We investigated the frequency of p53 mutations in 19 pediatric cases of therapy-related leukemia or myelodysplastic syndrome. Eleven children presented with acute myeloid leukemia, one with mixed-lineage leukemia, two with acute lymphoblastic leukemia, and five with myelodysplasia at times ranging from 11 months to 9 years after a primary cancer diagnosis. The primary cancers, which included 11 solid tumors and eight leukemias, were treated with various combinations of DNA topoisomerase II inhibitors, alkylating agents, or irradiation. Leukemic or myelodysplastic marrow specimens were screened for possible mutations by single-strand conformation polymorphism (SSCP) analysis of p53 exons 4 to 8. The only observed mutation was an inherited 2-basepair deletion at codon 209 in exon 6 that would shift the open reading frame, create a premature termination codon, and foreshorten the resultant protein. Prior therapy in this patient included DNA topoisomerase II inhibitors, alkylating agents, and irradiation. The secondary leukemia presented as myelodysplasia with monosomies of chromosomes 5 and 7 and abnormalities of chromosome 17. Although the primary cancer was an embryonal rhabdomyosarcoma and there was a family history of cancer, the case did not fulfill the clinical criteria for Li-Fraumeni syndrome. This study suggests that germline p53 mutations may predispose some children to therapy-related leukemia and myelodysplasia, but that p53 mutations otherwise are infrequent in this setting.

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The Tumor Suppressor gene, p53, located on the short arm of human chromosome 17p13.1 encodes a 53-kD nuclear phosphoprotein that functions as a negative regulator of cell proliferation.1-4 Wild-type p53 is a transcription-activating factor induced in late G1 to block cell-cycle progression, particularly in the presence of damaged DNA.5-7 Induction of p53 has been observed in the normal cellular response to DNA damage caused by exogenous mutagens, including certain anticancer drugs and UV and gamma radiation.7-11 Wild-type p53 leads to exit from the cell cycle and initiation of apoptosis in the presence of high levels of DNA damage,8,12 but permits growth arrest, DNA repair, and reentry into the cell cycle when low-level DNA damage has occurred.13 In contrast, mutant p53 abrogates the G1 block and permits a cell with damaged DNA to pass through S phase.11,14 Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by y-irradiation.15 Targets of p53 include genes that regulate genomic instability, the cellular response to DNA damage, and cell-cycle progression.16-18

The epipodophyllotoxins, anthracyclines, and dactinomycin are DNA-damaging anticancer drugs that induce double-stranded breaks in DNA by interaction with the nuclear enzyme, DNA topoisomerase II.19-27 The cytotoxic activity of these drugs involves a p53-dependent apoptotic cell death program.12,25 When induced by specific agents that cause DNA-strand breakage, such as y-radiation or etoposide, p53 exerts a dose-dependent effect on the initiation of apoptosis in mouse thymocytes.8

The same chemotherapy drugs that induce a p53-dependent apoptotic cell death program are occasionally associated with the development of leukemia as a second cancer. The Li-Fraumeni syndrome is specifically associated with germ-line p53 gene mutations.28 Moreover, patients with the Li-Fraumeni syndrome are predisposed to multiple primary cancers, which may include leukemia.29-32 These observations suggest that mutant p53 may contribute to leukemogenesis by decreasing cell death after exposure to DNA-damaging chemotherapy and irradiation. Nonetheless, the clinical relevance of coincidental expression of mutant p53 and altered mechanisms of DNA repair or apoptosis in the predisposition to or development of therapy-related leukemia has not been established. In the present study, we investigated the frequency of p53 mutations in therapy-related leukemia and myelodysplastic syndrome in a pediatric population.

Subjects and Methods

Identification of p53 mutations. Genomic DNAs extracted from leukemic or myelodysplastic marrow specimens were screened by the polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) method.33-34 The oligonucleotide primers have been reported.34 PCR fragments containing exons 4, exons 5 and 6, or exons 7 and 8 and incorporating [32P]dCTP were amplified using 100 ng genomic DNA as a template. Aliquots of PCR products were digested with restriction enzymes to reduce the fragment sizes and separate the exons.34 Reaction mixtures were diluted with loading buffer and denatured by heating at 90°C for 5 minutes. Exon 5/6
and exon 7/8 products were electrophoresed at 4°C in non-denaturing acrylamide at constant power. For analysis of exon 4, resolution was achieved by addition of a final concentration of 5% glycerol to the gel and electrophoresis at 26°C. Exons 4 through 8 were examined in all cases.

**Sequencing of a mutation suggested by screening.** A fresh aliquot of DNA from each sample with an abnormality suggested by band shifts on SSCP analysis was amplified with the same SSCP primers encompassing the abnormal region. Fragments were directly subcloned into a T-tailed pBSK vector (Stratagene, La Jolla, CA), and at least six clones were sequenced in both directions by the Sanger dideoxyxynucleotide method using Sequenase version 2.0 (US Biochemical, Cleveland, OH).

**p53 immunostaining of paraffin-embedded tissues.** Paraffin-embedded rhabdomyosarcoma tissue from patient no. 17 was examined for p53 expression by immunohistochemistry as described previously.35 Paraffin sections of 5-μm thickness were dried onto charged ProbeOn Plus slides (Fisher Biotech, Pittsburgh, PA), dehydrated at 80°C for 10 minutes, deparaffinized in xylene, and rehydrated in 100% ethanol and deionized water. The sections were then steam-treated with 10 mM/L sodium citrate (BioTek Solutions, Santa Barbara, CA) for 25 minutes at 90°C using an Isotemp Waterbath (Fisher Scientific, Pittsburgh, PA) for antigen retrieval. p53 protein expression was evaluated by the avidin-biotin complex (ABC) procedure with monoclonal antibody DO-7, which detects wild-type and mutant human p53 (DAKO Corp, Carpenteria, CA). Slides were stained on an immunohistochemistry stainer using standard buffer, biotinylated secondary antibody, and ABC-horseradish peroxidase reagents (BioTek Solutions). The DO-7 primary antibody was used at a 1:10 dilution. Slides were reacted with 3,3’ diaminobenzidine (DAB), counterstained with hematoxylin, and reviewed by light microscopy.

**RESULTS**

The p53 gene was examined in secondary leukemic marrow samples from 19 pediatric patients. Table 1 summarizes the demographic features, primary diagnoses, prior treatment regimens, French-American-British morphologies, p53 gene status, and karyotypes in the cases studied. The clinical features and karyotypes of cases no. 2 through 17 were previously described in a molecular analysis of the MLL gene in this patient population.36

Of 19 leukemias in the present study, 10 had a translocation of chromosome band 11q23 or del(11)(q23) by karyotype (patients no. 2 to 10 and 15). In five leukemias (patients no. 11 to 14 and 18) without cytogenetic detection of an abnormality of this region, there was molecular evidence of MLL gene involvement at band 11q23. Monosomies of chromosomes 5 and 7 were present in two cases (patients no. 17 and 20).36,37

In 15 cases, SSCP patterns were consistent with known polymorphisms at codons 36 or 72 in exon 4, and sequencing was unnecessary.34,35 SSCP analysis identified band-shift patterns that suggested either p53 mutations or different polymorphisms in two other cases (patients no. 4 and 17). In the leukemia of patient no. 4, sequencing of genomic subclones prepared from marrow DNA revealed the common polymorphism (Arg → Arg) at codon 213 in exon 6.36

Genomic DNA SSCP analysis of the myelodysplastic marrow of patient no. 17 and the peripheral blood of the father, who was unaffected by cancer, identified both a wild-type allele and an abnormal allele that was present in the germline (Fig 1A). Sequence analysis of individual genomic subclones prepared from the myelodysplastic marrow confirmed a 2-basepair deletion at codon 209 in exon 6 (Fig 1B). This genomic lesion shifts the open reading frame and results in a premature termination codon and truncated protein product. Although the abnormality was present in three of six subclones, loss of heterozygosity cannot be excluded, since the myelodysplastic marrow contained only 9% abnormal cells.

Patient no. 17 developed myelodysplastic syndrome 62 months after diagnosis of embryonal rhabdomyosarcoma of the buttok. Multimodality sarcoma treatment had included the alkylating agents, cyclophosphamide and cisplatinum, the DNA topoisomerase II inhibitor dactinomycin, and therapeutic radiation to the primary tumor and the lungs.37

The embryonal rhabdomyosarcoma and surrounding normal tissue showed an undetectable level of p53 protein by immunohistochemical analysis using DO-7 antibody staining (data not shown). Similarly, other sarcomas with mutations that cause premature truncation show undetectable p53 protein by immunostaining.40

The pedigree has been described elsewhere.37 The paternal grandfather died of a lymphoma, and a paternal great uncle had a melanoma. One maternal great grandfather was diagnosed with skin cancer, and the other maternal great grandfather died of an unspecified malignancy. Neither parent and none of three siblings have been diagnosed with cancer.37 Thus, the family history was not consistent with the clinical definition of Li-Fraumeni syndrome, which requires the presence of cancer in at least one first-degree relative of the patient with sarcoma.36,37,41

**DISCUSSION**

Nineteen pediatric secondary leukemias were examined to determine whether loss-of-function mutations of p53 might permit selection of cells carrying leukemogenic chromosomal translocations or deletions. The results of this analysis exclude somatic and germline p53 mutations in exons 4 through 8 in 18 cases. p53 mutations were notably absent in DNA topoisomerase II inhibitor–related cases with translocations at chromosome band 11q23 (Table 1). Cases no. 7, 11, 14, 17, and 20 presented as myelodysplasia rather than overt leukemia.36 The sensitivity of mutation detection by SSCP for the conditions and primers used has been evaluated. Serial dilution experiments indicated that 1 part mutant allele in 16 parts wild-type would be detectable,34 a relevant issue for somatic mutation detection when myelodysplastic specimens contain substantial normal cells. In cases no. 7, 11, and 14, there was molecular evidence of MLL gene rearrangement at the level of the Southern blot,36 which has similar or greater sensitivity. By morphology, the marrows in cases no. 14, 17, and 20 contained 21%, 9%, and 4% to 5% blasts, respectively. Thus, in each case classified as myelodysplastic syndrome, the percentage of abnormal cells approximated or exceeded the sensitivity of the method. Moreover, if a mutation were present in the germline, sensitivity of detection would not be an issue. Thus, somatic and

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germline p53 mutations were absent in four of five cases presenting as myelodysplasia (Table 1).

Nonetheless, germline gene mutations and the late effects of previous therapy together may contribute to the development of pediatric secondary cancers.\textsuperscript{24,25} We identified a child with paternal inheritance of a 2-basepair deletion in a highly conserved coding region of the p53 gene, who sequentially developed embryonal rhabdomyosarcoma and therapy-related myelodysplastic syndrome.

Monosomies of chromosomes 5 and 7, typical of alkylator-induced myelodysplasia,
were present (Table 1, patient no. 17). In addition, the der(17) and t(5;17)(p12;p11.2) chromosomal translocation are of interest, since p53 mutations in sporadic acute myeloid leukemia are frequently associated with cytogenetic abnormalities of chromosome 17. It is possible that the accumulation of cytogenetic abnormalities in the myelodysplastic marrow cells reflects the high mutation rate that results when there is functional loss of wild-type p53 as the cellular sensor of damaged DNA.

Inheritance of the codon 209 mutation, occurrence of two cancers in one child, the early age of onset, and the specific types of cancer may suggest a Li-Fraumeni family in which the father is thus far a silent carrier. Although this family does not fulfill the clinical criteria, molecular identification of the germline mutation in the father implicates either the father or the paternal grandfather, who was affected with lymphoma, as possible founding members.

The 2-basepair deletion at codon 209 changes the reading frame, creating a premature termination codon in exon 7. The predicted protein product would lack two of the evolutionarily conserved domains, one of the two SV40 large T-antigen binding sites, the nuclear localization signal, and the structural motifs required for oligomerization.

At the genomic DNA level, the mutation differs from germline p53 missense mutations at codons 133, 272, or 273 identified in leukemia. Missense mutations of both p53 alleles were detected in acute myeloid leukemia blast cells of one adult who was previously treated for breast cancer with melphalan and irradiation, but germline tissues were not examined for mutation. Three other germline p53 exonic deletions of 1 or 2 basepairs have been described to date, but none in association with leukemia.

Although sarcomas and leukemias are component tumors of the Li-Fraumeni syndrome, no p53 mutations were detected in secondary leukemic marrows of six other children in this series in whom the primary cancers were sarcomas (patients no. 4, 7, 9, 10, 13, and 19; Table 1). In cases no. 13 and 19, in which the family history was investigated, there were several adulthood cancers, but the pedigrees did not meet clinical criteria for Li-Fraumeni syndrome. These results may suggest that most children with therapy-related leukemia following the various sarcoma treatment regimens generally are not affected with Li-Fraumeni syndrome. Alternatively, since germline p53 mutations are identified in only 50% to 75% of classic Li-Fraumeni pedigrees, p53-
related upstream or downstream effectors of DNA repair may be abnormal in these cases.

p53 mutations were also absent in cases of therapy-related leukemia after neuroblastoma and peripheral neuroectodermal tumor treatment regimens. Both neuroblastoma and peripheral neuroectodermal tumor are pediatric cancers in which mutations of the p53 gene occur infrequently.20

Although it is possible that a fraction of mutations could be missed by only screening exons 4 through 8, a recent analysis of the distribution of over 2,000 p53 mutations in 53 different cancer types demonstrates that 95% of p53 mutations are clustered in the DNA binding domain between codons 120 in exon 4 and 290 in exon 8.51

Induction of wild-type p53 by exogenous DNA-damaging agents prompted this investigation of a possible role of p53 mutations in therapy-related leukemia and myelodysplasia. The germline deletion that we observed suggests that germ-line p53 mutations may predispose some patients to leukemia. This study raises questions about the predictive value of identifying such mutations in children with sarcomas without a family history of cancer. However, the lack of p53 mutations in 18 of 19 pediatric therapy-associated leukemias suggests that defects in other p53-related or p53-independent DNA-repair or apoptotic pathways may also be involved. These data are also consistent with earlier findings that hereditary and somatic p53 mutations are infrequent in the initial pathogenesis of most primary leukemias.33,45,55,62-64

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p53 MUTATIONS IN SECONDARY LEUKEMIA


The p53 gene in pediatric therapy-related leukemia and myelodysplasia

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