Nonhereditary p53 Mutations in T-Cell Acute Lymphoblastic Leukemia Are Associated With the Relapse Phase

By Michael H. Hsiiao, Alice L. Yu, Jo Yearygin, David Ku, and Martin Haas

We have previously reported that greater than 60% of human leukemic T-cell lines possess mutations in the p53 tumor suppressor gene. To determine whether T-cell acute lymphoblastic leukemia (T-ALL) patient samples possess p53 mutations, we screened peripheral blood- and bone marrow-derived leukemia samples, taken at diagnosis and at relapse, for p53 mutations. Exons 4 through 9 and selected intron regions of the p53 gene were analyzed using polymerase chain reaction–single-strand conformation polymorphism and direct sequencing. p53 mutations were found in 0 of 15 T-ALL diagnosis samples, as compared with 10 of 36 (28%) T-ALL relapse samples. To determine whether p53 mutations play a role in the recurrence (relapse) of T-ALL, two special groups of T-ALL patients were studied: (1) a group of 8 relapse patients whose disease was refractory to chemotherapeutic treatment, and (2) a group of 6 “paired” T-ALL cell samples from patients for whom we possess both diagnosis and relapse samples. Three of 8 relapsed patients (37.5%) whose disease was refractory to the reinduction of remission by chemotherapy possessed missense mutations of the p53 gene. All 3 cases had mutations in exon 5. Among the paired samples, 3 of 6 patients harbored p53 mutations at disease recurrence, but possessed only wild-type p53 alleles at diagnosis. One case had mutation on exon 4, 1 case in exon 5, and 1 case in exon 8 with loss of heterozygosity. These data clearly indicate that recurrence of T-ALL is associated with missense mutations in p53. Our results indicate that (1) mutations of p53 do occur in T-ALL in vivo, and such mutations are associated with the relapse phase of the disease; and (2) p53 mutation is involved in the progression of T-ALL. This conclusion is supported by our observation that the introduction of T-ALL–derived mutant p53 expression constructs into T-ALL cell lines further increases their growth rate in culture, enhances cell cloning in methylcellulose, and increases tumor formation in nude mice.

P53 BELONGS TO THE tumor suppressor class of genes whose function involves the negative regulation of cell growth. The loss of function of the p53 gene by deletion, mutation, or rearrangement may contribute to the genesis or progression of a wide variety of human cancers.1-3 Abnormalities in the p53 gene have been demonstrated in human carcinoma of the colorectum,3 liver,4-6 bladder,7 and ovary;8 in blast crisis chronic myelogenous and myelocytic leukemias9,10,11, in progressed adult T-cell leukemia12,13, and in osteogenic sarcomas.14,15 Several lines of evidence suggest that p53 mutation/deletion is a late event in the development of cancer, because (1) in carcinomas of the colorectum, chromosome 17p deletions are associated with the transition from benign adenoma to malignant carcinoma;16 (2) the progression of brain tumors is associated with a clonal expansion of cells that have acquired a mutation in the p53 gene;17 (3) the evolution of chronic-phase chronic myelogenous leukemia (CML) to myeloid blast crisis18-20 is associated with mutation of the p53 gene; and (4) the mutation of p53 and the loss of heterozygosity of chromosome 17p and 10 are associated with the progression of astrocytomas.21 However, mutations in p53 have also been found in the precancerous phases of adenocarcinomas and in adenomatous polyps of patients with familial polyposis coli,22 suggesting that p53 mutation may occur as an early event in carcinogenesis as well.

In our studies on the role of p53 in the pathogenesis of human T-cell acute lymphoblastic leukemia (T-ALL), we have shown that greater than 60% of T-ALL cell lines, all of which had been grown from relapse T-ALL cases, possess mutations on both p53 alleles,23 suggesting that p53 serves a critical role in the generation of the fully tumorigenic leukemic T cells. However, our experiments did not show whether these p53 mutations originated in vivo or whether the p53 gene had been mutated during cell line establishment in vitro, as has been shown to occur during the establishment of some rat embryo fibroblast cell lines.24 Gaidano et al25 and Jouveaux and Berger26 have reported that samples from T-ALL patients obtained at diagnosis harbor no mutations in the p53 gene. In all, these two groups studied 37 T-ALL diagnosis samples, all of which possessed only wild-type p53 alleles. Recently, we reported that T-ALL cell lines established in our laboratory from a patient sample taken at relapse possess the same p53 mutation found in the in vivo sample.27 Furthermore, no additional mutations of p53 occurred during establishment of the cell lines. These results suggest that the establishment of human T-ALL cell lines need not be associated with the induction of p53 mutations.

To study whether p53 mutations may be differentially associated with the relapse versus diagnosis phases of T-ALL, we used polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP)28 and direct sequencing of PCR-amplified fragments to study 15 cases of T-ALL at diagnosis and 36 cases at relapse. The results suggest that, in the patients studied, mutation of the p53 gene was associated with the clonal evolution that takes place during recurrence of the disease.

MATERIALS AND METHODS

T-ALL patient samples. Peripheral blood or bone marrow cells from 51 T-ALL patients at diagnosis or at relapse were donated in
p53 mutations in relapse acute T leukemia

RESULTS

p53 mutation is a frequent genetic change in relapse T-ALL

We used PCR-SSCP analysis to screen for p53 mutations in the genomic DNA of 51 patients with childhood acute lymphoblastic leukemia at different clinical stages (15 diagnosis T-ALL, 36 relapse T-ALL). Significant electrophoretic mobility shifts were detected in 10 of the 36 relapse T-ALL samples (28%), and in none (0 of 15) of the diagnosis T-ALL samples (0%). Figure 1 shows examples of PCR-SSCP analyses of exons 4 and 5. DNA extracted from a normal human placenta was used as normal control to show the germline bands. The T-ALL cell line CEM, which harbors a mutation in p53 codon 175, was used as a mutant control of exon 5 to show different mobility shift patterns under two different gel conditions (see legend to Fig 1 and Materials and Methods).

Direct sequence analysis was performed on the 10 samples found to be positive for p53 mutations based on significant SSCP shifts. Most of the mutations found were missense mutations with single-base changes that encoded single amino acid substitutions in the p53 protein (Table 1). The majority of the mutations found are located in exon 5. One patient, PJN, had an intron 4 mutation. Although its biologic significance has not been studied further, its detection shows that cells harboring this mutation were selected for; hence,
Table 1. Frequency and Nature of p53 Mutations in Primary T-ALL Samples

<table>
<thead>
<tr>
<th>T-ALL Patient Sample</th>
<th>SSCP Codon</th>
<th>Seq Change</th>
<th>aa Change</th>
<th>Genotype</th>
<th>% Blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnoses</td>
<td>0/15 (0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relapse 10/36 (28%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMN</td>
<td>Ex 4 110</td>
<td>CGT → CTT</td>
<td>Arg → Leu</td>
<td>Heterozygous</td>
<td>95</td>
</tr>
<tr>
<td>FAN</td>
<td>Ex 5 135</td>
<td>TGC → AGC</td>
<td>Cys → Ser</td>
<td>Heterozygous</td>
<td>92</td>
</tr>
<tr>
<td>ONN</td>
<td>Ex 5 135</td>
<td>TGC → AGC</td>
<td>Cys → Ser</td>
<td>Heterozygous</td>
<td>90</td>
</tr>
<tr>
<td>ASN</td>
<td>Ex 5 135</td>
<td>TGC → TAC</td>
<td>Cys → Tyr</td>
<td>Homozygous*</td>
<td></td>
</tr>
<tr>
<td>GCN</td>
<td>Ex 5 141</td>
<td>TGC → TTC</td>
<td>Cys → Phe</td>
<td>Heterozygous</td>
<td>91</td>
</tr>
<tr>
<td>SRN</td>
<td>Ex 5 161</td>
<td>GCC → ACC</td>
<td>Ala → Thr</td>
<td>Heterozygous</td>
<td>90</td>
</tr>
<tr>
<td>FMN</td>
<td>Ex 5 167</td>
<td>CAG → CGG</td>
<td>Gln → Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAN</td>
<td>Ex 5 175</td>
<td>AGC → ATG</td>
<td>Thr → Met</td>
<td>Homozygous*</td>
<td></td>
</tr>
<tr>
<td>BTN</td>
<td>Ex 8 282</td>
<td>CGG → GGG</td>
<td>Arg → Gly</td>
<td>Heterozygous</td>
<td>93</td>
</tr>
<tr>
<td>Pjn</td>
<td>Intron 4</td>
<td>C → T</td>
<td></td>
<td>Heterozygous†</td>
<td></td>
</tr>
</tbody>
</table>

* Wild-type allele at this locus undetectable on sequencing gel, but no LOH by codon 72 polymorphism (see Fig 7).
† Homozygous mutation verified by LOH determination (see Fig 7).
‡ C → T mutation occurs in intron 4 at position 13049 (Ref: Genebank sequence accession no. X54156).

This intronic mutation may possess biologic significance. Figures 2 and 3 show representative examples of the direct sequencing data. Figure 2 demonstrates two relapse T-ALL samples (GCN and HAN, respectively) with heterozygous missense mutations in exon 5. Interestingly, three separate cases possess mutations at codon 135. Two of these cases (FAN and ONN) possess a heterozygous mutation with nucleotide change from TGC to A/TGC (135YY6), whereas a homozygous mutation with nucleotide change from TGC to TAC (135YYs) was found in the peripheral blood-derived leukemic cells of patient ASN (Fig 3).

The apparent heterozygous nature of the mutations summarized in Table 1 may be caused by contamination of the leukemia samples with normal cells. Table 1 shows that at the time of DNA extraction the percent blast cells present in the patients’ cell samples was at least 90%. The minority (wild-type) alleles detected could not have been caused by contamination with normal cells at the less than 10% level, because in a mixture of mutant and wild-type DNA molecules the minority component is detectable on SSCP or sequencing gels only when present at the 20% level or higher. Hence, we conclude that the heterozygous nature of the mutations presented in Table 1 and in the corresponding figures represent mutations in one allele of the leukemia cells, whereas the other allele remains wild-type. To verify this conclusion, we have tested whether unrearranged (ie, germline) T-cell receptor (TCR) β-chain DNA could be demonstrated in the samples tested. Southern blots containing BamHI-digested genomic T-ALL DNA were hybridized with a probe specific for the human T β locus. All 6 samples showed rearranged TCRβ bands, whereas in only one (ONN) patient sample could a band of apparent germline size be shown (data not shown). Hence, the p53 mutations shown in Table 1 represent bona fide heterozygous mutations that are not caused by contamination of the leukemia cell samples with normal cells.

EXON 5

Fig 2. DNA sequence identification of exon 5 mutations detected by SSCP. Shown are codons 141 and 175 heterozygous mutations. Human placenta DNA serves as normal control.
p53 mutation is somatically acquired and is associated with the recurrence of T-ALL. Ten of 36 relapse T-ALL cases studied (28%) possess p53 mutations in the p53 gene. Because these are random T-ALL patient samples collected during a 5-year period, there is no means of determining whether the mutations found are germline or somatically acquired, nor what role, if any, p53 mutations play in the recurrence of T-ALL. To determine the pathologic significance of p53 mutations in the recurrence (relapse) of T-ALL, a group of 6 “paired” T-ALL cell samples from patients for whom we possess both diagnosis and relapse samples were studied. PCR-SSCP and direct sequencing showed that none of the samples taken at diagnosis possessed p53 mutations, whereas 3 of 6 (50%) of the “paired” T-ALL samples studied harbored missense mutations at the relapse phase of the disease (Table 2). Figure 4 shows that a mutation was present in the leukemic cells of patient SMN at relapse. This mutation was absent from SMN’s leukemic cells at diagnosis. PCR-direct sequencing data identify the nature of this mutation as a heterozygous missense mutation with a nucleotide change from CGT to CTT (110Arg → Leu). A heterozygous missense mutation with a nucleotide change from GCC to ACC (161Ala → Thr) was found in the leukemic cells of patient SRN at relapse but not at diagnosis (Fig 5). Furthermore, a homozygous missense mutation was detected in the leukemic cells of patient BTN at relapse but not at diagnosis. Figure 6 shows this mutation as found by SSCP (arrow). Direct sequencing data establish the nucleotide change at relapse, a codon change from CGG to GGG (282Arg → Gly).

We also studied whether p53 mutations were present in the cells of a group of 8 T-ALL relapse patients whose

### Table 2. p53 Mutations in Paired Diagnosis/Relapse T-ALL Patient Samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>SSCP (exon)</th>
<th>Identification of Mutation</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>AHN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SZN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SZN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMN/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMN/R</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: /D, patient sample obtained at diagnosis; /R, patient sample obtained at relapse.
*Homozygous/heterozygous mutations, genotype verified by codon 72 polymorphism (see Fig 7).
Loss of heterozygosity at the p53 locus is an infrequent event in relapse T-ALL. In human solid tumors, mutations of the p53 gene are frequently accompanied by the loss of heterozygosity of chromosome 17q13.1, the locus to which the p53 gene maps. We have previously reported that, in leukemia cell lines, both alleles of the p53 gene were often independently mutated without concomitant loss of heterozygosity at the 17q13.1 locus. To investigate whether loss of heterozygosity of p53 is a frequent event during recurrence of T-ALL, we used the known polymorphism at codon 72 of the human p53 gene.32-35 This polymorphism is encoded by sequences in exon 4 of the p53 gene. The frequency of the 72Gly- and the 72Arg-encoding alleles (CGC and CCC, respectively) in the human population is 0.68 and 0.32, respectively.36 Thus, 44% of the human population are heterozygous at this locus, whereas 46% and 10% encode homozy-
gous (arg/arg)- or homozygous (pro/pro)-containing p53 proteins, respectively.

We applied PCR-SSCP analysis of exon 4 to determine the polymorphic status at codon 72 in "paired" diagnosis and relapse leukemia samples taken from the same patients. Five of the 6 "paired" leukemia samples were heterozygous at codon 72 (4 of these are shown in Fig 7), whereas 1 of the 6 "paired" patient samples, LMN, showed the homozygous arg/arg genotype at this position (Fig 7). Only 1 of the 3 leukemia cases that harbored a p53 mutation at relapse (Table 2), patient BTN, also underwent loss of heterozygosity upon leukemia relapse, by losing the arg-encoding p53 allele. As can be seen, the band of the amplified 72<sup>rst</sup>-encoding alleles of patient BTN’s relapse sample is of double intensity compared with the cases that were heterozygous at this codon. The status of codon 72 in the alleles carried by the leukemia cell lines MOLT-4 (arg/arg) and CEM (arg/pro), which we have previously shown by immunoprecipitation to harbor alleles encoding homozygous (arg/arg) and heterozygous (arg/pro) p53 proteins, respectively, are shown as internal controls. Subsequent DNA sequencing has shown that the arginine allele was indeed lost during the recurrence (relapse) of disease in patient BTN (Fig 7, right).

**DISCUSSION**

We have previously shown that acute lymphoblastic T-cell leukemia cell lines, all of which were derived from relapse T-ALL patients, frequently harbor mutations in the p53 gene. In contrast, T-ALL patient samples have been reported to lack such mutations. This suggested that p53 mutations that are found in cell lines may have been induced during in vitro establishment of the lines. However, in vitro mutation of p53 during the establishment of acute leukemia cell lines seemed unlikely because we have found in other experiments that, during establishment of multiple T-ALL cell lines, the p53 gene remained stably unaltered when compared with its state in the patients’ leukemia cells. This discrepancy was resolved in the current experiments by screening 15 untreated (“diagnosis”) and 36 recurrent (“relapse”) T-ALL cases, 12 of which were donated, pairwise, by the same patients at the diagnosis and at the relapse phases of their disease.

Among the 15 diagnosis T-ALL cases studied here, none possess p53 mutations in exons 4 through 9. Adding these 15 diagnosis cases to those reported by a total of 52 diagnosis T-ALL cases have now been reported to lack p53 mutations, supporting the notion that mutation of the p53 gene is not a causal event in the induction of childhood T-ALL. In contrast to diagnosis T-ALL cases, among 36 relapse cases studied, 10 (28%) harbored mutations in the p53 gene. Most (9 of 10) of these mutant p53 alleles specified missense mutations that gave rise to nonconservative amino acid exchanges, whereas 1 occurred in intron 4. The majority of these mutations were mapped to the highly conserved, T-
antigen binding region of the p53 protein,38 suggesting that the mutations were biologically significant by conveying a selective growth advantage to the cells so endowed. In recent work (M. Hsiao, E. Dorn, J. Yeargin, and M. Haas, manuscript submitted), we have observed that mutated p53 genes that had been cloned from relapse T-ALL cells and constructed into retroviral expression vectors indeed possess dominant oncogenic activity when introduced into T-ALL cells, as assayed by in vitro and in vivo criteria. Thus, p53 dominant oncogenic activity when introduced into T-ALL diagnosis samples lacked detectable p53 mutations in exons 5 and 6. Doubt, that mutation of the p53 gene in relapse samples is evaluable polymorphic cases displayed the mutations were biologically significant by conveying a selectively advantageous growth advantage to the cells.6

The lack of p53 mutations in any of the “diagnosis” samples suggests, but does not prove beyond a reasonable doubt, that mutation of the p53 gene in relapse samples is specifically associated with the progression of the leukemic phenotype. To prove that p53 mutations in relapse leukemia represent clonal evolution events, we compared the status of the p53 gene in diagnosis and in relapse samples in a pairwise fashion. Six pairs of leukemia cell samples, each derived from the same patients at different points in the clinical course, were studied for the status of the p53 gene. All six pairs of relapse leukemia cells most probably play a biologically significant role in the disease.

The antigen binding region of the p53 protein, suggesting that the mutations were biologically significant by conveying a selective growth advantage to the cells so endowed. In recent work (M. Hsiao, E. Dorn, J. Yeargin, and M. Haas, manuscript submitted), we have observed that mutated p53 genes that had been cloned from relapse T-ALL cells and constructed into retroviral expression vectors indeed possess dominant oncogenic activity when introduced into T-ALL cells, as assayed by in vitro and in vivo criteria. Thus, p53 mutations in relapse T-ALL cells most probably play a biologically significant role in the disease.

The lack of p53 mutations in any of the “diagnosis” samples suggests, but does not prove beyond a reasonable doubt, that mutation of the p53 gene in relapse samples is specifically associated with the progression of the leukemic phenotype. To prove that p53 mutations in relapse leukemia represent clonal evolution events, we compared the status of the p53 gene in diagnosis and in relapse samples in a pairwise fashion. Six pairs of leukemia cell samples, each derived from the same patients at different points in the clinical course, were studied for the status of the p53 gene. All six pairs of relapse leukemia cells most probably play a biologically significant role in the disease.

The antigen binding region of the p53 protein, suggesting that the mutations were biologically significant by conveying a selective growth advantage to the cells so endowed. In recent work (M. Hsiao, E. Dorn, J. Yeargin, and M. Haas, manuscript submitted), we have observed that mutated p53 genes that had been cloned from relapse T-ALL cells and constructed into retroviral expression vectors indeed possess dominant oncogenic activity when introduced into T-ALL cells, as assayed by in vitro and in vivo criteria. Thus, p53 mutations in relapse T-ALL cells most probably play a biologically significant role in the disease.

The lack of p53 mutations in any of the “diagnosis” samples suggests, but does not prove beyond a reasonable doubt, that mutation of the p53 gene in relapse samples is specifically associated with the progression of the leukemic phenotype. To prove that p53 mutations in relapse leukemia represent clonal evolution events, we compared the status of the p53 gene in diagnosis and in relapse samples in a pairwise fashion. Six pairs of leukemia cell samples, each derived from the same patients at different points in the clinical course, were studied for the status of the p53 gene. All six pairs of relapse leukemia cells most probably play a biologically significant role in the disease.

Fig 7. Detection of allelic loss at the p53 locus in “paired” diagnosis/relapse patient samples by SSCP analysis of the polymorphic marker at codon 72. Cell lines MOLT-4 and CEM were used as controls. MOLT-4 cells are homozygous (codon72<sup>WT</sup>), whereas CEM cells are heterozygous (72<sup>mut</sup>/72<sup>WT</sup>) for this polymorphic marker.36 The leukemic cells of patient BTN were observed to undergo loss of heterozygosity at the p53 locus (left panel), which was verified by direct sequencing (right panel). Gel conditions for SSCP: 4% MDE gel.

(a) SSCP analysis of the polymorphic marker at codon 72. The leukemic cells of patient BTN were observed to undergo loss of heterozygosity at the p53 locus (left panel), which was verified by direct sequencing (right panel). Gel conditions for SSCP: 4% MDE gel.
lie in the nature of acute leukemia cells as compared with cells of malignant solid tumors. The generation of malignant human solid tumors requires a series of 6 to 8 or more genetic alterations in the same cell; the malignant cells are "transformed" and transplantable, and possess many in vitro characteristics of "tumor cells." In contrast, in acute leukemias, malignancy is engendered by cells that have undergone fewer genetic alterations and that have few, if any, characteristics of "tumor cells." Specifically, acute leukemia cells are "malignant," i.e. life-threatening, even though they merely display the characteristics of differentiation-inhibited, proliferating precursor cells (ibid), and may lack many or most of the in vitro criteria of "tumor cells." We propose that, in acute leukemia cells, mutation of each p53 allele provides the leukemic cell with a growth advantage through the elimination of normal suppressor functions and, especially, through the acquisition of dominant oncogenic functions (M. Hsiao, E. Dorn, I. Yezrin, and M. Haas, manuscript submitted, and Dittmer et al.). Thus, whereas the acquisition of each dominantly acting mutated p53 allele is significant for the potentiation of the leukemic phenotype of acute leukemia cells, in solid tumors a fully tumorigenic and metastatic phenotype necessitates the loss of each functional p53 allele, hence the frequent loss of the functional allele through LOH.

REFERENCES

30. Cabanillas F, Patthak S, Grant G, Hagemanter FB, Mel-augh-
Nonhereditary p53 mutations in T-cell acute lymphoblastic leukemia are associated with the relapse phase

MH Hsiao, AL Yu, J Yeargin, D Ku and M Haas