Rearrangements in the p53 Gene in Philadelphia Chromosome Positive Chronic Myelogenous Leukemia

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Molecular structural analysis of the p53 gene in patients with Philadelphia chromosome-positive chronic myelogenous leukemia (CML) indicates a significant incidence of gene rearrangements in patients at either accelerated phase or blastic crisis. Southern blot analysis of genomic DNA hybridizing with either genomic or cDNA p53 specific probes indicated that 30% of the CML patients at blastic crisis phase exhibited rearrangements, mostly mapping downstream to the first non-coding exon. This is compatible with the observation that the progression of CML from the chronic to the acute phase involves frequent aberrations in chromosome 17, to which the p53 oncogene has been mapped. Therefore, we suggest that one of the pathways of development of CML to the acute phase is associated with aberrations in the p53 nuclear oncogene.

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The p53 nuclear protein that was considered an oncogene has recently been shown to function as an anti-oncogene in normal cells. It was suggested that the wild type p53 protein functions as a suppressor oncogene, and that malignant transformation involves mutations in the gene that give rise to a mutated p53 protein, which has lost the capacity to suppress the outburst of the malignant phenotype.

In some primary tumors and established cell lines, p53 is expressed at high levels. However, it was found that myeloid-derived human cell lines are frequently p53 non-producers. For example, in HL-60, a p53 non-producer cell line established from a patient with acute promyelocytic leukemia, we found that the p53 gene was mostly deleted and significantly rearranged and, as a result, it failed to express a mature p53 protein molecule. Screening of primary human tumors also showed that while T and B lymphatic leukemias expressed high levels of p53 protein, malignancies of myeloid origin frequently lacked p53 expression. Compatible with the findings in humans, the p53 gene in cells derived from Friend erythroleukemic tumors in mice were found to exhibit a high incidence of rearrangements that either abrogated p53 protein synthesis or induced the expression of aberrant p53 protein forms, suggesting that the development of Friend leukemia in mice depends on p53 expression shut-off. All these findings support the conclusion that malignant transformation of cells of myeloid lineage may depend on p53 expression shut-off, possibly mediated by aberrations in the p53 gene structure.

The hypothesis that p53 functions in normal cells as an anti-oncogene is based mainly on the observation that the wild type p53 protein, which lacks any transforming activity in an in vitro assay, actively suppresses the transforming activity of other oncogenes. Indeed, it was found that while the mutated p53 protein forms induce transformation of primary embryonic cells in conjunction with the ras oncogene, the wild type cDNA codes for an inactive p53 protein. Furthermore, Finlay et al showed recently that the wild type p53 directly suppresses the transforming activity mediated by the E1A and the ras oncogenes, supporting the notion that under certain conditions, p53 may function as an anti-oncogene. This concept was further advanced by recent findings in colorectal carcinomas, which exhibit a high incidence of allelic deletions of the short arm of chromosome 17, to which the p53 gene was mapped. In these tumors, it was found that the remaining p53 allele was mutated at the highly conserved region of the p53 gene. These mutations coincide with the mutation of the murine p53 gene, suggesting that mutations in the p53 gene are involved in colorectal neoplasia, possibly through inactivation of the tumor suppression function of the wild type p53 gene.

To further elucidate the interrelationships between structural aberrations in the p53 oncogene and the development of tumors in humans, we focused on a malignancy which involves karyotypic changes in chromosome 17-Philadelphia chromosome (Ph) positive chronic myelogenous leukemia (CML). The natural clinical course of the disease—from the unstable chronic phase through the accelerated phase to the blastic crisis, which is accompanied by frequent alterations in chromosome 17—is well-suited for evaluating the role of the p53 oncogene in the progression of the disease.

RESULTS

p53 Gene polymorphism. Analysis of about 100 human primary tumors and established cell lines has revealed several restriction site polymorphisms in the human p53 gene. One interesting gene polymorphism is the existence of FnuDII restriction sites in exon No. 4 of the p53 gene.
Sequence analyses of various cDNA clones that we isolated indicate that this site is created by a shift of a C to a G, coding for a proline or an arginine, respectively, at codon 72, leading to the expression of a faster- or a slower-migrating protein on polyacrylamide gels.45,46 We found a random distribution of this gene polymorphism in p53 producer primary tumors and established cell lines, suggesting that the protein properties encoded by this gene polymorphism are not directly associated with any specific malignancy. Another gene polymorphism we discovered was a BamHI restriction site, found at the 5' region upstream to the first p53 noncoding exon. Again, this polymorphism was randomly distributed, at a rather lower incidence, and was not correlated with any specific malignancy. A third polymorphic site, which turned out to be important in detecting rearrangements in the Ph positive CML patients, was the polymorphic BglII site in the first noncoding large intron, situated 3 kb downstream from the first BglII site. Figure 1b is the restriction map of the p53 gene, with the polymorphic sites outlined.

Using Southern blot analysis, Fig la compares genomic DNA exhibiting a BglII polymorphic site with genomic DNA lacking that site. Detailed restriction analysis clearly shows that in both DNAs the patterns formed by the various fragments generated by a number of restriction enzymes were identical. The only difference detected was a second BglII polymorphic site. We found that 30% of the DNAs from either healthy donors or CML patients contained the polymorphic BglII site on one of the alleles only, and therefore, they exhibited two BglII fragments, 12 kb and 9 kb in size. In none of the DNAs examined did we detect the polymorphic BglII site in both alleles. Seventy percent of the DNAs did not contain the polymorphic BglII, and therefore, restriction gave rise to a 12 kb fragment only. In all genomic DNAs tested, the 3 kb BglII fragment, which maps to the 3' end of the gene, was evident.

**Southern blot analysis of DNA obtained from CML patients in blastic crisis.** In the present study we scored 53 Ph positive CML patients from several hospitals in Israel. To permit the detection and characterization of the entire p53 gene, our screening strategy consisted of cutting high molecular genomic DNA with the BglII restriction enzyme and probing both with the 3.8 kb EcoRI, which we isolated from a genomic library (probe A), and with the p53-H19 cDNA clone, which we isolated from a cDNA library (probe B). For further detailed analysis, we used a cDNA Rsal-Rsal fragment (592 bp), which probes exons 2 to 4 specifically (probe C). This fragment, which was isolated from the pSP-H-19 expression vector, contains at the 5' end 92 bp of the pSP65 vector as well as 94 bp of the first exon. Of the 53 patients, 39 were in the chronic phase and 14 were in either blastic crisis or accelerated phase. The phase of the disease was determined by standard clinical and hematological criteria, as well as by cytogenetic analysis (see legend to Table 1).

One of the 39 patients at the chronic phase exhibited rearrangements in the p53 gene; four of the 14 patients in
the p53-H19 probe. Although the distribution of the rear-
end of the gene seemed to be intact. Indeed, all patients
primary embryonic fibroblasts (data not shown). In general,
specific
of DNA from patient S.A. were amplified, whereas DNA of patient Y.H. contained only single copies of the corresponding fragments.

The novel 8 kb BgII fragment detected by probe B hybridized with neither probe A nor probe C (Fig 1). This suggests that the 8 kb fragment shares homology with the central part of the p53 gene, bordering at the 3' end of exon 4 and the 5' end of exon 10. In these DNAs, no additional alterations were found after hybridization with probe A, suggesting that the 5' end of the gene is intact. Figure 3 represents an example of a detailed Southern analysis of DNA obtained from S.A., showing the pattern of the BgII derived fragment hybridizing with the various p53 probes. In this example, DNA was first hybridized with p53-H19 (probe B), showing the amplified fragments on top of other expected bands. The blot was stripped and then hybridized with probe A, indicating the 5' end, large BgII fragment. Finally, the blot was hybridized with probe C, which failed to bind to the 8 kb fragment. In the last hybridization, the residual band (top band), representing the 5' end of the gene, was also evident, because the last stripping was not efficient. In DNA of patient B.A., who was still in chronic phase, a smaller rearranged BgII fragment was evident (6 kb). The same pattern was also found in the DNA of patients Y.B. and B.S., both in blastic crisis. This smaller BgII fragment hybridized with the full-length p53-H19 and with probe C, suggesting that it was generated by deletions in either the first large intron or in sequences downstream of exon 4.

The DNA of patient Y.B. is interesting in the sense that it contains both the 8 kb fragment that does not hybridize with probe C and the 6 kb fragment that does. Moreover, it contains only a single copy of the 12 kb BgII fragment, which suggests the existence of rearrangements in at least one allele at the 5' end of the p53 gene (Fig 4). Using an additional probe specific for exon 5 and 6 (a PvuII-Bst36I, 237 bp insert) we could detect the novel BgII 8 kb fragment, suggesting that the rearrangement in the p53 gene occurred

<table>
<thead>
<tr>
<th>Phase of Disease</th>
<th>No. of Patients</th>
<th>BgII Polymorphism</th>
<th>Rearrangements†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>39</td>
<td>30%</td>
<td>1/39</td>
</tr>
<tr>
<td>Accelerated and blastic crisis</td>
<td>14</td>
<td>50%</td>
<td>4/14</td>
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The diagnostic criteria for CML were the conventional criteria and included history and physical examination, a typical peripheral blood and bone marrow picture with increased myeloid elements, low alkaline phosphatase activity in circulating neutrophils and the presence of Ph in the metaphases of marrow cells. Transformation from chronic phase to accelerated phase or blastic crisis was determined by standard clinical and hematological criteria as well as by cytogenetic analysis. Blood samples were collected from several hospitals in Israel. Leukemic cells were isolated from the buffy coat of peripheral blood and high molecular weight DNA was extracted.

* BgII polymorphism was determined by digesting genomic DNA with BgII and probing with the p53-H19 cDNA clone.

† Various genomic blots were probed with the p53-derived fragments described in detail in the legend to Figure 1.

Blastic crisis had rearrangements. Figure 2 depicts the five cases which exhibited rearrangements in the p53 gene. In each of the experiments, the pattern of BgII-derived fragments of DNA from a patient in blastic crisis was compared with that of DNA of another CML patient, analyzed under the same conditions. A summary of this analysis, showing the specific bands detected with p53-H19, is presented in Fig 2. In each of these experiments, we also compared genomic DNA obtained from established human cell lines or normal primary embryonic fibroblasts (data not shown). In general, all CML patients who exhibited rearrangements in the p53 gene contained the BgII polymorphic site. Moreover, the 3' end of the gene seemed to be intact. Indeed, all patients tested had an intact 3 kb BgII fragment hybridizing with the p53-H19 probe. Although the distribution of the rearranged BgII fragments of the various patients seemed rather random, we noticed the appearance of two novel BgII fragments. Digestion of DNA obtained from patients S.A. and Y.H. showed a novel fragment of about 8 kb, in addition to the three typical BgII fragments detected with the p53-H19 (12, 9, and 3 kb long, respectively). Judging by the hybridization intensities, the 9 kb fragment and the novel 8 kb fragment of DNA from patient S.A. were amplified, whereas DNA of patient Y.H. contained only single copies of the corresponding fragments.

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![Fig 2. Rearrangements in the p53 gene of CML patients. High molecular weight DNA was digested with BgII, fragments were separated through a 0.8% agarose gel, blotted onto a nitrocellulose paper and hybridized with probe B. (see Results). * DNA obtained from CML patients at blastic crisis; ** DNA obtained from a CML patient in the chronic phase.](image-url)
REARRANGEMENTS IN THE p53 GENE

Fig 3. Southern blot analysis of CML patients using various p53-specific probes. High molecular weight DNA obtained from CML patient at blastic crisis (S.A.*) and from patients at chronic phase (H.A., Y.E.) were digested with BglII and hybridized with probes A, B, and C (see Results). The blot was first hybridized with probe B, then after exposure the blot was denatured in NaOH to remove probe B. Second hybridization was done with probe A, and finally, probe C was used. Arrows, DNA bands.

in sequences bordering between exon 4 and exon 5. It should be added that in several patients we have further extended our analysis by using other restriction enzymes, which again confirmed the existence of rearrangements in the p53 gene. Figure 5 illustrates an example of Southern blot analysis of genomic DNA obtained from a CML patient (B.A.) that was restricted with EcoRI and a combination of BamHI and BglII, in addition to the BglII used routinely. The pattern of fragments obtained by using this variety of enzymes strongly supports the conclusion that the DNA is rearranged and that BglII analysis is not representing a single point mutation but rather an authentic p53 gene rearrangement.

Because our analysis was performed on DNA extracted from mixed populations of cells containing a variable percentage of leukemic cells, we could not exclude the possibility that the non-rearranged BglII fragments were derived from DNA of the normal population, whereas the rearranged BglII fragments were derived from DNA of the leukemic cells. The alternative possibility—that leukemic cells contain both intact and rearranged p53 sequences—is also valid.

Fig 4. Detailed genomic analysis of DNA obtained from a patient with CML. High molecular weight DNA obtained from CML patient at blastic crisis (Y.B.*), a patient at a chronic phase (T.C.), and from a transformed fibroblastic cell line (SV-80) were digested with BglII and hybridized with either probe B or probe C (see Results).

Fig 5. Southern blot analysis of DNA obtained from CML patients using several restriction enzymes. High molecular weight DNA obtained from CML patients B.A. and S.T. as well as DNA from SV-80 transformed cell line were digested with several enzymes and hybridized with probe B.
Although we screened a rather small number of CML patients (53), the distribution of p53 rearrangements in CML patients at blastic crisis (4 of 14) is significant (Table 1). The fact that we also detected p53 rearrangements in a patient at chronic phase (B.A.) implies that clinical staging in this group of patients and appearance of rearrangements do not always coincide. The alternative possibility, provided DNA analysis has a higher resolution efficiency than clinical monitoring, is that patient B.A., who was diagnosed as being in the chronic phase, may at the time of testing have been about to enter into the accelerated phase of the disease. To further evaluate the significance of these findings, we shall increase the number of patients analyzed and follow changes in individuals as the disease progresses.

**DISCUSSION**

The p53 nuclear protein is overproduced in a large number of tumor cells and has been found to be directly associated with the induction of the malignant process. In agreement with the concept that malignant transformation is a multi-stage process, it can be assumed that the oncogenic p53 protein, activated by various cellular signals, may in turn induce or suppress the expression of other cellular genes involved in this chain of reactions. In certain cell lineages, malignancies may involve p53 overproduction, whereas in others p53 may function as an anti-oncogene. Malignant transformation in these cases might be conditioned by p53 expression shut-off.

The multiple facets of the biological activity of p53 make it an attractive model system for an in-depth investigation of the interrelationships between oncogene activity and the neoplastic process. p53 might be an example of a normal regulatory protein, the perturbation of the expression of which can lead to transformation. In the present study we found p53 gene rearrangements which are most likely to interfere with the expression of the gene in these tumors. At this stage of our study, it is not clear whether rearrangements in the p53 gene in these malignancies induce shut-off of p53 expression or whether these translocations induce the appearance of a novel mRNA product that may account for the progression of the disease. If these rearrangements cause p53 down-regulation, then our findings are compatible with the conclusion that p53 functions as an anti-oncogene in the chronic phase of the disease. Therefore, the progression of the disease depends on inactivation of the p53 nuclear oncogene. This agrees with the concept recently suggesting that progression of the malignant process may be dependent on the inactivation of the wild type p53 gene.

Chronically myelogenous leukemia is a clonal disorder arising from a neoplastic transformation at the level of the pluripotent hematopoietic stem cell. The typical clinical course of CML involves progression from the chronic protracted phase, through the accelerated phase, to the blastic crisis. This natural history of CML makes the disease well-suited for the study of human tumor progression.

CML is characterized cytogenetically by a reciprocal translocation between chromosomes 9 and 22, t(9:22) (q34:q11), which results in a 22q+ chromosome or Ph and a 9q− chromosome. In this translocation, sequences of the abl protooncogene and of the breakpoint cluster region (bcr) gene on chromosomes 9 and 22, respectively, join to form a bcr-abl fusion gene on the Ph. An 8.5 kb mRNA is transcribed from the hybrid gene and is translated into a fusion protein (bcr-abl) of 210 kd. This protein is believed to play an important role in the pathogenesis of CML, but apparently not in the progression from a chronic phase to a more advanced one. While it is well established that the chronic phase is directly associated with the rearrangements of the abl oncogene, no consistent aberrations in known oncogenes coincide with the blastic crisis phase. The molecular events leading to the inevitable malignant evolution from a chronic to an acute leukemia phenotype remain largely unexplored. However, the frequent and nonrandom cytogenetic changes detected with disease progression (in 80% of patients) suggest that superimposed secondary genetic events account for the evolution to blastic crisis.

Numerous surveys have shown a remarkable consistency in the type of chromosomal alterations superimposed on the Ph, most commonly two copies of the Ph, trisomy 8 (+8) and an isochromosome 17 (i17q).

Additional genetic alterations, most probably involving structural changes in other oncogenes, may provide a “second hit,” which causes loss of differentiation potential by the leukemic cells and their transition to blastic phase. Liu et al were the first to report such activation. Using gene transfer and tumorigenesis assays, they detected mutant alleles of ras proto-oncogenes in chronic phase and, mainly, in blastic crisis. In addition, amplification and rearrangements of c-myc have been found in the acute phase of CML patients and may play a role in the progression of the disease.

In the present study we screened the p53 gene structure in CML patients and found rearrangements in the p53 gene in about 30% of patients at blastic crisis. The rearrangements which we detected were rather gross, and it is possible that the actual incidence of p53 gene alterations was higher but that, under the resolution conditions used, we were unable to detect minor aberrations or mutations occurring in CML patients at the acute phase.

In agreement with this, we advance the hypothesis that rearrangements in the p53 oncogene may be an important facet in the progression from the chronic phase, in which the typical Ph rearrangement is exhibited, to the more severe phase, with additional genetic aberrations exhibited as rearrangements in the p53 gene.

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Rearrangements in the p53 gene in Philadelphia chromosome positive chronic myelogenous leukemia [see comments]

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