



Novel p53 splice site mutations in three families with Li-Fraumeni syndrome

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Germline mutations in the p53 tumor suppressor gene predispose to a variety of cancers in families with Li-Fraumeni syndrome. Most germline p53 mutations observed to date cause amino acid substitutions in the protein's central sequence-specific DNA binding domain. Outside this conserved core region, however, we found novel alterations in sequences that regulate precursor mRNA splicing in three Li-Fraumeni syndrome families. Two splice site mutations affected the consensus sequence at the splice donor sites of introns 1 and 9, and produced unstable variant transcripts in normal cells. A third mutation at the splice acceptor site of intron 9 generated splicing at a cryptic acceptor site in intron 9. These splice site alterations emphasize the need to examine both noncoding and untranslated regions of the p53 gene for germline mutations in Li-Fraumeni syndrome families. *Oncogene* (2000) 19, 4230–4235.

Keywords: p53; germline mutation; Li-Fraumeni syndrome; alternate splicing

Introduction

Families with Li-Fraumeni syndrome (LFS) tend to develop childhood sarcomas, brain tumors, leukemias, adrenocortical carcinomas, and early-onset breast cancers, often as multiple primary neoplasms (Li and Fraumeni, 1969; Li *et al.*, 1988; Birch *et al.*, 1994). Germline mutations have been identified in the tumor suppressor gene, p53, in over 50 per cent of LFS families (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Varley *et al.*, 1997a). Germline and somatic p53 mutations usually develop in the peptide's central domain involved in sequence-specific DNA binding (Hainaut *et al.*, 1997; Beroud and Soussi, 1998; Sedlacek *et al.*, 1998). Most alterations cause substitutions of evolutionarily conserved amino acid residues that make direct contact with DNA, or maintain the conformational structure of the protein's DNA binding surface (Soussi *et al.*, 1990; Cho *et al.*, 1994). Outside this core region, deleterious p53 changes tend to be nonsense or frameshift mutations that cause premature protein translation termination (Ishioka *et al.*, 1995; Hainaut *et al.*, 1997; Beroud and Soussi, 1998; Sedlacek *et al.*, 1998). In addition, mutations that

cause alternate splicing of precursor mRNA have been described in six LFS kindreds (Warneford *et al.*, 1992; Felix *et al.*, 1993; Jolly *et al.*, 1994; Frebourg *et al.*, 1995; Varley *et al.*, 1997b, 1998a,b). In this study we describe three additional LFS families with inherited p53 splice site mutations.

Results

Three families (1–3) were found to have germline heterozygous p53 alterations at exon-intron junctions (Figure 1). Members of family 1 developed diverse cancers that characterize LFS. In family 2, two children and their father developed sarcomas. Family 3 had two women with breast cancers, one of whom subsequently developed melanoma. No germline p53 mutations in the coding regions were detected in these families.

The three families had different p53 splice site mutations. Family 1 had a deletion of a single G nucleotide at the exon 9-intron 9 junction (IVS9+1-delG). This alteration has two possible consequences: (1) deletion of G in codon 331 (CAG) causing a frameshift and premature truncation of p53 translation at codon 344; or (2) loss of one of two invariant nucleotides (GT) necessary for correct pre-mRNA processing that results in aberrant splicing (Padgett *et al.*, 1986; Shapiro and Senapathy, 1987). In family 2, a single G→T substitution was detected at the 5' end of intron 1 (IVS1+1G>T), causing a change in one of the invariant dinucleotides (GT) involved in the splicing of precursor mRNA. Family 3 had a G→C substitution at the 3' end of intron 9, which altered the invariant AG at the splice acceptor site (IVS9–1G>C).

To evaluate the effects of each splice site alteration, leukocyte-derived mRNAs from one mutation carrier in each of the three families were examined for alternate splicing by reverse transcription (RT)–PCR. No splice variants were detected in the cDNAs of tested individuals from families 1 and 2. Furthermore, the cDNA sequence from subject IV.6 of family 1 with a germline IVS9+1delG mutation showed no deletions at the 3'-end of exon 9 (data not shown). The splice site alterations in both cases probably caused alternate splicing with resulting variant transcripts that were unstable and subsequently degraded. Therefore, expression of a common polymorphism at codon 72 in exon 4 (proline substituted for arginine) was sought in families 1 and 2 (Matlashewski *et al.*, 1987; Ara *et al.*,

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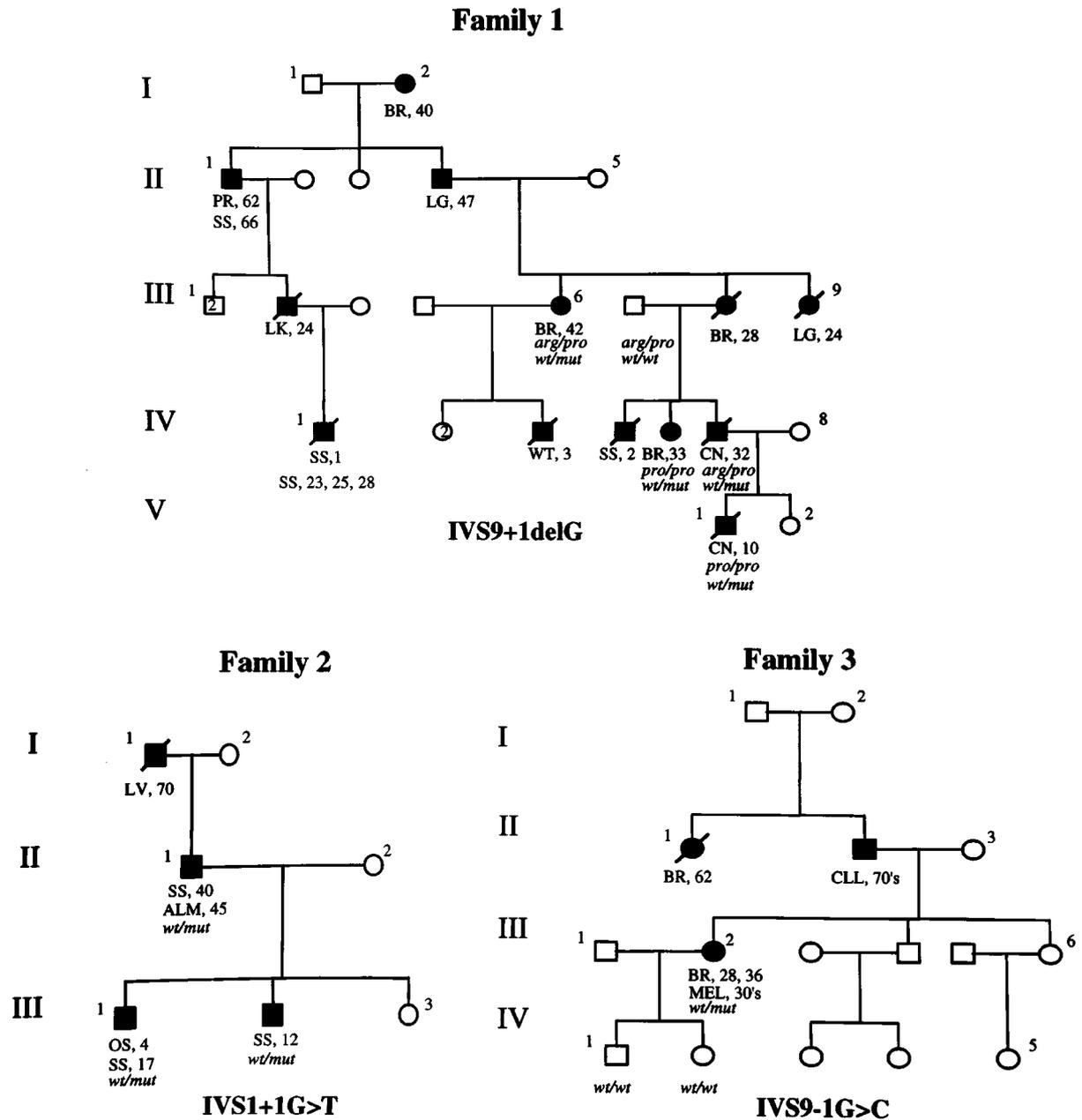


Figure 1 Pedigrees of three LFS families showing affected individuals with heterozygous germline p53 splice site mutations (*wt/mut*) and age at cancer diagnosis. Tumor types: ALM, angioliomyoma; BR, breast cancer; CLL, chronic lymphocytic leukemia; CN, central nervous system (brain) tumor; LG, lung cancer; LK, leukemia; LV, liver cancer; MEL, melanoma; OS, osteosarcoma; PR, prostate cancer; SS, soft tissue sarcoma; WT, Wilms' tumor. In family 1, carriers of splice site mutations and their codon 72 (*arg/pro*) genotypes are shown. Each germline p53 splice site mutation is shown below the corresponding family pedigree

1990). Individuals III.6 from family I and II.1 from family 2 were heterozygous for the polymorphism in analysis of genomic DNA. However, the codon 72 sequences were wild-type in the cDNA of both splice site mutation carriers, suggesting absence of the transcript associated with proline at codon 72. Furthermore, the missing allele in family 1 co-segregated in four affected family members with the deletion at the exon 9-intron 9 junction (Figure 1).

In family 1, normal epidermal keratinocytes (strain LiF-Ep) cultured from a skin biopsy of breast cancer patient III.6 expressed only the wild-type transcript as observed in normal leukocytes from the same donor (Figure 2). In contrast, an immortalized cell line derived from these cells (LiF-Ep/TERT-1) (Dickson *et al.*, 2000) showed at least three additional RT-PCR

products from variant transcripts along with the expected 580 bp amplicon. Nucleotide sequence analysis of a 506 bp PCR variant demonstrated exon 9 skipping. These findings suggest that the intron 9 splice site deletion produced alternate-spliced products that are unstable in normal cells.

In family 3 with a mutation at the intron 9-exon 10 border (Figure 3), at least three PCR products from alternate-spliced transcripts larger than the expected 376 bp were detected in the cDNA. Sequence analysis of the 420 bp variant revealed splicing at a cryptic splice site that led to inclusion of 44 bp of intron 9 into the coding sequence. This variant transcript had a new termination signal (UGA) at codon 359. In addition, RNA isolated from individual III.2 in family 3 was analysed in a yeast p53 functional assay (Flaman *et al.*,

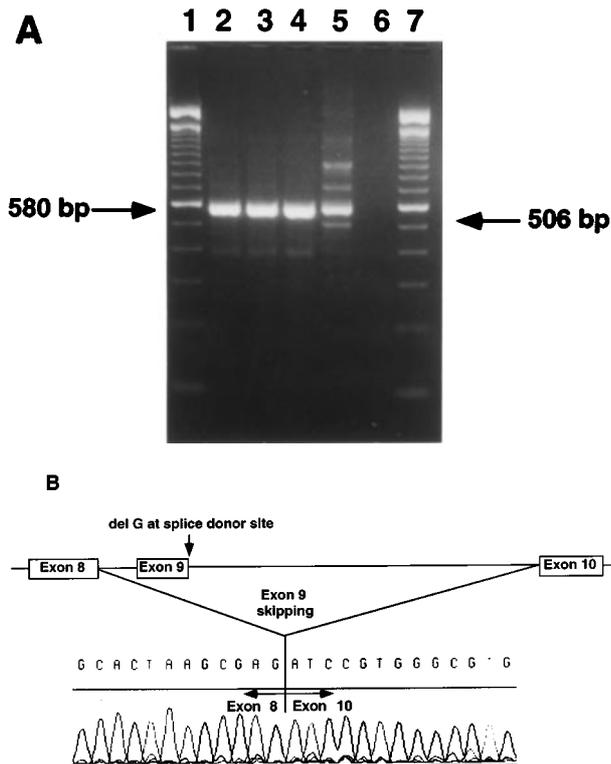


Figure 2 Alternate splicing of epidermal keratinocyte cell line-derived mRNA from family 1. (a) RT-PCR analyses of leukocyte-derived RNAs from two controls (lanes 2 and 3), and both normal (LiF-Ep, passage 5) (lane 4) and hTERT-immortalized (LiF-Ep/TERT-1, passage 25) (lane 5) epidermal keratinocyte cell line-derived RNAs from individual III.6 with a p53 splice site mutation. Amplification of exons 6/7-11 yields an expected 580 bp PCR product and three additional products in lane 5. A 100 bp DNA ladder (lanes 1 and 7) and a no DNA control (lane 6) are also shown. (b) Exon 9 skipping detected in 506 bp variant PCR product in lane 5 with cDNA sequence at the site of alternate splicing

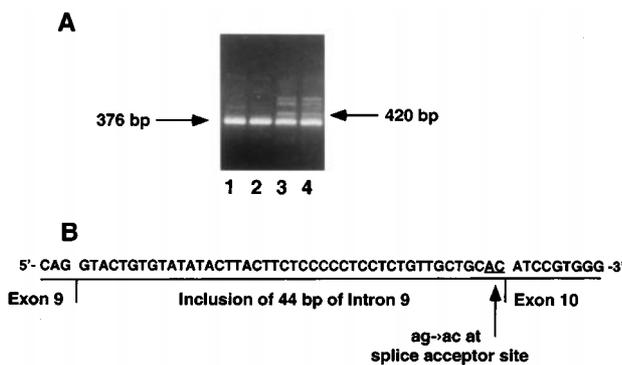


Figure 3 Alternate splicing of mRNA from family 3 with germline p53 mutation at intron 9 splice acceptor site. (a) Leukocyte-derived RNAs from two controls (lanes 1 and 2) and family 3 member III.2 (lanes 3 and 4) were assessed by RT-PCR. The reverse transcription reactions were performed with a p53-specific primer E11bR (see Table 3) in lanes 1-3, and poly-dT primer in lane 4. The PCR products span exons 8-11 in lanes 1-4 with an expected amplicon size of 376 bp and at least three additional products in lanes 3 and 4. (b) The cDNA sequence of an alternate-spliced variant, generating an amplicon of 420 bp in lane 4, is shown. The splice site mutation is illustrated below the cDNA sequence

scription of the ADE2 gene cloned into a reporter plasmid, generating white colonies with wild-type p53 and red colonies with mutant p53. Roughly half of the colonies were red indicating that the mutant allele in family 3 was transcriptionally inactive (data not shown).

Discussion

We identified novel germline p53 splice site mutations in three LFS families. In a review of the literature, six of nearly 200 families with germline p53 mutations had alterations that induce aberrant precursor mRNA splicing (Sedlacek *et al.*, 1998) (Table 1). Together with the three kindreds reported here, alterations causing defects in mRNA splicing account for approximately 5% of germline p53 mutations. Two of the three p53 splice site mutations in this report involve the invariant intronic dinucleotides, GT and AG, that are critical for pre-mRNA processing. The third mutation, IVS9 + 1delG, is a deletion of one of a pair of contiguous G bases, i.e., an invariant intronic G in the splice donor site and an upstream G, the final base of codon 331 (CAG) of exon 9. This final coding G nucleotide is also highly conserved at splice donor sites, so a deletion might weaken the signal for splicing at this exon-intron junction (Padgett *et al.*, 1986; Shapiro and Senapathy, 1987).

Among the five germline mutations previously reported in six families, three were substitutions that altered invariant dinucleotides and generated alternate-spliced transcripts (Table 1). Two other distantly related families showed a silent mutation in codon 125 (ACG→ACA) at the exon 4-intron 4 junction, generating three aberrant-spliced products (Warneford *et al.*, 1992; Varley *et al.*, 1998a). This substituted coding G nucleotide is highly conserved, as described in family 3 of our study. The fifth mutation involved an 11 bp deletion of the intron 5 branch point and polypyrimidine tract sequences affecting splicing (Felix *et al.*, 1993); normal cells from this patient expressed predominantly normal transcript and very low levels of mutant, suggesting instability of alternate-spliced mRNA.

Similarly, the two splice donor site mutations in our families probably generated variant transcripts that were degraded, as shown by the absence of mononuclear leukocyte-derived alternate-spliced products assessed by RT-PCR. Furthermore, cDNA analysis of the heterozygous genotype of codon 72 in individuals from families 1 and 2 with splice site mutations confirms the loss of expression of one allele. Other genes that exhibit reduced levels or complete loss of mRNA from alleles with splice site mutations include protein C in type I protein C deficiency, methionine synthase, and type XVII collagen in generalized atrophic benign epidermolysis bullosa (Soria *et al.*, 1996; Darling *et al.*, 1998; Wilson *et al.*, 1998).

Unlike normal leukocytes or the normal keratinocyte strain LiF-Ep cultured from an affected member of family 1, alternate-spliced products were detected by RT-PCR in an immortalized cell line derived from LiF-Ep (Figure 2). The immortalized cell line was karyotypically stable and was able to undergo normal differentiation and form a normal epidermis in grafts

1995). The p53 cDNA was cloned into a yeast expression vector. Expressed p53 activated the tran-

Table 1 Summary of eight germline p53 mutations causing aberrant splicing

Family (ref)	Mutation location	Sequence change	Aberrant splicing events
Present study			
1	Codon 331/Intron 9 splice donor site	deletion G	Exon 9 skipping
2	Intron 1 splice donor site	GT→TT	Alternate spliced transcripts undetected
3	Intron 9 splice acceptor site	AG→AC	Use of cryptic acceptor site in intron 9
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4 (Varley <i>et al.</i> , 1997b, 1998b)	Intron 3 splice acceptor site	AG→AA	Exon 4 skipping, use of cryptic acceptor site in exon 4
5 (Frebourg <i>et al.</i> , 1995)	Intron 4 splice donor site	GT→AT	Exon 4 skipping, use of cryptic donor site in exon 4
6 (Jolly <i>et al.</i> , 1994)	Intron 5 splice acceptor site	AG→CG	Exon 6 skipping
7* (Warneford <i>et al.</i> , 1992; Varley <i>et al.</i> , 1998a)	Codon 125 at intron 4 splice donor site	ACG→ACA	Intron 4 inclusion, use of cryptic donor sites in exon 4 and intron 4
8 (Felix <i>et al.</i> , 1993)	Intron 5 branch point and polypyrimidine tract	deletion 11 bp	Exon 6 skipping

*Represents two distantly related families

of cultured cells to athymic mice (Dickson *et al.*, 2000). Also, the normal p53 allele acquired a new alteration in the immortalized line (Dickson *et al.*, 2000), however, it seems likely that the inherited mutant allele produced the alternate products. Either the variant transcripts were stabilized in the immortalized line, or reduced levels of p53 mRNA from the spontaneously-mutated allele competed less in RT-PCR amplification of low levels of alternate-spliced p53 mRNA from the germline-mutated allele.

Splice site mutations often cause frameshifts that generate new stop codons. Premature stop codons can induce nonsense-mediated mRNA decay, which reduces or eliminates deleterious gene products (Jacobson and Peltz, 1996; Maquat, 1996). This mechanism of mRNA degradation can explain the loss of variant transcripts due to the splice donor site mutation in intron 9 in family 1. Although the splice donor site mutation in intron 1 of family 2 is in the 5' untranslated region, splicing at potential cryptic sites within intron 1 could produce an upstream translation initiation AUG codon and a frameshift that causes nonsense-mediated mRNA decay. Additionally, a large deletion has been described in an LFS family that removes 167 bp spanning part of exon 1 and intron 1 (Varley *et al.*, 1997b). Although consequences of this deletion have not been reported, splicing is likely perturbed because the intron 1 splice donor site is eliminated by the deletion.

In family 2, splicing may have occurred at sites within or flanking sequences that hybridize with the oligonucleotides used for PCR, resulting in failure to amplify variant transcripts. Two potential cryptic splice donor sites exist at the 5'-end of exon 1 (5'-CACCGTCCAGGGAGCAGGTAG-3', where underlined nucleotides mark potential cryptic donor sites) upstream from the forward primer sequence used for RT-PCR of mRNA from family 2. Use of these alternate donor sites would generate truncated exon 1 sequences either 4 or 17 bp in length. However, the size of most vertebrate exons appears to be confined to a certain range, with an observed average size of 137 bp (Berget, 1995). Berget has proposed that during

precursor mRNA processing exons are recognized by the pairing and interaction of spliceosome complexes across exons. In the case of first exons, interactions occur between factors that bind the 7-methyl-guanosine cap and the first intron splice donor site. Steric hindrance between assemblies at the ends of exons would prevent the definition of very short exons, so that splicing at the two putative cryptic donor sites far upstream in exon 1 is unlikely. Furthermore, it is not clear whether a shorter exon 1 would have any effect on p53 expression, since the translation initiation codon occurs downstream in exon 2.

The splice acceptor site mutation (IVS9-1G>C) in family 3 generated at least three new products as shown by RT-PCR (Figure 3). Sequencing one of the variants revealed use of a cryptic splice site in intron 9, which resembles the consensus acceptor site sequence CAG/G of higher eukaryotes (the 3'-end invariant dinucleotides are underlined) (Padgett *et al.*, 1986; Shapiro and Senapathy, 1987). Although products from the other variant transcripts were not sequenced, they are all larger than the expected PCR product, suggesting splicing at other cryptic acceptor sites in intron 9.

Our findings highlight the need to examine all p53 exons and all exon-intron borders, including untranslated regions, when screening for germline p53 mutations in LFS families. In addition, the loss of allele expression through transcript instability with certain splice site mutations illustrates the limitations of RNA-based assays in this screening process.

Materials and methods

Ascertainment

The three families (1–3) in this report are part of our series of 80 LFS kindreds ascertained over the last three decades. Affected probands and their relatives consented to participate in our Institutional Review Board-approved research protocol, release relevant medical records and donate blood samples for laboratory studies.

Cell lines

Normal epidermal keratinocytes cultured from family 1 member III.6 (strain LiF-Ep) and an immortalized cell line (LiF-Ep/TERT-1) derived from LiF-Ep following transduction to express hTERT (the catalytic subunit of telomerase), and which was found to have acquired loss of p53 protein expression and function after immortalization, have been described (Dickson *et al.*, 2000). Cells were maintained as exponentially growing cultures in GIBCO keratinocyte serum-free medium, as described (Dickson *et al.*, 2000).

Sequencing analysis

Genomic DNA was isolated from peripheral blood leukocytes using Qiagen DNA isolation kits. All 11 p53 exons (through the stop codon), including some intron sequence flanking each exon, were individually analysed by direct sequencing of PCR products. DNA was sequenced with an automated ABI PRISM 377 DNA sequencer using the ABI PRISM™ dye-primer cycle sequencing chemistry (PE Corporation). The primers used for PCR of genomic DNA are listed in Table 2. All primer pairs, including those used for RT-PCR (Table 3), were designed with alternate forward and reverse M13 tails to facilitate dye-primer sequencing of both DNA strands. PCR conditions were as follows: 94°C for 4 min; 10 cycles of 94°C, 65°C (decrease 1°C each cycle), and 72°C for 20 s each; 25 cycles of 94°C, 55°C, and 72°C for 20 s each; 72°C for 6 min. All alterations were confirmed by sequencing both DNA strands. Mutations were identified according to the recommendations of the Nomenclature Working Group for human gene mutations (Antonarakis, 1998).

RT-PCR analysis

Mononuclear leukocytes were isolated from freshly collected blood by ficoll gradient centrifugation. Total RNA was isolated from mononuclear cells by the acid-guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA from family 1 was also isolated from both primary and transformed epithelial cell lines. Reverse transcription reactions were run with 1–2 µg of total RNA using the Superscript Preamplification System (Life Technologies, Inc.) according to manufacturer's instructions with two modifications. The recombinant ribonuclease inhibitor, rRNasin (Promega), was added to each reaction at a final concentration of 1 unit/µl. Also, reactions were performed with both poly-dT and random hexamer primers together for the cDNA synthesis in families 1 and 2, whereas poly-dT alone or a p53-specific primer (E11bR, see Table 3) was used for family 3. The cDNA was amplified in two rounds of PCR to look for alternate-spliced products. The oligonucleotide primer sets used for each round of PCR are shown in Table 3: first round PCR primers E6/7F + E11bR and nested PCR primers E8F + E10R for leukocyte-derived cDNA, first round PCR primers E4bF + E11bR and nested PCR primers E6/7F + E11R for epithelial cell line-derived cDNA from family 1 with an IVS9+1delG/intron 9 donor site mutation; first round PCR primers E1F + E7R and second round PCR primers E1F + E2R and E1F + E4R for family 2 with an IVS1+1G>T/intron 1 donor site mutation; first round PCR primers E6/7F + E11bR and nested PCR primers E8F + E11R for family 3 with an IVS9-1G>C/intron 9 acceptor site mutation.

Assessing loss of allelic expression

cDNAs were synthesized from one member of families 1 and 2, who carry a germline p53 mutation and are heterozygous for the codon 72 polymorphism at the level of genomic DNA

Table 2 p53 primers used for PCR

		Primer sequences
Exon 1	forward	5'-GCTCAAGACTGGCGCTAAAA-3'
	reverse	5'-GTGACTCAGAGAGGACTCAT-3'
Exon 2	forward	5'-GAAGCAGCCATTCTTTCT-3'
	reverse	5'-GGTCCCCAGCCCAACCCTT-3'
Exon 3	forward	5'-GGAGCCGAGTCAGATCCTA-3'
	reverse	5'-GGTCCCCAGCCCAACCCTT-3'
Exon 4	forward	5'-CAACGTTCTGGTAAGGACAA-3'
	reverse	5'-GCCAGGCATTGAAGTCTCAT-3'
Exon 5	forward	5'-GCCGTGTTCCAGTTGCTTTA-3'
	reverse	5'-AGGAGGGGCCAGACTAAGA-3'
Exon 6	forward	5'-AGCGCTGCTCAGATAGCGAT-3'
	reverse	5'-TAAGCAGCAGGAGAAAGCCC-3'
Exon 7	forward	5'-AAGGCGCACTGGCCTCATCTT-3'
	reverse	5'-GGCTGGATGGGTAGTAG-3'
Exon 8	forward	5'-GACCTGATTTCTTACTGCCT-3'
	reverse	5'-TCCTCCACCCTTCTT-3'
Exon 9	forward	5'-GGAGACCAAGGGTGCAGTTAT-3'
	reverse	5'-GCCCAATTGCAGGTAAAC-3'
Exon 10	forward	5'-GGTACTGAAGTGCAGTTTCT-3'
	reverse	5'-CAGCTGCCTTTGACCATGAA-3'
Exon 11	forward	5'-CCAGCCTTAGGCCCTTCAA-3'
	reverse	5'-TGTCAGTGGGGAACAAGAA-3'

Table 3 p53 cDNA primers used for RT-PCR

cDNA primers	Primer sequences
E1F	5'-CTGGGCTCCGGGACACTTT-3'
E1bF	5'-CACCGTCCAGGGAGCAGGTA-3'
E4F	5'-CTCCTGGCCCCGTGTCATCTT-3'
E4bF	5'-GTCTGGGCTTCTTGCATTCT-3'
E4cF	5'-CCCTTGCCGTCCCAAGCAAT-3'
E6/7F	5'-TGAGGTGGCTCTGACTGTA-3'
E8F	5'-GGAAGAGAATCTCCGCAAGA-3'
E2R	5'-CGGCTCCTCCATGGCAGTGA-3'
E4R	5'-TCGTCCGGGGACAGCATCAA-3'
E7R	5'-CGCCCATGCAGGAAGTGTTA-3'
E7bR	5'-GGTGGTACAGTCAGAGCCAA-3'
E10R	5'-GAGTTCCAAGGCTCATTTCA-3'
E11R	5'-CTGTCAAGTGGGGAACAAGAA-3'
E11bR	5'-CCAGTGCAGGCCAACTTGT-3'

(Matlashewski *et al.*, 1987; Ara *et al.*, 1990). These cDNAs were amplified in two rounds of PCR using the following primers: first round PCR primers E1bF + E10R and nested PCR primers E4cF + E7bR. The second round PCR products were sequenced as described above.

Yeast p53 functional assay

RNA was isolated from fresh blood as described above in the DFCI laboratory and analysis of p53 transcriptional activity was performed by OncorMed, Inc. (Gaithersburg, MD, USA) according to Flaman *et al.* (1995).

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