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Advances in Brief

Complex Replication Error Causes *p53* Mutation in a Li-Fraumeni Family[1]

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Abstract

We demonstrated a germline *p53* replication error in two generations of a Li-Fraumeni family affected with liposarcoma, adrenocortical carcinoma, and osteosarcoma. The trinucleotide repeat mutation changed 5′-AGT GTG GTG GTG-3′ at codons 215–218 to 5′-AGT TGG TTG GTG GTG-3′. The predicted protein would be elongated by one amino acid (val^{216}→trp leu) without a change in charge. Detection of *p53* in the adrenal tumor by immunostaining suggested that the mutant protein was expressed. Persistence of the mutation in the germline may suggest a defect in DNA repair in the family member first affected. This is the first report where germline transmission of replication-damaged *p53* trinucleotide repeats is associated with the Li-Fraumeni syndrome.

Introduction

The Li-Fraumeni syndrome is a rare autosomal dominant disorder characterized by increased cancer susceptibility and early onset of multiple forms of cancer. The clinical definition requires an individual with a sarcoma diagnosed before age 45, a first degree relative with cancer before age 45, and another first or second degree relative with a sarcoma diagnosed at any age or any cancer before age 45 (1). Constituent cancers of the syndrome include breast carcinoma, soft tissue sarcomas, osteosarcoma, acute leukemias, brain tumors, adrenocortical carcinoma, and possibly other cancers (2, 3).

Germline mutations in the *p53* tumor suppressor gene account for the increased susceptibility to cancer in some Li-Fraumeni families (reviewed in Ref. 4). Germline *p53* mutations are also found in individuals with multiple primary cancers, sporadic cancers that are constituent tumors of the Li-Fraumeni syndrome, and familial breast cancer (5–11). At the molecular level, 53% of germline *p53* mutations are G:C to A:T transitions at CpG dinucleotides, which are naturally occurring sites of methylation in the genome (12). Because the spontaneous deamination of 5-methylcytosine forms thymine, it has been suggested that these mutations are endogenous and that the genomic sequence is the mutagen (13, 14).

Not all endogenous mutations in the Li-Fraumeni syndrome can be attributed to this mechanism. In the present study, we analyzed the germline sequence of the *p53* gene in one Li-Fraumeni family. A mutation involving trinucleotide repeats within the coding region was vertically transmitted in two generations. The mutation suggests a replication error and defective DNA repair. This is the first example of germline transmission of replication-damaged *p53* trinucleotide repeats in a Li-Fraumeni family. These results indicate that trinucleotide repeats within the *p53* coding region may also be endogenous mutation sites in the Li-Fraumeni syndrome.

Materials and Methods

Screening for *p53* Mutations. The referring physician obtained parental informed consent to collect peripheral blood specimens for molecular analysis. Peripheral blood genomic DNAs from the affected father (III-8), daughter (IV-4), son (IV-5), and normal mother (III-9; Fig. 1) were screened by the PCR/single-strand conformational polymorphism method (7, 15). Oligonucleotide primers have been reported (15). PCR fragments containing exon 4, exons 5 and 6, or exons 7 and 8 and incorporating [α-^{32}P]dCTP were amplified from 100 ng genomic DNA. Aliquots of these products were digested with restriction enzymes to reduce the fragment sizes and separate the exons. Reaction mixtures were diluted with loading buffer and denatured by heating at 90°C for 5 min. The exon 5/6 and exon 7/8 products were electrophoresed at 4°C in nondenaturing acrylamide at constant power. Resolution of exon 4 was achieved by the addition of a final concentration of 5% glycerol to the gel and electrophoresis at 26°C.

Characterization of the *p53* Mutation in a Li-Fraumeni Family. Peripheral blood genomic DNAs from the affected father (III-8), daughter (IV-4), and son (IV-5; Fig. 1) were directly sequenced. Genomic DNA from the normal normal mother (III-9) was control. PCR products were amplified from 1 μg of genomic DNA using exon 5 sense primer 5′-CCTGCCCTCAACAAGATG-3′ and intron 6 antisense primer 5′-TTAGCCTCGTAAGCTTCAGT-3′. Heminested PCR amplification of 1/250 volume of these products was performed using exon 5 sense primer 5′-ATGCGAATTCCAACTGGCCAAGACCTGCCCTGTG-3′ and the same intron 6 antisense primer. Products of the heminested reaction were isolated and purified from low-melting temperature agarose (FMC Corp.). Direct dideoxy sequencing with exon 6 primer 5′-TATCCGAGTGGAAGGAAATT-3′ was as described (7).

The *p53* mutation was further verified by sequencing individual genomic subclones prepared from peripheral blood DNA of the affected son (IV-5). One μg genomic DNA was PCR amplified in three separate reactions using intron 4 sense primer 5′-TGCAGAATTCTGACTTTCAACTCTGTCTCCT-3′ and intron 6 antisense primer 5′-GATCAAGCTTCCAGAGACCCCAGTTGCAAAC-3′, which contained restriction sites. Pooled PCR products were cleaved with *Eco*RI and *Hin*dIII, agarose gel-purified, and then ligated into pGEM-7Zf+ (Promega) for transformation of DH5α cells (Bethesda Research Laboratories). Individual genomic subclones were sequenced by the dideoxy method with *p53* exon 6 sense primer 5′-TATCCGAGTGGAAGGAAATT-3′.

p53 Immunohistochemistry. Paraffin-embedded adrenocortical carcinoma cells of Patient IV-4 were examined for *p53* expression by immunohistochemistry. Archival specimens of the other tumors were not available. Paraffin sections of 5-μm thickness were dried onto charged ProbeOn Plus slides (Fisher Biotech), dewaxed at 80°C for 10 min, deparaffinized in xylene, and rehydrated in 100% ethanol and deionized water. The sections were then steam treated with 10 mM sodium citrate (BioTek Solutions) for 25 min at 90°C using an Isotemp waterbath (Fisher Scientific) for antigen retrieval. p53 protein expression was evaluated by the avidin-biotin complex procedure with mAb DO-7, which detects wild-type and mutant human p53 (DAKO Corporation). Slides were stained on an immunohistochemistry stainer using standard buffer, biotinylated secondary antibody, and avidin-biotin complex-horseradish peroxidase reagents (BioTek Solutions). The DO-7 primary antibody was used at 1:10 dilution. Slides were reacted with 3,3′-dimethylaminoazobenzene, counterstained with hematoxylin, and reviewed by light microscopy.

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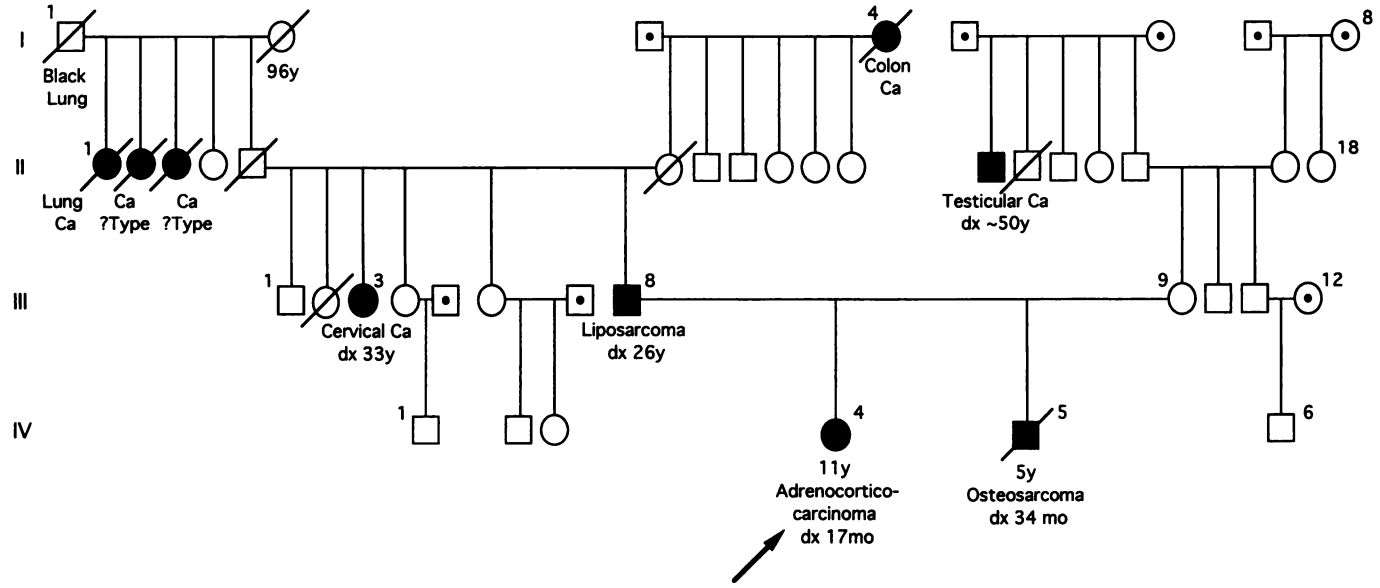

Fig. 1. Pedigree of Li-Fraumeni family with germline *p53* replication error. *Open box* or *circle*, unaffected male or female; *solid*, affected; *slash mark*, deceased; *dot* in *center* of *open box* or *circle*, history unknown. Age is shown in years. *Arrow*, the proband.

Positive staining for p53 protein was defined as visible brown nuclear staining. The intensity of p53 immunostaining was graded as faint (1+), moderate (2+), or intense (3+). The number of positive cells was visually quantitated as the percentage of brown-staining nuclei.

Results

Family History. The proband (IV-4), a 17-month-old white female, presented with accelerated linear growth, clitoromegaly, and pubic hair. Laboratory testing revealed markedly elevated testosterone and dehydroepiandrosterone-S. Abdominal ultrasound and computed tomography scans showed a left adrenal mass. At surgery, a 5.5 x 4.5 x 4.0 cm adrenocortical carcinoma was resected. One hilar and one subcapsular sinus node were positive for tumor. No tumor was found elsewhere in the abdomen. Adjuvant chemotherapy was with cyclophosphamide and doxorubicin. The patient remains well with normal growth, development, and hormone levels 8 years following diagnosis.

Patient IV-5, the younger brother of Patient IV-4, presented with chest pain, dyspnea, and a chest wall mass at 34 months of age. Imaging studies showed destruction of the right 5th rib, pleural and hilar calcifications, and a large pleural effusion. Osteosarcoma was diagnosed on biopsy of the chest wall primary. Metastases were present in the pelvis, spine, and femurs. The disease responded to intensive chemotherapy. Upon chest wall reconstruction 21 months from diagnosis, viable tumor was present in resected ribs. Eight months later, the patient died from his disease.

The father of these children (III-8) had survived a liposarcoma of the spermatic cord diagnosed at 26 years old. It became evident that this was a Li-Fraumeni family. The pedigree is shown in Fig. 1.

Characterization of *p53* Mutation in a Li-Fraumeni Family. Peripheral blood from the affected father (III-8), daughter (IV-4), son (IV-5), and normal mother (III-9) and paraffin-embedded adrenocortical carcinoma from the daughter (IV-4) were obtained (Fig. 1). Single-strand conformational polymorphism analysis of peripheral blood genomic DNAs revealed a wild-type allele and a germline abnormal allele with a band shift that localized to exon 6 in the three affected family members and the normal pattern in the mother (Fig. 2). Direct sequencing of peripheral blood genomic DNA PCR products identified a mutation that changed the sequence 5′-AGT GTG

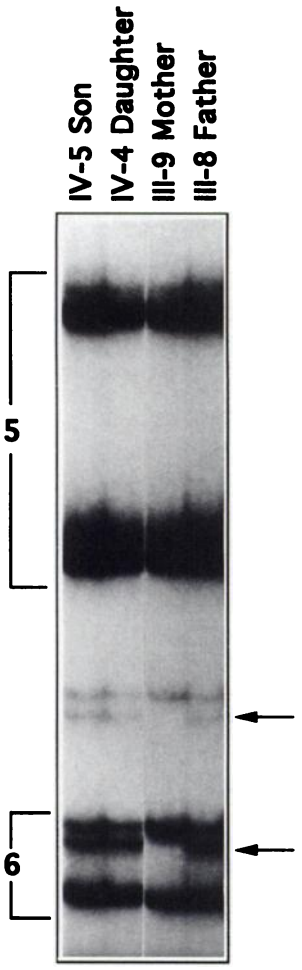

Fig. 2. Localization of germline mutation in Li-Fraumeni family to *p53* exon 6 by genomic DNA PCR/single-strand conformational polymorphism (7, 15). Samples are total peripheral blood white cell DNAs from the father with liposarcoma (III-8) (*Lane 4*), proband daughter with adrenocortical carcinoma (IV-4) (*Lane 2*), son with osteosarcoma (IV-5) (*Lane 1*), and unaffected mother (III-9) (*Lane 3*). p53 exons 5 and 6 are *bracketed*. *Arrows* show the band shift. Specimen from mother shows the normal pattern.

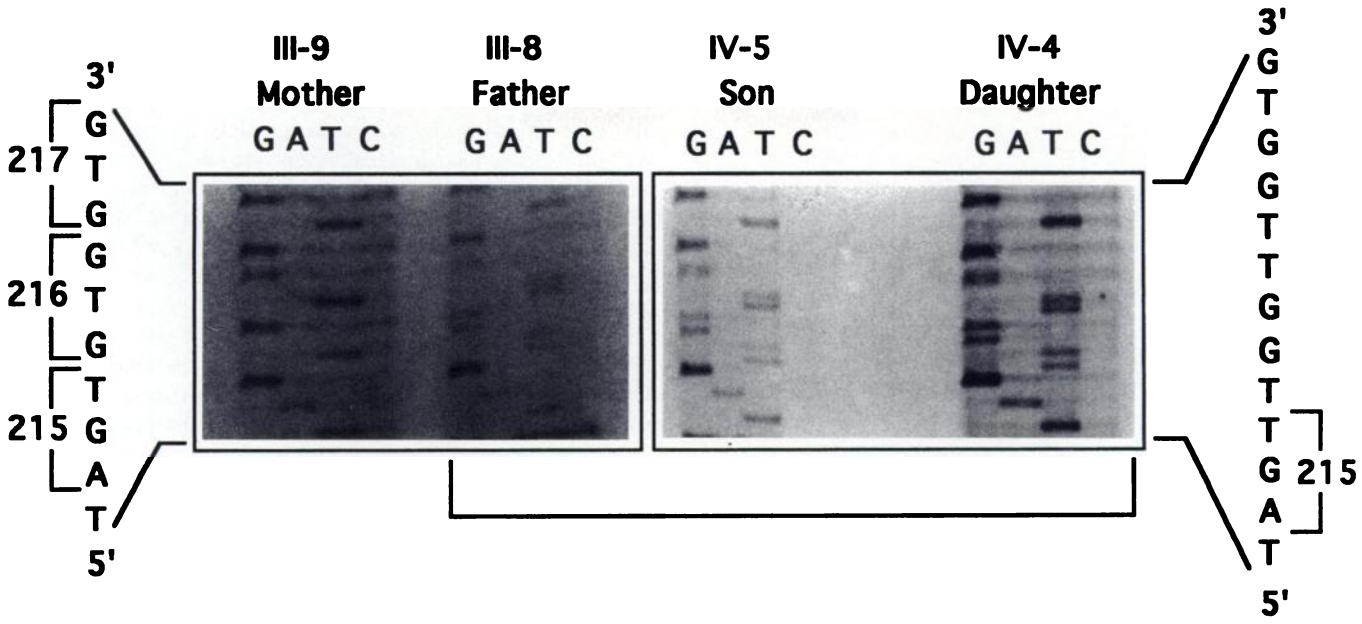

Fig. 3. Characterization of germline *p53* replication error by direct genomic sequencing (7). Samples are total peripheral blood white cell DNAs from the unaffected mother (III-9), affected father (III-8), affected son (IV-5), and affected proband daughter (IV-4). Specimen from mother shows the normal sequence.

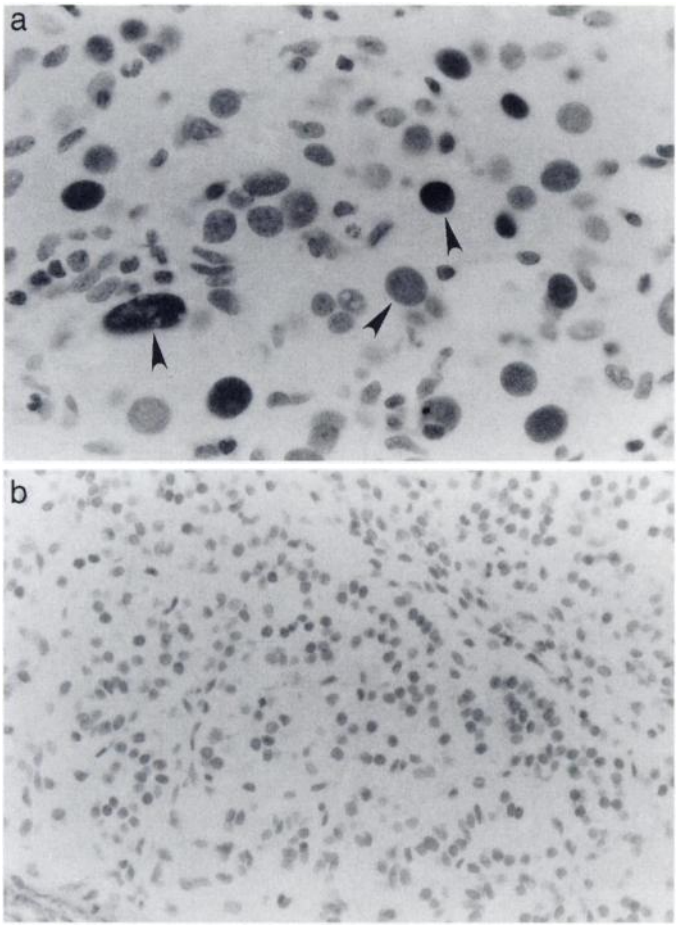

Fig. 4. Immunohistochemical evaluation of p53 protein with DO-7 antibody, which recognizes wild-type and mutant conformations (DAKO Corp.). In *a*, approximately 50% of adrenocortical cells stain positive with nuclear localization of p53. *Arrows* demonstrate variable intensity of p53 immunostaining from 1+ to 3+ (hematoxylin counterstain, × 563). In *b*, p53 protein is not detected in adjacent nonneoplastic cells in the adrenal cortex. The uniform nuclear staining is from the hematoxylin counterstain (× 375).

GTG GTG-3′ at *p53* codons 215–218 to 5′-AGT TGG TTG GTG GTG-3′ in the three affected family members and the normal sequence in the mother (Fig. 3). Sequencing of individual genomic subclones prepared from peripheral blood DNA of the affected son (Patient IV-5) identified the wild-type sequence and the same abnormal sequence.

p53 Protein Expression. The DO-7 mAb recognizes wild-type and mutant conformations of human p53 protein (16). Using DO-7, p53 immunostaining was detected within nuclei of adrenocortical carcinoma cells of Patient IV-4. In contrast, immunostaining of adjacent normal adrenal tissue with the same antibody was negative (Fig. 4). The number of p53-positive adrenocortical carcinoma cells was variable within the tumor and ranged from 25–50% overall, but in focal areas, greater than 50% of nuclei stained positive. The intensity of immunostaining was from 1+ (faint) to 3+ (intense) within the tumor, but most of the positive cells showed 2+ (moderate) immunostaining.

Discussion

The family we describe fulfills the clinical definition of the Li-Fraumeni syndrome. The father (III-8) was diagnosed with a liposarcoma at age 26, his daughter, who was the proband (IV-4), with an adrenocortical carcinoma at 17 months and his son (IV-5) with osteosarcoma at 34 months of age. The same mutation affecting GTG repeats at *p53* codons 215–218 in exon 6 were present in the germline of these family members. The precise changes in the wild-type trinucleotide repeats that resulted in the mutant sequence cannot definitively be determined. However, three single-base insertions, or one single-base and one two-base insertion, are both possibilities (Fig. 5). Alternatively, combined deletion and insertion are candidate events (Fig. 5). In any case, the mutation duplicates three adjacent bases.

In contrast, most germline *p53* mutations are missense mutations that cause amino acid substitutions at codons 245–258 in exon 7 (5, 8, 14, 17, 18). Other germline missense mutations occur in evolutionarily conserved regions of exons 4–9 (5, 8, 14, 17, 18). Base substitutions in splice donor or splice acceptor regions and base substitutions creating stop codons have been reported in the germline (6, 19–21).

Insertions and deletions account for approximately 10% of somatic

NORMAL:

5'-AGT GTG GTG GTG-3'

MUTANT:

5'-AGT **TG**G T**T**G GTG GTG-3'

5'-AG**T** T**G**G **T**TG GTG GTG-3'

5'-AGT **TG**G T**T**G GTG GTG-3'

5'-AGT **TG**G **T**TG GTG GTG-3'

5'-AG**T** T**G**G **T**TG GTG GTG-3'

5'-AGT **GTG** **TGG** **TTG** GTG GTG-3'

5'-AGT **G**T**GG** **T**TG GTG GTG-3'

Fig. 5. Possible alterations that could produce the mutant sequence, including multiple nonconsecutive insertions (*large bold*), or combined deletion (*small shadowed*) and insertion (*large bold*). For comparison, the normal sequence at codons 215–218 is shown at *top*.

and germline *p53* mutations (12, 22). In two sporadic cancers, somatic *p53* deletions removed one complete GTG repeat at codons 216–218 (22). Single-base insertions involving mononucleotide C repeats at codons 71–72 or 151–152 were present in the germline in two families (6). Germline deletions of one base pair at codons 257 or 307, and deletion of two base pairs involving mononucleotide A repeats at codons 209–210, have been described (6, 23, 24). We reported a germline 11-bp deletion at an intron 5 polypyrimidine branch point sequence (7). A combined deletion and insertion spanning codons 108–111 has also been reported (25). Since *p53* insertions often duplicate adjacent bases and deletions involve repeats of identical nucleotides, it has been suggested that these mutations occur by replication slipped mispairing (22). In this model, the replication fork becomes misaligned at short direct repeats. Like the C:G to A:T transitions at CpG dinucleotides, insertions and deletions at short direct repeats are considered endogenous mutations (22).

The mutation we describe differs from the common germline *p53* mutations and augments more atypical germline *p53* mutations because two or three nonconsecutive insertions, or combined deletion and insertion at trinucleotide repeats, may have been involved. In addition, the expansion did not involve a whole repeat. These differences raise questions about the mutagenic structural features of the GTG repeats. This is the first report where germline transmission of replication-damaged p53 trinucleotide repeats has been associated with the Li-Fraumeni syndrome. The defect(s) whereby *p53* replication errors remain uncorrected are unknown (26).

At the protein level, the mutation would add a nonpolar amino acid at codon 216 (val→trp leu) but would not affect the local charge. Immunohistochemical staining of the adrenocortical carcinoma cells suggests expression of the mutant protein. The insertion of three bases without a frame shift and the conservative nature of the amino acid substitution/insertion may explain the transmissibility of this mutation in the germline and compatibility with life in two generations. Moreover, transmission in the germline suggests a growth advantage or selection to retain the mutant, elongated protein.

The *p53* replication error in this family may suggest that a defect in DNA repair was present in the member first affected. Wild-type *p53* is induced and blocks late G_1 progression in response to exogenously damaged DNA. *p53* prevents entry into the S phase of the cell cycle, and the damage is repaired or there is apoptosis (reviewed in Ref. 27). Although there is evidence that wild-type *p53* also blocks G_2-M progression (28), a role of *p53* in the repair of DNA damage caused by S phase replication errors has not yet been described. Failure to repair the replication error in the father (III-8) may suggest that both copies of wild-type *p53* are required to repair *p53* mutations caused by replication errors. It is possible that resultant protein elongation in the DNA-specific binding region between *p53* codons 115–295 (29) prevents *p53* from interacting with other genes involved in DNA repair.

Alternatively, this *p53* mutation may be similar to microsatellite instability, where mismatch repair gene abnormalities are associated with defective DNA repair and numerical expansion or contraction of short direct repeats (30–34). The maternal grandmother (I-4) of the father (III-8) is of interest because mismatch repair gene abnormalities and the replication error phenotype are associated with hereditary nonpolyposis colon cancer (30–34).

The pedigree in Fig. 1 delineates the pattern of tumors in this family and identifies other members who had cancer. Although cervical and colon cancers in the lineage of the father are not constituent tumors of the Li-Fraumeni syndrome, others have demonstrated that inherited *p53* mutations are associated with additional types of cancer (5, 6, 20, 35, 36). Cancer in the father's 33-year-old sister (III-3) would be consistent with autosomal dominant inheritance. Alternatively, it is possible that these cancers were not due to the *p53* mutation, that an inherited mismatch repair gene defect was involved, or that they were sporadic.

This new class of inherited mutations involving replication-damaged *p53* trinucleotide repeats advances our understanding of genetic heterogeneity in the molecular pathogenesis of the Li-Fraumeni syndrome. Future investigation will reveal the functional and structural properties of *p53* harboring such mutations. Moreover, the defect in DNA repair allowing persistence in the germline of replication-damaged *p53* trinucleotide repeats remains to be determined.

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