samples with p53 gene mutations, five showed loss of a 17p cytogenetically, and only one lymphoid crisis case exhibited normal chromosome 17 homologues. Thus, mutations of the p53 gene were closely associated with myeloid crisis with loss of a 17p (four mutations in ten samples), in contrast to myeloid crisis with normal chromosome 17 homologues (zero in 13) or lymphoid crisis (one in seven). Our results also suggest that alterations of the p53 gene might occur after loss of a 17p during the course of chronic myelogenous leukemia.

INTRODUCTION

CML3 is characterized by the Philadelphia translocation, which results in a genetic recombination between the c-abl protooncogene on chromosome 9 and the breakpoint cluster region (bcr) gene on chromosome 22 (1). The chimeric bcr-abl mRNA and a resulting hybrid protein play an important role in pathogenesis of CML (2, 3). Clinically, CML is characterized by its biphasic course, i.e., CP and BC. In some cases, a transitional phase, AP, is observed between these two phases. Blast crisis is usually inevitable and fatal, although the disease is well controlled during the chronic phase. A few studies on the activation of the ras gene in blast-crisis of CML were reported (4, 5); however, little is known about the genetic events responsible for blastic transformation.

The p53 protein was first identified as a protein that binds to the T antigen, a polypeptide expressed in cells transformed by SV40 (6). At that time, this protein was regarded as an oncogene in transformed cells (7). However, recent studies have disclosed that not the wild-type p53 could transform cells cooperating with activated oncogenes, such as the ras genes (8), and that the wild-type p53 could act as a tumor suppressor (9). The p53 gene was mapped on 17p13 (10), and mutations of the p53 gene with loss of normal allele are now regarded as important steps in the development of a variety of human malignancies, including hematological ones (11-13).

Iso(17q), which results in loss of a short arm of chromosome 17 (17p), is well known to emerge in BC of CML, and several reports suggested the involvement of the p53 gene in the transition from CP to BC (14-19). To elucidate the incidence of loss of a 17p and p53 gene mutations in each phase of CML, and correlation between these two events, we analyzed karyotypes and p53 gene mutations in exons 2 to 11 by SSCP analysis (20) and direct sequencing. We also examined CP samples for polymorphism at the p53 gene locus using PCR and analyzed LOH on chromosome 17p in AP and BC samples (21).

MATERIALS AND METHODS

Patients. We studied 118 bone marrow or peripheral blood samples obtained from 92 patients with CML in various clinical phases (80 in CP, 7 in AP, and 31 in BC). The diagnosis of CML was made on the basis of clinical features, hematological data, and the Philadelphia chromosome (Ph+). Among 92 patients, 4 were Ph'-negative CML, and 3 other patients were unavailable for cytogenetic analysis. In these 7 patients, rearrangements of the bcr gene were detected by Southern blotting.4 In 24 patients, both cytogenetic and molecular analyses were performed in more than 2 phases, i.e., CP and BC (18 patients), CP and AP (4 patients), and CP and AP and BC (2 patients). The types of blast crisis in 31 cases were as follows: 16 myeloid; 7 lymphoid; 3 basophilic; 1 megakaryocytic; 1 myelomonocytic; 1 erythroid; 1 mixed-phenotype crisis; and 1 unknown. All samples were collected after obtaining informed consent.

Cytogenetic Analysis. Bone marrow or peripheral blood cells were cultured overnight, without mitogen, in RPMI 1640 supplemented with 15% fetal bovine serum. Chromosome preparations were prepared according to our standard procedures (22), and the trypsin-Giemsa technique was applied for G-banding. Identification of chromosomes and karyotype designation were done according to the International System for Human Cytogenetic Nomenclature (1991).

PCR-SSCP Analysis. High-molecular-weight DNA was extracted from each sample according to the method of Sambrook et al. (23). PCR and subsequent SSCP analysis were carried out as described by Orita et al. (20) and Toguchida et al. (24), with minor modifications. In brief, 12 sets of primers, 2 sets for each of exons 4 and 5 and one set for each of exons 2, 3, and 6 to 11, were used to amplify these exons including small segments of neighboring intron sequences. Each fragment was amplified by PCR using Gene ATAQ Controller (Pharmacia LKB) in the presence of [α-32p]dCTP. The amplified fragments of exons 2, 4, and 10 were digested into two fragments with restriction enzymes BamHI,MspI, and Alul, respectively. A few microliters of the PCR reaction mixtures were diluted and mixed with denaturing dye. After denaturation at 94°C for 3 min, samples were applied to a 6% polyacrylamide gel with or without 10% glycerol and electrophoresed at room temperature. The gel was dried and exposed to Kodak X-Omat film at −80°C for 0.5 to 3 days with an intensifying screen.

Received 6/26/92; accepted 9/24/92.

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1 This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CML, chronic myelogenous leukemia; CP, chronic phase; AP, accelerated phase; BC, blast crisis; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity.

4 H. Nakai and S. Misawa, unpublished results.
Clinical samples additional translocations involving chromosome 17, i.e., der(5)t(5;17)(pl 5;q 11), dic(14;17)-(pl 1,pl 1), and der(17)t(17;?)(pl 1;?), were sequenced to identify mutations. One g of genomic DNA was amplified by asymmetric PCR and then purified by using ultrafiltration kits (SUPREC-02; Takara Shuzo Co.). The AccII site was digested into two fragments of 160 and 99 base pairs, and electrophoresed on a 3% agarose gel. An allele having an polymorphism in exon 4 of the p53 gene, a 259-base pair fragment was amplified by PCR according to the method of de la Calle-Martin et al. (25). The reaction mixtures were then incubated overnight at 59°C in the presence of 20 units of AccII, followed by electrophoresis on an 8% polyacrylamide gel containing 7 M urea. Sequencing was performed on both sense and antisense strands to confirm mutations.

PCR-LOH Analysis. To detect the AccII polymorphism in exon 4 of the p53 gene, a 259-base pair fragment was amplified by PCR according to the method of de la Calle-Martin et al. (25). The reaction mixtures were then incubated overnight at 59°C in the presence of 20 units of AccII and electrophoresed on a 3% agarose gel. An allele having an AccII site was digested into two fragments of 160 and 99 base pairs, while another allele without any AccII site showed a 259-base pair fragment.

### Table 1: Chromosome findings in 92 patients examined

<table>
<thead>
<tr>
<th>Clinical phase</th>
<th>No. of samples analyzed</th>
<th>No. of individuals with additional changes</th>
<th>No. of individual aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>77</td>
<td>4 (5%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+Ph&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>AP</td>
<td>7</td>
<td>6 (86)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+i(17q)</td>
</tr>
<tr>
<td>BC</td>
<td>31</td>
<td>24 (77)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>i(17q)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three other samples were examined but inadequate for karyotyping.
<sup>b</sup> Numbers in parentheses, percentage.
<sup>c</sup> Number of samples with additional changes/number of samples analyzed.

**Sequencing.** The samples showing aberrantly migrating bands in the PCR-SSCP analysis were sequenced to identify mutations. One μg of genomic DNA was amplified by asymmetric PCR and then purified by using ultrafiltration kits (SUPREC-02; Takara Shuzo Co.). The dideoxy-mediated chain-termination reactions were carried out using AccII labeled primers, and T7 Sequencing Kit (Pharmacia LKB), followed by electrophoresis on an 8% polyacrylamide gel containing 7 M urea. Sequencing was performed on both sense and antisense strands to confirm mutations.

**PCR-SSCP Analysis and Sequencing.** We first examined exons 5 to 8 of the p53 gene in all of 118 samples by PCR-SSCP analysis and subsequent DNA sequencing, and we found point mutations in only 5 BC samples. Then, all of 7 AP and 31 BC samples, and 25 of 80 CP samples, were analyzed on exons 2 to 4 and 9 to 11, where the p53 gene mutations were rarely found according to the previous reports (12, 13). In total, p53 gene mutations were found in one AP and 5 BC samples (Fig. 1) but in none of the CP samples. G:C to T:A transversions at codons 175 and 248 were seen in 2 BC samples (Fig. 2, A and B), and a T:A to T:A transversion at codon 203 was found in one BC sample (Fig. 2C). These 3 point mutations were missense mutations. Sample 37-BC showed a G:C to C:G transversion at exon 4-exon 5 junction.

### Table 2: Loss of a 17p, p53 gene mutation, and prognosis in CML

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Phase (lineage)</th>
<th>Loss of a 17p&lt;sup&gt;*/i(17q)&lt;/sup&gt;</th>
<th>p53 gene mutation</th>
<th>Survival (mo)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>M/55</td>
<td>CP</td>
<td>None</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AP</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 11/11&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>203</td>
</tr>
<tr>
<td>36</td>
<td>M/65</td>
<td>CP</td>
<td>None</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AP</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 20/50&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>37</td>
<td>F/49</td>
<td>CP</td>
<td>None</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(basophilic)</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 29/29&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>51</td>
<td>F/55</td>
<td>CP</td>
<td>None</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(myeloid)</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 6/6&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>52</td>
<td>F/74</td>
<td>BC</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 5/16&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>55</td>
<td>M/36</td>
<td>BC</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 34/39&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>M/46</td>
<td>BC</td>
<td>dic(14;17)(p11;p11)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>M/37</td>
<td>BC</td>
<td>der(17)(17;7)(p11;7)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>F/55</td>
<td>BC</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 9/14&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>64</td>
<td>F/45</td>
<td>BC</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 3/5&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>M/46</td>
<td>CP</td>
<td>None</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 11/11&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>i(17q)&lt;sup&gt;c&lt;/sup&gt; 11/11&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>19</td>
<td>F/65</td>
<td>BC</td>
<td>None</td>
<td>CGT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96-base pair deletion</td>
</tr>
</tbody>
</table>

<sup>a</sup> Proportion of metaphases carrying loss of a 17p among total metaphases analyzed.
<sup>b</sup> Survival after the onset of blastic transformation.
<sup>c</sup> ND, not detected.
<sup>d</sup> This sample was obtained at the time of diagnosis of AP.
<sup>e</sup> This sample was obtained at the terminal stage of disease.
<sup>f</sup> Although cytogenetic analysis was not performed, PCR-LOH analysis revealed loss of one p53 allele.
<sup>g</sup> The patient died during the accelerated phase.

### RESULTS

**Chromosome Findings.** Karyotypes were obtained in 115 samples of 89 patients. The Ph<sup>1</sup> chromosome was found in 85 patients, 8 of which showed variant Ph<sup>1</sup> translocations. Additional chromosome abnormalities were rarely found in CP (5%) but frequently observed in AP and BC (86% and 77%, respectively) (Table 1). An isochromosome for the long arm of chromosome 17, i(17q), was the most common structural change (43% of AP and 23% of BC samples). Unbalanced translocations involving chromosome 17, which also resulted in the loss of the distal portion of 17p, were observed in 3 other BC samples. In summary, the loss of at least the distal portion of 17p was observed in 10 of 31 BC samples. The proportion of metaphases carrying these aberrations varied from 31% to 100% in each sample (Table 2).

**PCR-SSCP Analysis and Sequencing.** We first examined exons 5 to 8 of the p53 gene in all of 118 samples by PCR-SSCP analysis and subsequent DNA sequencing, and we found point mutations in only 5 BC samples. Then, all of 7 AP and 31 BC samples, and 25 of 80 CP samples, were analyzed on exons 2 to 4 and 9 to 11, where the p53 gene mutations were rarely found according to the previous reports (12, 13). In total, p53 gene mutations were found in one AP and 5 BC samples (Fig. 1) but in none of the CP samples. G:C to T:A transversions at codons 175 and 248 were seen in 2 BC samples (Fig. 2, A and B), and a T:A to T:A transversion at codon 203 was found in one BC sample (Fig. 2C). These 3 point mutations were missense mutations. Sample 37-BC showed a G:C to C:G transversion at exon 4-exon 5 junction.
Fig. 1. PCR-SSCP analysis of exons 5 to 8 and 10. The middle lane of each panel exhibits aberrantly migrating bands. In Samples 37-BC, 51-BC, 36-BC, and 31-BC, normally migrating bands are faint or absent. Samples 19-BC and 3-AP exhibit both aberrant and normal bands.

Fig. 2. Point mutations identified in CML-BC sample. In A, B, and C, the sequence of normal control is shown at the left, and the sequence of the samples at the right. A, sequencing of exon 7 in Sample 31-BC demonstrating a mutation at codon 248 (CGG→CAG); B, exon 5 in Sample 51-BC showing a point mutation at codon 175 (CGC→CAC); C, exon 6 in Sample 36-BC revealing a mutation at codon 203 (GTG→GAG); D, sequence of antisense strand of exon 5 including neighboring intron 4 in Sample 37-BC. A point mutation at a splice acceptor site preceding exon 5 is seen (antisense, tCATG→tgATG). The unique dinucleotides, AG, at a splice acceptor site are replaced by AC.

From these observations, we presumed that a G:C to C:G transversion and a C insertion occurred at codon 273 (CGT→CCCT) on one allele. To confirm this result, we cloned the PCR product into pCR 1000 vector using a TA Cloning Kit (Invitrogen Co.) and performed conventional sequencing. As expected, we obtained two types of clones: one showed a completely normal sequence; and another showed a mutant sequence as we presumed above (Fig. 3).
Sample 3-AP, obtained at the terminal stage of disease, had aberrantly migrating bands in addition to normal ones in exon 10. Through agarose gel electrophoresis of the amplified PCR fragments of this sample, we found two fragments of different length; one was of the expected normal length, but the other was shorter than normal. DNA sequencing of this shorter fragment, purified by agarose gel electrophoresis, revealed a 96-base pair deletion spanning from codon 344 in exon 10 to the 26th base of the 5' side of intron 10 (data not shown). In the normal length fragment, no mutation was detected. Another sample of this case, obtained at the time of diagnosis of AP, was normal length fragment, no mutation was detected. Another sample, 3-AP, which was obtained at the time of diagnosis of AP, was also analyzed in the same manner. Interestingly, this sample showed i(17q) but no p53 mutation.

The results are summarized in Table 2. Chronic phase samples of these cases with mutations, except for Case 19, were examined by both PCR-SSCP and sequencing analysis and showed no mutation (data not shown).

**PCROH Analysis.** In this PCROH analysis, since we compared the restriction fragment length polymorphism pattern of leukemic cells in CP with those in AP or BC, only the LOH which occurred in the course of transition from CP to AP or BC was detected.

In 24 cases where samples in CP were available for PCROH analysis, 9 of them were informative. Among these 9 cases, one case in AP (Sample 3-AP) and one in BC (Sample 37-BC) showed LOH at the p53 locus (Fig. 4). Both cases carried an i(17q), and no case showed LOH at the p53 locus without a gross aberration of chromosome 17. As for Sample 3-AP, the same p53 allele was lost at the onset of AP and at the terminal stage; however, only the sample at the terminal stage had p53 gene mutation.

Correlation among p53 Gene Mutations, Clinical Phase, and Karyotypes. As described above, the p53 gene was mutated more frequently in BC (5 of 31 samples) and AP (one of 7) than in CP (none of 80), and p53 gene mutations were closely associated with the loss of a 17p. In BC, p53 gene mutations were observed in 4 of 10 samples with the loss of a 17p, but in only one of 21 samples without the loss of a 17p (Table 3). In addition, these 4 samples with a p53 gene mutation and loss of a 17p were in myeloid crisis. In contrast, p53 mutation was rare in those without the loss of a 17p, even though they were in myeloid crisis (none of 13). Only one sample in lymphoid crisis showed a p53 gene mutation without the loss of a 17p. These findings suggest that p53 gene mutations might occur mostly in myeloid crisis accompanied by the loss of a 17p.

**DISCUSSION**

We detected p53 gene mutations in 5 of 31 BC and one of 7 AP samples, and they were closely associated with the loss of a 17p. Codons 175, 248, and 273, which were mutated in our series, are within the conserved domains of the p53 protein (26) and commonly mutated in human cancers (12, 13). All of them contain CpG dinucleotides, and two of them showed a G:C to A:T transition. Because CpG dinucleotides are likely to be methylated, spontaneous deamination of 5-methylcytosine residues might lead to such a mutation (27). Sample 37-BC had a point mutation at a splice acceptor site. Although we did not analyze mRNA of this case, similar mutations at splicing sites of the p53 gene were reported previously in a CML blast crisis cell line, lung cancer, colorectal cancer, a T-cell leukemia cell line, and so on (13, 28).

Isochromosome 17q was observed in about 30% of blast crisis of CML according to previous reports (29); however, the frequency of loss of the p53 allele has not been reported. In the present study, chromosome analysis and PCROH analysis suggested that the loss of one p53 allele was mainly caused by
In accordance with previous reports on CML, alterations of the \( p53 \) gene were frequently observed in BC but rarely in AP (14–18). Our results not only supported them but also elucidated close correlation between mutation and specific karyotype, loss of a 17p. A similar correlation was reported in patients with acute myelogenous leukemia (36). Feinstein et al. (19) recently reported that, in all 6 cases of CML-BC with loss of one \( p53 \) allele, the remaining \( p53 \) allele was also inactivated by mutation or loss of transcription. In our study, although loss of a 17p was frequently observed in myeloid crisis, about half of those did not carry \( p53 \) mutation. Taking their study into consideration, loss of function of the normal \( p53 \) might occur more frequently than we actually observed if we analyzed expression of the \( p53 \) gene. In addition, Feinstein et al. speculated that \( p53 \) allele loss precedes mutation (19), and the results of our serial analysis also support their speculation about the order of these two events. Therefore, it is possible that \( p53 \) gene dosage affects the growth of leukemic cells at an early stage of AP or BC before mutation occurs, although we have no biological evidence for it at this time.

ACKNOWLEDGMENTS

The authors thank Professors K. Kashima and T. Abe for critical reading of the manuscript, Dr. S. Yokota for technical advice, and Dr. H. Fujii and Dr. C. Shimazaki for providing us with samples and clinical data.

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