Analysis of p53 Mutations in a Large Series of Lymphoid Hematologic Malignancies of Childhood


Mutations of p53 have been reported in several types of hematologic malignancies.1,5 Therefore, we analyzed a large series of lymphoid (330 cases) and a smaller series of myeloid (29 cases) malignancies of childhood for p53 mutations by single-strand conformational polymorphism (SSCP) following polymerase chain reaction. Samples with abnormal SSCP were reamplified and analyzed by direct sequencing method. p53 mutations were detected within the known mutational hotspots (exons 5 to 8) in 8 of 330 lymphoid malignancies, and in none of 29 myeloid malignancies, showing that the frequency of p53 mutations in childhood lymphoid malignancies was very low (8 of 330 cases [2%]). Four of these patients had very aggressive, fatal acute lymphocytic leukemia (ALL). None of 13 infants and none of 48 patients with T-lineage leukemia had detectable p53 mutations in their ALL cells. Exceptionally, p53 mutations were comparatively frequent in a small sample of B-cell non-Hodgkin’s lymphomas (2 of 8 cases). Mutations were detected in samples from two patients with ALL at relapse; these were not detected in samples at initial diagnosis from the same patients, suggesting that p53 mutations may be associated with progression to a more malignant phenotype. Seven of eight alterations of p53 were missense mutations, and seven of eight samples may be heterozygous for the mutant p53, indicating that p53 protein may act in a dominant negative fashion.

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HE p53 GENE IS位於 chromosome 17 at band p13.1.1-3 and it codes for a nuclear DNA-binding phosphoprotein with properties of a transcriptional activator.4 Alterations of the human p53 gene have been implicated in the pathogenesis of a wide variety of malignancies, including cancers of bone, lung, esophagus, breast, colon, brain, and liver.4°° Moreover, germline p53 mutations have been demonstrated in Li-Fraumeni syndrome; patients with this syndrome develop several types of malignancies, including breast cancers, sarcomas, brain tumors, and leukemias/lymphomas.12—14 These p53 mutations probably inactivate the tumor suppressor function of the p53 protein, contributing to the development of human malignancies.

Mutations of p53 have been reported in several types of hematologic malignancies.14-45 Previously, we reported that p53 mutations were frequent in adult T-cell leukemia (ATL) and may play a role in progression from chronic to acute phase.21 Further data suggest seven groups of hematologic malignancies with moderate frequency of p53 mutations: (1) Burkitt’s type acute lymphocytic leukemia (ALL) (L3) (39%)18,20; (2) Burkitt’s lymphoma (32%)18,20,24; (3) ATL (44%)21,23,24; (4) acquired immunodeficiency syndrome (AIDS)-related lymphoma (22%)18,33; (5) myeloid blast crisis (BC) of chronic myelogenous leukemia (CML) (27%)12,33,37; (6) acute leukemia with chromosome 17p deletion (32%)20,36; and (7) Richter’s transformation of B-cell chronic lymphocytic leukemia (42%).18 The incidence of p53 gene mutations in childhood hematologic malignancies is unclear; therefore, we analyzed a large series of lymphoid and a small number of myeloid malignancies of childhood for p53 mutations to determine if the mutations (1) localized to a specific subtype; (2) clustered to specific codons or regions; (3) had a specific alteration such as G:C → A:T transitions; or (4) correlated with clinical information.

MATERIALS AND METHODS

Patients. All cell samples used in this study were collected from pediatric patients in Germany from 1987 to 1990 after the individuals and/or their parents provided informed consent. The 330 ALL and non-Hodgkin’s lymphoma (NHL) cases were treated in the prospective multicenter ALL/NHL Berlin-Frankfurt-Münster (BFM) trial.46 Moreover, we included 27 children with acute myelogenous leukemia (AML) and 2 with pediatric CML blast crisis. As part of the BFM trial, all cases were immunophenotyped (W.D.L.) and immunogenotyped (C.R.B.) as specified below.

Immunophenotyping. Fresh bone marrow (BM) and/or peripheral blood (PB) samples of patients containing >80% blasts were isolated by standard Ficoll-Hypaque density gradient centrifugation, and surface expression was identified by an indirect immunofluorescence (IF) assay, as previously described.46-50 Nonspecific binding was avoided by adding heat-inactivated, 10% rabbit serum (GIBCO/BRL, Eggenstein, Germany) in the first and second incubation. Cells were evaluated for IF by epifluorescence Zeiss microscope or by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Background fluorescence, determined by using nonreactive monoclonal antibodies

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Specifically, the PCR mixture contained 100 ng of DNA, 10 pmol/L of each of the primers, 250 pmol/L of each of the four deoxynucleotides, 0.05 U/µL of Taq DNA polymerase (GIBCO BRL Inc, Gaithersburg, MD), 3 µCi[^32P]dCTP (3,000 Ci/mmol) in 20 µL of the specified buffer with 1.5 mmol/L MgCl₂. Thirty-five cycles of denaturing for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extending for 60 seconds at 72°C were performed in a MicroCycler (Eppendorf Inc, Fremont, CA). After amplification, PCR samples were diluted 10-fold in the loading buffer containing 20 mmol/L EDTA, 96% formamide, plus 0.05% each of bromophenol blue and xylene cyanol. The products were heated to 95°C for 5 minutes and chilled on ice. Dilutions (1 µL/lane) were separated on a 6% nondenaturing polyacrylamide gel with or without 10% (vol/vol) glycerol running at 300 V for 16 hours at room temperature. The gel was dried and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at ~80°C for 16 hours. For each experiment, positive and negative DNAs and water controls were run in parallel.

**Direct sequencing of PCR-amplified fragments.** Direct sequencing of p53 from PCR products were performed. Samples of DNA with altered migration by SSCP were reamplified as described above except that the reaction volume was 100 µL and the[^32P]dCTP was omitted. The amplified product was gel purified using either the freeze-fracture technique or electroelution method. The purified, double-stranded DNA was sequenced directly using deoxyribonucleotides and T7 DNA polymerase (Sequenase Kit Version 2.0: US Biochemicals, Cleveland, OH) or by ds DNA Cycle Sequencing System using PCR with Taq DNA polymerase (GIBCO BRL). All amplified exons were sequenced in both the sense and antisense directions.

**RESULTS**

**Analysis of p53 mutations.** A total of 359 cases were examined, including 312 of several types of ALLs (188 common ALLs [c-ALLs], 46 T-cell ALLs [T-ALLs], 43 pre-B-cell ALLs [pre-pre-B-ALLs], 33 pre-B-cell ALLs [pre-B-ALLs], 2 pre-T-cell ALLs [pre-T-ALLs], 18 T- and B-NHLs, 27 AMLs, and 2 CMLs in BC (Table I). Thirteen of these cases were infants and 19 samples were obtained at relapse. In 10 cases, we studied both materials derived at diagnosis and relapse. Altered migration of p53 was observed by single-strand conformational polymorphism (SSCP) analysis in 8 of 359 cases, including 5 of 312 (2%) ALLs (3 of 188 [2%] c-ALLs, 1 of 43 [2%] pre-pre-B-ALLs, and 1 of 33 [3%] pre-B-ALLs), 3 of 18 (17%) NHLs

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. Positive/ No. Examined</th>
<th>Frequency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lymphocytic leukemia</td>
<td>5/312</td>
<td>2</td>
</tr>
<tr>
<td>c-ALL</td>
<td>2/189</td>
<td>2</td>
</tr>
<tr>
<td>Pre-B ALL</td>
<td>1/33</td>
<td>3</td>
</tr>
<tr>
<td>Pre-pre-B ALL</td>
<td>1/43</td>
<td>2</td>
</tr>
<tr>
<td>T-ALL</td>
<td>0/46</td>
<td>0</td>
</tr>
<tr>
<td>Pre-T ALL</td>
<td>0/2</td>
<td>0</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>3/18</td>
<td>17</td>
</tr>
<tr>
<td>B-NHL</td>
<td>2/8</td>
<td>25</td>
</tr>
<tr>
<td>T-NHL</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td>0/27</td>
<td>0</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia in BC</td>
<td>0/2</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 1.** p53 Mutations in Childhood Hematologic Malignancies
P53 MUTATION IN CHILDHOOD LEUKEMIAS/LYMPHOMAS

Exon 5

Exon 7

Exon 8

Fig 1. SSCP analysis of p53 mutations in lymphoid malignancies of childhood. Radiolabeled, PCR-amplified fragments corresponding to exons 5, 7, and 8 after denaturation and electrophoresis on a 6% acrylamide gel containing 10% glycerol are shown in each panel. N, negative control; P, positive control. Case 1 shows an aberrant pattern of bands for exon 5; cases 3 and 5 show abnormal bands for exon 7; and case 6 has altered bands for exon 8. Numbers correspond to the patient numbers in Table 2; unlabeled lanes represent patient samples with normal pattern of bands.

including 2 of 8 (25%) B-NHLs and 1 of 10 (10%) T-NHLs and none of either 27 AMLs or 2 CMLs in BC (Table 1 and Fig 1). We analyzed 8 L3 neoplasias, including 2 B-ALLs and 6 B-NHLs, and detected altered p53 migrations in 2 of them (25%). Seven of the 8 samples with altered p53 migration showed not only altered bands but normal migrating bands, whereas a remaining sample (case no. 4, Table 2) had only altered bands, indicating homozygosity or hemizygosity for the p53 mutations. From 4 of 5 cases (nos. 2, 3, 4, and 8) who had a p53 mutation in lymphoid malignant cells and are still alive, we could obtain DNA samples in complete remission, and confirmed that they had only germline configuration of the p53 sequences, indicating that the p53 mutations occurred somatically in malignant cells. For case no. 4, in whom a hemizygous or homozygous p53 mutation was detected, we could also analyze a DNA sample from the mother of the patient, and only germline p53 sequences were detected.

Sequencing of samples that were abnormal by SSCP confirmed that each had a p53 mutation. These were detected in exon 5 at codons 142 and 175; in exon 7 at codons 240, 245 and 248; and in exon 8 at codons 273, 282, and 297 (Table 2 and Fig 2). Seven of the 8 alterations were base substitutions resulting in missense mutations. One was an insertion of C at codon 142 causing a frameshift generating a new stop codon (ATG) 16 bp downstream. Six of 8 alterations were C:G to T:A transitional mutations; and five of these occurred at CpG sites (Table 2).

Immunogenotypic and clinical characterization. All the samples were not only immunophenotyped but also immunogenotyped. The immunogenotypic data of the p53 mutation-positive cases are listed in Table 3. In 10 cases, in which both materials at initial diagnosis and at relapse were analyzed, the immunogenotype of the two cases (nos. 1 and 3) exhibiting p53 mutations at relapse showed the same recombination pattern at initial diagnosis. This result proves that the same leukemic cell population, and not an independent minor subclone, acquired the p53 mutation during the clinical course, and that p53 alterations were not germline mutations in these cases.

Clinical data of p53 mutation-positive patients are shown in Table 4, no tendency was detected concerning either age or sex. We did not detect any p53 mutations in 13 infants’ samples. Concerning outcome in the eight p53 mutation-positive cases, four were very aggressive: case no. 7 died at day 38 after diagnosis; case no. 6 developed bone marrow relapse at 5 months from diagnosis and died; case no. 5 also relapsed at 25 months after diagnosis and died; and case no. 4 relapsed in the central nervous system (CNS) at 6 months from diagnosis. The remaining four patients have remained

Table 2. p53 Mutations in Eight Children With Lymphoid Malignancies

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Codon</th>
<th>Exon</th>
<th>Base</th>
<th>Mutational Change (amino acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Pre-pre-B-ALL</td>
<td>142</td>
<td>5</td>
<td>CCT—CCCT</td>
<td>C insertion</td>
</tr>
<tr>
<td>2</td>
<td>c-ALL</td>
<td>175</td>
<td>5</td>
<td>CTC—CAC</td>
<td>Arg—His</td>
</tr>
<tr>
<td>3</td>
<td>Pre-B-ALL</td>
<td>240</td>
<td>7</td>
<td>AGT—CTG</td>
<td>Ser—Arg</td>
</tr>
<tr>
<td>4</td>
<td>B-NHL</td>
<td>245</td>
<td>7</td>
<td>GCC—AGC</td>
<td>Gly—Ser</td>
</tr>
<tr>
<td>5</td>
<td>c-ALL</td>
<td>248</td>
<td>7</td>
<td>CCG—CAG</td>
<td>Arg—Gln</td>
</tr>
<tr>
<td>6</td>
<td>B-NHL</td>
<td>273</td>
<td>8</td>
<td>CTT—CTG</td>
<td>Arg—Cys</td>
</tr>
<tr>
<td>7</td>
<td>T-NHL</td>
<td>282</td>
<td>8</td>
<td>CCG—TGG</td>
<td>Arg—Trp</td>
</tr>
<tr>
<td>8</td>
<td>c-ALL</td>
<td>287</td>
<td>8</td>
<td>CAC—TAC</td>
<td>His—Tyr</td>
</tr>
</tbody>
</table>

* In case no. 1, insertion of C at codon 142 generated a new stop codon (TGA) 16 bp downstream, due to a frame shift.
Fig 2. Analysis by PCR-direct sequencing of the p53 gene in lymphoid malignancies of childhood. Samples interpreted as positive in the PCR-SSCP assay were subjected to genomic sequence analysis to confirm and characterize the p53 mutation. Coding strands are shown for exons 5 and 7. Each mutation is matched to a control sequence in (A) and (C). (A) Patient no. 1 shows a C insertion at either the first or second nucleotide of codon 142 (CCCT, arrow). Extra bands can be seen after insertion in addition to normal bands; (B) The reaction samples of different individuals are grouped so that a mutation can be easily recognized; all of the As, Cs, Gs, and Ts of three patients are run by each other. The insertion of patient no. 1 creates a frameshift beginning at the base marked with the arrow. (C) Patient no. 3 shows A → C change (arrow) at the first nucleotide of codon 240 resulting in a change from serine (AGT) to arginine (CGT). Patient no. 4 shows a G → A change (arrow) at the first nucleotide of codon 245 resulting in a change from glycine (GGC) to serine (AGC). Numbers correspond to the patient nos. in Table 2; unlabeled samples on (B) represent patient samples with normal pattern of bands.

Table 3. Immunogenotypes of Eight Children With p53 Mutation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>IgH</th>
<th>Igκ</th>
<th>Igλ</th>
<th>TCRβ</th>
<th>TCRγ</th>
<th>TCRδ</th>
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<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<tr>
<td>2</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<tr>
<td>4</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<tr>
<td>5</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>6</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<tr>
<td>7</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>8</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
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</tbody>
</table>

Abbreviations: IgH, Ig H gene; Igκ, Ig κ gene; Igλ, Ig λ gene; TCRβ, T-cell receptor β gene; TCRγ, T-cell receptor γ gene; TCRδ, T-cell receptor δ gene; G, germline; R, rearranged.

* Numbers correspond to the patient numbers in Table 2.

DISCUSSION

We examined 359 childhood hematologic malignancies for alterations of p53 gene using PCR-SSCP analysis followed by direct sequencing of amplified DNA of those samples with abnormal SSCP migration. Mutations were detected in 8 of 330 (2%) lymphoid malignancies within the p53 mutational hotspots, and none in myeloid leukemias (AML and CML-BC). p53 alterations were determined not to be germline mutations by analyzing DNA samples in complete remission in four of eight positive cases, and by analyzing both DNA samples at initial diagnosis and at relapse in two of eight positive cases. In total, five of eight positive cases were determined to have somatic p53 mutations. In two of three remaining cases, no specific family history was obtained to suggest inheritance of a p53 mutation. Positive cases include two relapsed ALL patients, who did not have a p53 mutation at initial diagnosis. Immunogenotyping by examining rearrangements of Ig and TCR genes showed that the same leukemic cell population developed the p53 mutation during the clinical course. p53 mutations were also found in three malignant lymphomas with aggressive phenotype: one T-NHL with early death at day 38 after diagnosis, and two B-NHL with either bone marrow or CNS relapse. These results indicate that p53 mutations are very rare events in primary lymphoid malignancies of childhood, and are correlated in several positive cases with disease progression to a more malignant phenotype. In previous smaller studies, p53 mutations were detected in 5% to 28% of primary ALL samples;14,25 most were B-cell origin (5% to 21%). They were also found in 0% to 19% of primary...
malignant lymphomas, mainly in Burkitt's lymphomas (13% to 37%).

One prior report studying p53 mutations by RNase protection and SSCP analysis focused on a small cohort of childhood ALL. The frequency of p53 mutations was relatively low (4 of 25) and two of these children probably had the Li-Fraumeni syndrome, indicating that the mutations were inherited from their parents. Thus, the number of acquired p53 mutations might have been two. Furthermore, three of those four cases were relapsed ALLs (one T-ALL and two pre-B-ALLs). Although the total number of cases examined by Felix et al was too small to determine the frequency of p53 mutations, their results were compatible with ours. Taken together, the two studies indicate that (1) p53 mutations are rare in childhood ALL compared with adulthood ALL; and (2) p53 mutations may play a role in progression of lymphoid malignancies to a more aggressive phenotype, including relapsed phase of leukemias and malignant lymphomas with aggressive phenotype.

Several reasons may explain why adult as compared with childhood lymphoid malignancies appear to have a higher frequency of p53 mutations. First, Burkitt's type ALL (L3) is more frequent in adults and this subtype of ALL has a relatively high frequency of p53 mutations. Of note, two of those four cases were relapsed ALLs (one T-ALL and two pre-B-ALLs). Although the total number of cases examined by Felix et al was too small to determine the frequency of p53 mutations, their results were compatible with ours. Taken together, the two studies indicate that (1) p53 mutations are rare in childhood ALL compared with adulthood ALL; and (2) p53 mutations may play a role in progression of lymphoid malignancies to a more aggressive phenotype, including relapsed phase of leukemias and malignant lymphomas with aggressive phenotype.

Concerning clinical information, no tendency was identified between p53 mutations and either age or sex. Although we examined 13 infants' cases, no p53 mutation was detected in this cohort. In most of the eight p53 positive cases, data of family history were either not available or did not suggest any specific history of either Li-Fraumeni syndrome or clustering of malignancies.

The analysis of the location and type of mutations can provide clues to the etiology of the tumor and to the function of specific regions of p53. Two types of base substitutions have been analyzed: transitions in which a purine is substituted for a pyrimidine or vice versa. In this study, six of eight mutations were G:C to A:T transitional mutations, and five of these occurred at CpG dinucleotides. This pattern of mutation results from spontaneous deamination at 5-methylcytosine residues. This suggests that p53 mutations in these hematologic malignancies may not be induced by exogenous carcinogens. The alterations that we found at codons 175, 248, and 273 are known mutational hotspots in many lymphoid malignancies and point mutations at codons 245 and 282 are mutational hotspots reported in some hematologic diseases. Other than these commonly mutated codons, two point mutations were newly detected at codons 240 (A:T to G:C transversion) and 297 (G:C to A:T transition), and one insertion of C at codon 142, which generated a new stop codon (ATG) 16 bp downstream, due to a frameshift.

In this study, seven of eight samples had both the wild-type and mutant p53 allele. This may reflect either contamination of the sample with normal hematopoietic cells or heterozygosity of the p53 alleles in the leukemic cells. But we believe that the former is unlikely, because those cell samples contained 88% to 96% morphologically malignant cells. Seven of eight alterations of p53 were missense mutations, and the resulting protein typically has a prolonged half-life. The p53 forms tetramers, and mutant p53 may bind wild type p53 and may act in a dominant negative fashion to provide the cells with a growth advantage. This is in vivid contrast to another tumor suppressor gene, APC, which is closely associated with colon cancer. Mutations of
ACPC are usually frameshift mutations presumably resulting in a short-lived, truncated protein.\textsuperscript{90} One explanation for the frequency of p53 point mutations in human cancer is that in the heterozygous state, mutant p53 protein can partially inhibit the function of wild-type p53 providing these cells with a growth advantage.

ACKNOWLEDGMENT

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