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

Hiroyuki Nakai, Shinichi Misawa, Junya Toguchida, David W. Yandell, and Kanji Ishizaki

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 isochromosome 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

CML 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 brief study 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 blast 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 oncoprotein due to an increased level of p53 in various transformed cells (7). However, recent studies have disclosed that not the wild-type, but mutant 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).

Isochromosome 17q, which results in 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 (Ph1). Among 92 patients, 4 were Ph1-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. 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 erythroblastic; 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.
Clinical samples additional translocations involving chromosome 17, i.e., der(5)t(5; 17)(pl 5;q I 1), dic(l 4; 17)- (pl 1,pl 1), and der(17)t(17;?)(pl 1;?). PCR-SSCP analysis were sequenced to identify mutations. One #g of using ultrafiltration kits (SUPREC-02; Takara Shuzo Co.). The performed on both sense and antisense strands to confirm mutations. dideoxy-mediated chain-termination reactions were carried out using genomic DNA was amplified by asymmetric PCR and then purified by electrophoresis on an 8% polyacrylamide gel containing 7 M urea. Sequencing was one third to one half of the purified PCR products, 32p-labeled primers, to the method of de la CaUe-Martin thep53 were then incubated overnight at 59~ in the presence of 20 units of while another allele without any phase analyzed changes +Ph ~ +8 i(17q) + 19 Others CP 77 a 4 (5) b, c 0 0 0 0 4 AP 7 6 (86) 2 4 3 (43) d 1 2 BC 31 24 (77) 9 11 7 (23) 6 16 e Sequencing. The samples showing aberrantly migrating bands in the PCR-SSCP sequence were identified. 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 one third to one half of the purified PCR products, 32p-labeled primers, and T7 Sequencing Kit (Pharmacia LKB), followed by electrophoresis on 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.

RESULTS

Chromosome Findings. Karyotypes were obtained in 115 samples of 89 patients. The Ph1 chromosome was found in 85 patients, 8 of which showed variant Ph1 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 a 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 116 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 A:T transitions at codons 175 and 248 were seen in 2 BC samples (Fig. 2, A and B), and a T:A to A:T 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 one of its p53 genes.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Phase (lineage)</th>
<th>Loss of a 17p &lt;proportion*&gt;</th>
<th>p53 gene mutation</th>
<th>Survival (mo)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>M/55</td>
<td>CP</td>
<td>None</td>
<td>i(17q) &lt;11/11&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>36</td>
<td>M/65</td>
<td>AP, BC (myeloid)</td>
<td>None</td>
<td>i(17q) &lt;8/8&gt;</td>
<td>248</td>
</tr>
<tr>
<td>37</td>
<td>F/49</td>
<td>CP</td>
<td>None</td>
<td>i(17q) &lt;20/50&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>51</td>
<td>F/55</td>
<td>CP, BC (myeloid)</td>
<td>None</td>
<td>i(17q) &lt;11/11&gt;</td>
<td>175</td>
</tr>
<tr>
<td>52</td>
<td>F/47</td>
<td>BC (myeloid)</td>
<td>None</td>
<td>i(17q) &lt;5/16&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>55</td>
<td>M/36</td>
<td>AP, BC (basophil)</td>
<td>None</td>
<td>i(17q) &lt;3/4&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>M/46</td>
<td>BC (erythroblastic)</td>
<td>None</td>
<td>i(17q) &lt;7/18&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>M/37</td>
<td>BC (myeloid)</td>
<td>None</td>
<td>der(17)(q17;p11) &lt;7/18&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>F/55</td>
<td>BC (myeloid)</td>
<td>None</td>
<td>i(17q) &lt;9/14&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>64</td>
<td>F/45</td>
<td>BC (myeloid)</td>
<td>None</td>
<td>i(17q) &lt;3/3&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>M/46</td>
<td>CP</td>
<td>None</td>
<td>i(17q) &lt;9/11&gt;</td>
<td>273</td>
</tr>
<tr>
<td>19</td>
<td>F/65</td>
<td>BC (lymphoid)</td>
<td>None</td>
<td>CGT—CCCT</td>
<td>Arg—Pro frameshift</td>
</tr>
</tbody>
</table>

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

Table 1 Chromosome findings in 92 patients examined

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

Cases with loss of a 17p, p53 mutation, or both were summarized in this table.
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).

the splice acceptor site of intron 4 (Fig. 2D), which might cause abnormal splicing of mRNA. In these 4 samples, the normal bands were faint or not present on the autoradiograms, indicating loss of normal alleles.

In Sample 19-BC, the sequence analysis on both sense and antisense strands revealed that doublet bands appeared from the position of codon 273, and on the sense strand, two unexpected bands were present on the C lane within codon 273.
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 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).

PCR-LOH Analysis. In this PCR-LOH 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 PCR-LOH 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 PCR-LOH analysis suggested that the loss of one p53 allele was mainly caused by...
Table 3 Summary of the loss of a 17p and p53 gene mutation in CML.

<table>
<thead>
<tr>
<th>Clinical phase</th>
<th>Cases with loss of a 17p</th>
<th>Cases with normal 17s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples examined</td>
<td>p53 mutation</td>
</tr>
<tr>
<td>Chronic phase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Accelerated phase</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Blast crisis</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Myeloid crisis*</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Others*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Basophilic, megakaryocytic, myelomonocytic, erythroblastic, and mixed-phenotype crises are included.

b Seven cases of lymphoid crisis are included.

gross aberrations of chromosome 17 detectable by conventional chromosome analysis. Although it was reported that, in some cases, one p53 allele was lost in spite of normal chromosome 17 homologues (16, 19, 30), our observations suggest that such cases might be few in CML-AP or BC. Therefore, the frequency of loss of one p53 allele may be almost equal to that of cytogenetically detectable loss of a 17p. In many types of tumors, mutations of the p53 gene are coupled with loss of the corresponding normal allele, indicating loss-of-function mutation of this gene. If this is also the case in CML, the p53 gene mutation may be closely associated with myeloid crisis, but not in lymphoid crisis, since an i(17q) can be detected frequently in myeloid crisis but rarely in lymphoid crisis.

It is an intriguing matter when the p53 gene is altered in the progression of diseases. In this regard, recent studies indicate that alterations of the p53 gene may precede loss of one p53 allele in colorectal tumors and gliomas (31, 32), or mutation may occur concomitantly with allelic loss in lung cancers or brain tumors (33, 34). Our results suggest, however, that loss of a 17p may precede p53 mutation, at least in CML, based on the following findings. (a) In our series, most of the p53 mutations accompanied loss of a 17p, while about two thirds of AP and BC samples with loss of a 17p had no alterations within exons 2 to 11 of the p53 gene. (b) Cases 31 and 36 carried an i(17q) without p53 mutations in AP, but later, evolution to BC was accompanied with mutation of the p53 gene. (c) In Case 3, both chromosome and PCR-LOH analyses revealed that most cells lost one p53 allele without p53 mutation at the time of diagnosis of AP; however, at the terminal stage, a portion of the leukemic cells showing loss of the same p53 allele had a mutated p53 allele.

Baker et al. (31) suggested that cells with p53 gene point mutation would have a stronger growth advantage than those with loss of one p53 allele but without p53 gene point mutation. This hypothesis was based on the recent finding that the mutant p53 protein negates the function of the wild-type p53 through exerting a dominant negative effect (35). Our results also led to a similar speculation; i.e., a blastic clone with a hemizygous 17p but without a p53 gene mutation tends to have a limited expansion; however, once the p53 gene is altered on the residual 17p, the leukemic clone may become more aggressive and dominate over the other clones. This is predicated upon the following observations. (a) In all 4 BC samples that had both a p53 gene mutation and loss of a 17p, the proportion of metaphases carrying loss of a 17p was more than 90%. In contrast, the proportion varied from 31% to 100% in BC samples with loss of a 17p but without p53 gene mutation. (b) Among the cases with loss of a 17p, survival periods after blastic transformation of these 4 cases with p53 mutation were shorter (0.5 to 2 mo; average, 1.4 mo) than those of the others (3 to 20 mo; average, 8.9 mo).

In accordance with previous reports on CML, alterations of the p53 gene were frequently observed in BC but rarely in CP (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 allelic 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.

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