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# Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia

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ABSTRACT **Molecular mechanisms responsible for the clinical progression of chronic myelocytic leukemia to its accelerated phase or to blast crisis have not been defined. We found alterations of the p53 gene (p53 is a 53-kDa nuclear protein) including deletions and rearrangements in 8 of 34 patients in blast crisis and 1 of 4 patients in the accelerated phase, but in only 1 of 38 patients in the chronic phase of chronic myelocytic leukemia. Only two other examples of p53 gene alterations were found among 203 patients with hematologic malignancies and solid tumors. Transcripts of the p53 gene were uniformly found in chronic-phase cells, but gene expression was variable in blast crisis, and transcripts were reduced or undetectable in 10 of 16 patients. Heterogeneous alterations in the structure and expression of the p53 gene appear to be relatively frequent in blast crisis and may be involved in the evolution of disease.**

Chronic myelocytic leukemia (CML) is a clonal disorder of pluripotent hematopoietic stem cells with a biphasic or triphasic clinical course. An initial chronic phase is usually followed by acceleration of disease and ultimately by an acute blast-crisis phase characterized by increasing cellular proliferation, maturation arrest involving cells of one or more hematopoietic lineages, and karyotypic clonal evolution (1). Occasionally, an accelerated phase of rising leukocyte counts and progressive splenomegaly precedes overt blast crisis. The leukemic cells of more than 90% of CML patients have the Philadelphia chromosome anomaly (2), which is characterized at the molecular level by fusion of sequences of the *ABL* protooncogene from chromosome 9 with sequences from the *BCR* gene on chromosome 22 (3), resulting in the formation of a chimeric *BCR–ABL* transcript and protein (4–6). Although activation of *ABL* is thought to be important in the pathogenesis of the chronic phase of CML, the genetic changes responsible for the transition to blast crisis are unknown. In general, the levels of the fused BCR mRNA vary considerably among individual patients and do not correlate with stages of disease (6). Also, with occasional exceptions, the genomic structures of the *ABL* and *BCR* genes are identical in both phases of the disease. On the other hand, the presence of additional nonrandom cytogenetic changes, most notably trisomy of chromosome 8 or 19, isochromosome 17q (i17q), and an additional Philadelphia chromosome (7), as well as the presence of occasional mutations or amplification of protooncogenes in the acute phase (8, 9), suggest that further genetic events may be important in evolution of CML to blast crisis. To test this hypothesis, we investigated leukemic cells from 34 blast-crisis, 4 accelerated-phase, and 38 chronic-phase patients for abnormalities of protooncogenes and compared the findings with those in more than 200 patients with malignancies of other types. In addition to the expected *BCR/ABL* rearrangements, alterations in the p53 gene (p53 is a 53-kDa nuclear protein) were the only frequent molecular abnormality observed in blast-crisis patients.

## MATERIALS AND METHODS

Bone marrow or peripheral blood samples (if the blast-cell count exceeded 60%), lymph nodes, and tumor biopsies were collected and kept frozen at −70°C. The diagnosis of CML was based on standard clinical and hematologic criteria. For the blast-crisis samples markers such as myeloperoxidase, Sudan black, periodic acid/Schiff reagent, nonspecific esterase with fluoride sensitivity, and acid phosphatase were analyzed. Additional determinants of phenotype included the reactivity of the blasts to a panel of lineage-specific monoclonal antibodies and the activity of terminal deoxynucleotidyltransferase determined by indirect immunofluorescence.

DNA and RNA were isolated from fresh frozen leukemic cells and analyzed by Southern and Northern blotting as described (10, 11). DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, or *Bgl* II was separated by electrophoresis on 0.8% agarose gels, transferred to coated nylon filters, hybridized to nick-translated $^{32}$P-labeled probes, and autographed with Kodak intensifying screens. Hybridization probes (Fig. 1*A*) were as follows: a, pR4-2 [a cDNA probe encompassing exons 2–10 and parts of exons 1 and 11 of p53 (12)]; b, 3.7-kilobase (kb) *Eco*RI fragment encompassing the first exon of p53; c, a 0.7-kb *Hin*dIII fragment from intron 1; d, a 0.9-kb *Eco*RI–*Bam*HI fragment encompassing exon 11 (12). Southern blots of restriction digests of DNA from leukemic cells were also hybridized with probes for *MYC, MYB, FMS, HRAS, ERBB2, INT2, ERBB1, SIS*, and a probe for the gene encoding the A chain of platelet-derived growth factor as described (14). A 1.2-kb *Hin*dIII–*Bgl* II *BCR* fragment was obtained from Oncogene Science, Mineola, NY.

A 109-base-pair fragment of the first exon of the *NRAS* gene was amplified by the polymerase chain reaction (15), using synthetic oligodeoxynucleotides of 20 bases spanning the 5′ and 3′ ends of the target sequence, *Thermus aquaticus* DNA polymerase, and 30 cycles with the following periods: 95°C (1 min), 55°C (0.5 min), and 72°C (1.5 min). The amplified fragments were electrophoresed on 3% Nusieve agarose, electroeluted, purified on Elutip D (Schleicher & Schuell), and ethanol-precipitated. Sequencing was as described (16) using Sequenase, version 2.0 (United States Biochemical), a third primer from the sense or antisense strands labeled with T4 kinase, and [$\gamma$-$^{32}$P]ATP. The dried 10% polyacrylamide gel was exposed to film overnight without screen intensification. Statistical analysis was done by

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Abbreviations: CML, chronic myelocytic leukemia; AML, acute myeloid leukemia.

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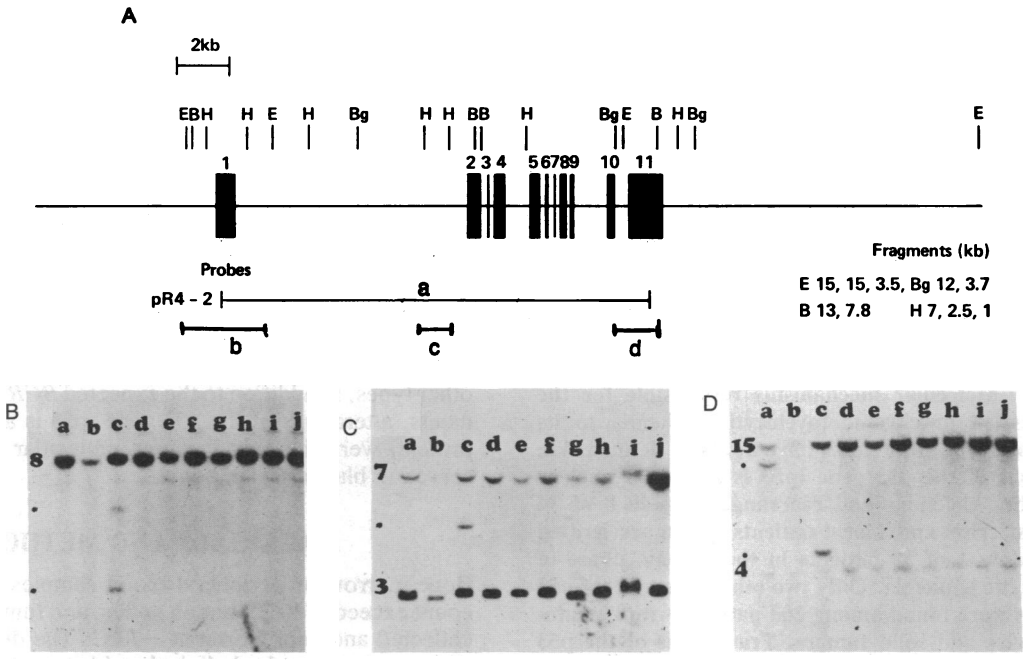


FIG. 1. (*A*) Map of the human p53 gene (12) showing probes and expected germ-line restriction fragments. Probe a is a p53 cDNA clone generously provided by E. Harlow (13). Probe b is a 3.7-kb *Eco*RI fragment obtained from a human genomic library. Probe c is a 0.7-kb genomic *Hin*dIII fragment from the first intron. Probe d is a 0.9-kb genomic fragment, obtained by *Eco*RI and *Bam*HI digestion of the plasmid HU7-1 (11). (*B–D*) Southern blots of *Bam*HI-, *Hin*dIII-, and *Eco*RI-digested DNA, respectively, hybridized with probe a. Lanes a–j are DNAs from patients 3, 4, 5, 20, 1, 22, and 24, a patient with lymphoblastic extra-medullary blast crisis, a patient with acute leukemia, and a normal control, respectively. Numbers indicate size of expected germ-line fragments in kb.

the $\chi^2$ method. Studies with recombinant DNA molecules conformed to the standards outlined by the Recombinant DNA Research Guidelines of the National Institutes of Health (July 1976).

## RESULTS

**Alterations in the p53 Gene in Blast Crisis.** We found structural abnormalities of the p53 gene in 8 of 34 patients with CML in blast crisis. The observed alterations were heterogeneous and included rearrangements, deletions, and additional restriction sites (Figs. 1–4). In addition, 10 of 16 patients with and without detectably altered p53 genes had no detectable p53 transcripts.

The distribution among the various forms of blast crisis is shown in Table 1. By using the various probes shown in Fig. 1*A* and restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Bgl* II abnormalities of p53 gene structure were detected in 8 blast-crisis patients and in 1 of 4 accelerated-phase patients. Detected alterations were rare in patients with chronic-phase CML (1 of 38 patients) and other hematologic malignancies (1 of 88 patients). The differences in frequency of p53 gene alteration in blast-crisis relative to chronic-phase CML or other hematologic disorders were statistically significant (Table 2). p53 gene alterations were seen only with a myeloid- or a mixed-phenotype blast crisis (Table 1); however, only 3 patients with a lymphoid blast crisis were studied.

The normal hybridization patterns with a full-length probe and DNAs digested with one of three restriction enzymes are shown in lane j of Fig. 1 *B–D* (13). In five patients (patients 4–8) additional bands were detected with the full-length probes. In these cases the novel bands were also detected with the 3′ probe d, but not with 5′ probes b and c (data not shown), indicating that a rearrangement at the 3′ end of the gene had occurred (Figs. 1 *B–D*, lanes c, and 2, lanes a and c, as examples). In two of these patients (patients 4 and 5) abnormalities were detected with multiple restriction enzymes, suggesting gross structural alterations (this can be seen for patient 5 in Fig. 1 *B–D*, lanes c). The remaining three patients (patients 6, 7, and 8) showed abnormalities with only one restriction enzyme, indicating more subtle abnormalities such as point mutations. Two of these latter patients (patients 6 and 7) had apparently similar gene alterations (Fig. 2, lanes a and c) but were dissimilar in p53 mRNA expression (see below) and in rearrangements of their *BCR* genes. In these two cases, three major *Eco*RI bands were detected with probe a; the expected germ-line bands of 15 and 3.7 kb and a rearranged fragment of 1.8 kb. No rearrangements were

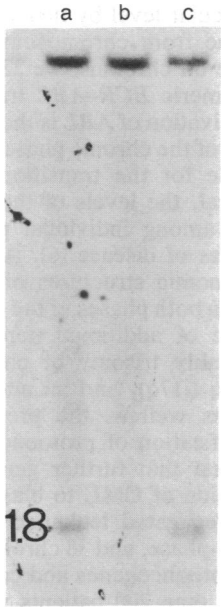


FIG. 2. DNAs from patients 6 and 7 (lanes a and c) and from a myeloid leukemia cell line, GDM-1 (lane b), were digested with *Eco*RI and probed with probe d containing exon 11 sequences. Rearranged 1.8-kb bands are visible in lanes a and c.

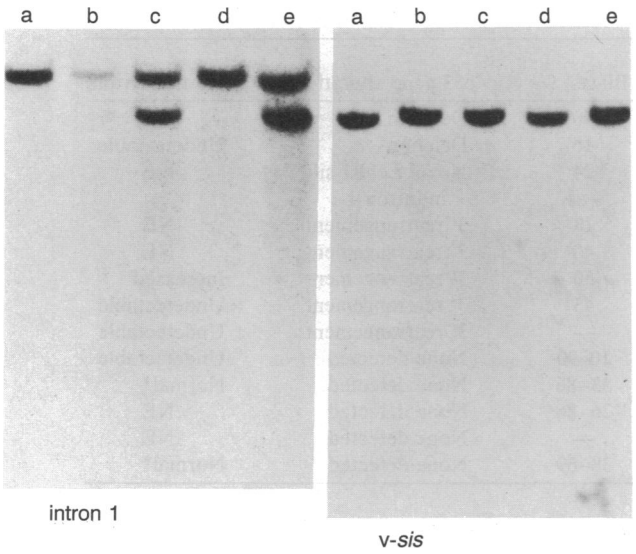


FIG. 3. DNAs digested with *Bgl* II and hybridized with probe c show a polymorphism of the p53 gene with either a doublet of 12- and 9-kb bands (heterozygous) or a single band (homozygous). Lanes a–e are from patients 20, 1, 22, and 24, and a patient with chloroma, respectively. The intensity of the hybridization signal with DNA from patient 1 (lane b) is reduced when compared to the same sample hybridized with v-*sis*, indicating loss of p53 gene sequences.

detected with probes b or c with multiple restriction enzymes. Probe d hybridized as expected to the germ-line 15-kb fragment but also hybridized as to the rearranged 1.8-kb fragment (Fig. 2, lanes a and c), suggesting creation of an *Eco*RI restriction site at the 3′ end of the gene. The restriction enzyme sites at the 3′ end of p53 depicted in Fig. 1*A* are only approximate as indicated by Lamb and Crawford (12); therefore, in these two patients we cannot definitely exclude a rearrangement on the 3′ side of the *Bgl* II site that is on the 3′ side of exon 11.

Patient 8 had an additional 1.5-kb *Bam*HI band detected with probes a and d, but not with probe c, suggesting a 3′ alteration of the gene. This was not further characterized.

A single patient (patient 3) had a novel *Eco*RI restriction site in the first intron of the p53 gene. Digestion with *Eco*RI revealed a 12-kb fragment in addition to the germ-line 15- and 3.7-kb fragments when Southern blots were hybridized with probes a and c (Fig. 1*D*, lane a). The same probes hybridized with DNA digested with *Bam*HI (Fig. 1*B*, lane a), *Hin*dIII

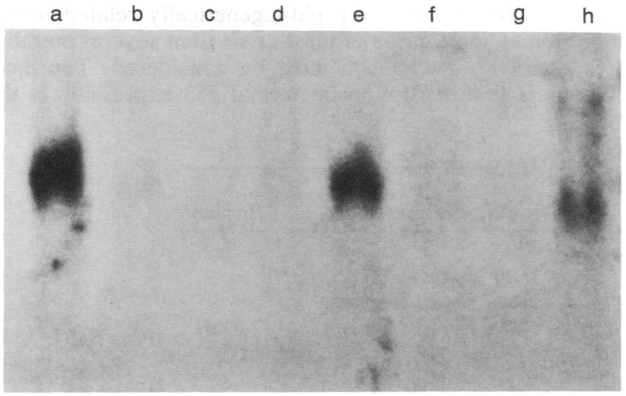


FIG. 4. Northern blot analysis of p53 mRNA. Lanes a–h are, respectively, from patients 6, 2, 7, and 8, a patient with a myeloproliferative disorder, patient 10, a blast-crisis cell line, and an acute leukemia line. Lanes b, c, d, and f from fresh blast-crisis cells and lane g from a blast-crisis cell line show reduced transcripts. Lane a from patient 6 with a 3′ alteration of the p53 gene shows a high level of transcripts relative to the acute leukemia cell line (lane h).

(Fig. 1*C*, lane a), or *Bgl* II or *Xba* I (data not shown) revealed no additional bands. It is possible that the additional *Eco*RI band reflected a restriction fragment length polymorphism; however, a similar hybridization pattern was not seen in 18 normal tissue samples and was seen in only 3 patients among the 245 patients with malignancies not involving blast crisis. Two of these samples were from patients with variants of CML: one in chronic phase and one in accelerated phase. In these samples the novel bands were of approximately the same size; however, in the chronic-phase cells, the density of the two *Eco*RI alleles was unequal suggesting an uneven distribution of the alleles in the cell population, consistent with an acquired abnormality. Unfortunately, normal tissue was not obtained from these patients because of bleeding defects, and the question of a restriction fragment length polymorphism cannot be definitively resolved, although we favor the view that it is an acquired mutation.

In cells of two patients (patients 1 and 2; Table 1) the intensity of the hybridizing signal with multiple p53 probes was less than normal, suggesting loss of at least one allele of the gene. The reduced hybridizing intensity in patient 1 was seen with probes c (Fig. 3, lane b) and also with probes b and d, indicating that one allele was completely deleted. Some signal is to be anticipated from residual or transfused nonblastic cells. In another patient (patient 2), loss of p53 sequences was suggested with probe a and was confirmed by a reduced signal intensity from one band of a *Bgl* II restriction fragment length polymorphism (ref. 17 and unpublished observations). The gene deletions in these two patients were not the result of loss of the whole chromosome 17, as hybridization with another chromosome 17 probe for *ERBB2* yielded a normal signal. Blast cells from 5 patients were found to have karyotypic alterations of chromosome 17, with i(17q) being present in 4 of them. None of these patients had rearranged p53 genes by Southern blotting.

**p53 Transcripts.** Cells from 26 CML patients were available for RNA analysis by Northern blotting including 16 in blast crisis, 7 in chronic phase, and 3 in accelerated phase. HeLa cells, GDM-1 cells (an acute leukemic cell line), and JOSK-M cells (a CML blast-crisis cell line) served as references. All chronic-phase cells from 7 patients showed 2.5-kb p53 transcripts at levels 3- to 5-fold greater than HeLa cells. Ten of 16 blast-crisis and 2 of 3 accelerated-phase patients had greatly reduced or undetectable levels (Fig. 4). The accelerated-phase patients had less than 6% blast cells and were, therefore, comparable to chronic-phase cell populations. Five blast-crisis and 1 accelerated-phase patient had levels of transcripts approximating those of chronic-phase cells. One blast-crisis patient (patient 6) with a putative 3′ rearrangement of the p53 gene had elevated levels of transcripts (Fig. 4, lane a). The acute leukemia cell line had abundant transcripts, whereas the blast-crisis line had no detectable p53 transcripts. Studies on seven additional myeloid blast-crisis cell lines revealed transcripts in only 3 (unpublished observations). No unusual-sized p53 transcripts were detected. Correlations between abundance of transcripts and p53 gene alterations are seen in Table 1.

**p53 in Other Diseases.** Because of the unusual frequency of rearrangements of the p53 gene in CML, we analyzed DNA from 203 patients with malignancies, which included 52 patients with acute leukemia (including 3 with Philadelphia-chromosome-positive acute lymphoblastic leukemia), 12 patients with myelodysplastic syndrome, 8 with multiple myeloma, 14 with lymphoma, 1 with polycythemia vera, 1 with lymphoepithelioma, 74 with adeno- or squamous carcinoma, 11 with soft tissue sarcoma, 3 with germ-cell tumors, 21 with neuroendocrine tumors, and 6 with childhood tumors (Table 2). Rearrangements of the p53 gene were found in osteogenic sarcomas and are restricted to the first intron of the gene in that disease (11). Of the remaining patients, structural alter-

Table 1. Alterations of the p53 gene in blast crisis of cml

| Patient | Phenotype | Leukocytes, no. $\times 10^{-9}$ per liter | Blasts, % | p53 gene alteration | p53 transcripts |
|---|---|---|---|---|---|
| 1 | Myeloid | 180 | 40 | Deletion | NE |
| 2 | Mixed | 100 | 16 | Deletion | Undetectable |
| 3 | Myeloid | 140 | 24 | Novel *Eco*RI site in intron 1 | NE |
| 4 | Myeloid | 174 | 18 | 3′ rearrangement | NE |
| 5 | Myeloid | 101 | 40 | 3′ rearrangement | NE |
| 6 | Myeloid | 228 | 50 | 3′ rearrangement | Increased |
| 7 | Undifferentiated | 29 | 43 | 3′ rearrangement | Undetectable |
| 8 | Uncertain | — | — | 3′ rearrangement | Undetectable |
| 9–15 | Myeloid | 40–480 | 10–90 | None detected | Undetectable |
| 16–19 | Myeloid | 38–236 | 33–86 | None detected | Normal* |
| 20–27 | Myeloid or mixed | 23–100 | 26–86 | None detected | NE |
| 28–31 | Uncertain | — | — | None detected | NE |
| 32–34 | Lymphoid | 36–111 | 38–69 | None detected | Normal* |

NE, not examined.
*Relative to chronic-phase cells.

ations of the p53 gene were seen only in 1 other patient with a myeloblastic chloroma. These results indicate that the high frequency of p53 gene abnormalities observed in blast crisis of CML is not a general phenomenon in human malignancy.

**Other Genetic Abnormalities.** No abnormalities of nine other protooncogenes were seen in blast-crisis cells by Southern blotting with appropriate restriction enzymes and molecular probes (14). Because of a report of *NRAS* mutations in blast crisis (9), we examined 26 patients by DNA amplification and direct sequencing. An abnormality was found in codon 13 of this gene in a single patient with myeloid blast crisis.

## DISCUSSION

Many observations suggest a role for the p53 gene in oncogenesis, cellular proliferation, and regulation of the cell cycle (18–21). Elevated levels and prolonged half-life of the p53 protein occur in most transformed cell lines. Protein encoded by the p53 gene can complement activated *RAS* genes in *in vitro* transformation of primary rodent cell cultures and can immortalize such cells (18). The synthesis of p53 increases in resting cells after stimulation with mitogen and stimulated cells can be blocked from entering S phase by microinjection of p53-specific antibody (21). Although these studies indicate that expression of the p53 gene is associated with cell proliferation, lack of expression of the p53 gene due in most cases to genomic rearrangements occurs in several transformed human and rodent cell lines, including HL-60 leukemic cells and osteogenic sarcoma cell lines (11). Several mouse erythroleukemia cell lines transformed *in vivo* by Friend leukemia virus show rearrangement and altered expression of the p53 gene. Whereas high levels are seen in some cell lines, others fail to express the protein or express truncated forms due to either genomic rearrangements or to a transcriptional block (22, 23). Our results in some cases of blast crisis are similar to those in mouse erythroleukemia cells, with both showing a heterozygous pattern of p53 gene alteration and expression. The decreased levels of p53 expression seen in about two-thirds of blast-crisis patients are probably significant. In 7 patients we found no detectable transcripts and no obvious gene rearrangement, suggesting more subtle mutations in transcription control regions of the gene. We do not know whether normal myeloblasts transcribe p53. However, several myeloblast cell lines do have transcripts, and gene expression is sometimes increased in blast cells of acute myeloid leukemia (AML) (24). Smith *et al.* (25) found p53 transcripts in 19 of 34 AML patients. However, it may be relevant that approximately 44% of AML patients do not have detectable p53 protein and this may correlate with their ability to form differentiated colonies *in vitro* (25). These observations indicate that some primitive myeloid blast cells can express the gene, whereas others do not. The defect in AML has not been defined (24, 25).

Alterations of p53 may be one of several genetic changes that accompany progression of CML. *MYC* amplification (8) and *NRAS* gene mutations at codons 12 and 13 also occur (9), but we found these to be rare events. Assuming that alterations of the p53 gene are pathogenetically related to the evolution of some cases of blast crisis then several possibilities as to the mechanism must be considered. The most obvious is that disruption of normal p53 expression is the

Table 2. p53 gene alterations in diverse malignant diseases

| Disease | No. DNAs analyzed | No. with altered p53 gene | p53 gene alteration | *P* value* |
|---|---|---|---|---|
| CML | | | | |
| Blast crisis | 34 | 8 | Heterogeneous | |
| Accelerated | 4 | 1 | Novel *Eco*RI fragment | |
| Chronic | 38 | 1 | Novel *Eco*RI fragment | 0.007 |
| Acute leukemia | 52 | 0 | NE | <0.001 |
| Myelodysplastic | 12 | 0 | NE | 0.05 |
| Other hematologic malignancies | 24 | 1 | Novel *Eco*RI fragment | 0.05 |
| Other solid tumors | 115 | 4 | Gene duplication in breast cancer; no structural alterations | 0.0002 |

NE, not examined.
*Statistical comparison of CML blast crisis with other disorders by the $\chi^2$ method.

common factor uniting the various structural alterations in the blast crisis of CML as is seen in mouse erythroleukemia. In the mouse model, p53 appears to be acting as a negative regulator of cell growth since the p53 gene is a common target for functional inactivation during the course of Friend virus-induced erythroleukemia (26). The data presented here for human CML are consistent with this proposal. Much remains to be learned about the role of the p53 gene product in proliferating cells. The alterations of this gene in some cases of blastic crisis of CML may provide a useful system for analysis and may have utility in the clinic for predicting the emergence of the second lethal phase of CML in some patients and for timing therapeutic interventions.

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