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Genetics

Germ-line mutations of the p53 tumor suppressor gene in patients with high risk for cancer inactivate the p53 protein

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ABSTRACT **Germ-line mutations in the p53 tumor suppressor gene have been observed in patients with Li–Fraumeni syndrome, brain tumors, second malignancies, and breast cancers. It is unclear whether all of these mutations have inactivated p53 and thereby provide an increased risk for cancer. Therefore, it is necessary to establish the biological significance of these germ-line mutations by the functional and structural analysis of the resulting mutant p53 proteins. We analyzed the ability of seven germ-line mutant proteins observed in patients with Li–Fraumeni syndrome, second primary neoplasms, or familial breast cancer to block the growth of malignant cells and compared the structural properties of the mutant proteins to that of the wild-type protein. Six of seven missense mutations disrupted the growth inhibitory properties and structure of the wild-type protein. One germ-line mutation retained the features of the wild-type p53. Genetic analysis of the breast cancer family in which this mutation was observed indicated that this germ-line mutation was not associated with the development of cancer. These results demonstrate that germ-line p53 mutations observed in patients with Li–Fraumeni syndrome and with second malignancies have inactivated the p53 tumor suppressor gene. The inability of the germ-line p53 mutants to block the growth of malignant cells can explain why patients with these germ-line mutations have an increased risk for cancer. The observation of a functionally silent germ-line mutation indicates that, before associating a germ-line tumor suppressor gene mutation with cancer risk, it is prudent to consider its functional significance.**

Mutation of the p53 tumor suppressor gene is currently the most frequent molecular alteration in human cancers (1, 2). These changes are mostly missense mutations primarily found at evolutionarily conserved amino acid residues (1, 2). Ninety-eight percent of the 280 base substitution mutations reported so far in malignant tumors are clustered between amino acid residues 110 and 307 (out of a total of 393 amino acids) (2). In most of the human cancers analyzed, both p53 alleles are inactivated, suggesting that loss of wild-type function is important in carcinogenesis (1). Recent work has indicated that mutations of the p53 gene can occur not only at the somatic level but also in the germ line. These findings are important because the detection of germ-line p53 mutations should allow the identification of subjects at high risk to develop cancer. Germ-line p53 mutations were initially reported in patients with the rare Li–Fraumeni syndrome, a family cancer syndrome in which affected relatives develop a diverse set of malignancies, including breast carcinomas, sarcomas, and brain tumors (3–5). Analysis of germ-line p53 mutations outside Li–Fraumeni syndrome has revealed that these germ-line mutations occasionally can be observed in patients with brain tumors (6) and sarcomas (28), patients who have developed a second primary neoplasm (29), and those with familial breast cancer (30, 31). The involvement of a germ-line p53 mutation in the development of cancer can be demonstrated by the cosegregation of the mutant allele with cancer in the affected family members. This has only been possible to do in three families with Li–Fraumeni syndrome (3–5). As new germ-line p53 mutations are detected in pedigrees with a limited familial history and few available samples, the genetic analysis is sometimes impossible to perform. When linkage analysis is not possible, it is difficult to determine if missense mutations have a biological significance or instead represent previously undetected rare polymorphisms.

The functional and structural properties of p53 have been extensively analyzed (1). One of the main properties of the wild-type protein is the ability to inhibit the growth of malignant cells in culture (7–13). Mutations observed in human sporadic tumors (like the mutations at codons 143 and 273) have been shown to disrupt this growth inhibitory property (7, 10, 12, 13). Furthermore, most of the p53 mutations observed in human tumors induce structural modifications of the wild-type protein (1).

In this study, we analyzed the biological properties of germ-line p53 mutants to determine if these mutations had inactivated the wild-type p53 protein and therefore provided a high risk for cancer. Missense mutations at codons 181, 245, 248, 252, 258, 273, and 282 were selected because they were observed in patients with different clinical presentations (Li–Fraumeni syndrome, second malignancies, familial breast carcinoma) and because they were distributed between the conserved regions III and V of the p53 protein (see Table 1). Although some of these mutations had already been observed in sporadic tumors (14), the only mutation to have been partially characterized was the mutation at codon 273 (1, 10, 15).

MATERIALS AND METHODS

Constructs of Vectors Expressing the Mutant p53 Proteins. Oligonucleotide-directed mutagenesis of the p53 cDNA was performed in the phage M13mp19 with the Amersham kit using the following oligonucleotides: 181, 5′-CTGAG CAGTG CTCAT GG-3′; 245, 5′-TCCGG TTCAT GCAGC CCATG C-3′; 248, 5′-GGATG GGCCT CCAGT TCATG C-3′; 252, 5′-ATGAT GGTGG GGATG GGCCT CCGG-3′; 258, 5′-GGAGT CTTTC AGTGT G-3′; 282, 5′-CTGTG CGCCA GTCTC TCC-3′. The mutant cDNA was then substituted for the wild-type cDNA in the plasmid pC53-SN (7). In this plasmid, the p53 cDNA is under the transcriptional

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Abbreviation: SV40, simian virus 40.

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control of the cytomegalovirus promoter enhancer and the neomycin-resistance gene is under the control of the simian virus 40 (SV40) promoter enhancer. The complete coding region derived from the mutant M13 clone was entirely sequenced to ensure that the mutant cDNA contained only the desired mutation. The mutant p53 protein at codon 273 was expressed by the plasmid pSVXRI (10).

Cell Culture and Transfection. The Saos-2 cell line was obtained from the American Type Culture Collection, and the cells were grown as described (10). Plasmid DNA (10 μg) expressing wild-type or mutant p53 protein was transfected using the calcium phosphate procedure into Saos-2 cells at 50–70% confluence in 10-cm plates. For stable transfections, the cells were incubated with the DNA/phosphate precipitate during 4 hr, split 1:3 the next day, and refed with medium containing 500 μg of Geneticin (G418; GIBCO) per ml 48 hr after the transfection. The plates were stained with 50% methanol/10% acetic acid/0.25% Coomassie blue after 3 weeks of selection in G418-containing medium and the numbers of colonies were counted. For cotransfection experiments, 10 μg of p53 construct and 10 μg of plasmid pSVlac0 [expressing SV40 large T antigen (16)] were transfected into Saos-2 cells as indicated.

Antibodies, Immunoprecipitations, and Western Blot Analysis. PAb122 recognizes an epitope located between amino acids 370 and 378 of p53. PAb240 recognizes an epitope located between amino acids 156 and 335. The monoclonal antibody PAb416 is directed to the large T antigen of SV40. The monoclonal antibody directed against heat shock proteins was obtained from Amersham. Cells were metabolically labeled with 300 μCi of [^{35}S]methionine (NEN; 670 mCi/mmol; 1 Ci = 37 GBq) in 2 ml of methionine-free Dulbecco's modified Eagle medium plus 2% dialyzed fetal calf serum for 2 hr. Lysates and immunoprecipitations were performed as described (3). The immunoprecipitated proteins were separated on a 10% polyacrylamide gel. Western blot analysis of the immunoprecipitated proteins was performed after transfer of the proteins to Immobilon membranes (Millipore).

Sequencing Reactions and PCR Amplification. Sequencing reactions were performed according to the dideoxy chain-termination method using the Sequenase 2.0 kit (United States Biochemical). A 129-base-pair (bp) fragment was generated by PCR amplification from genomic DNA extracted from paraffin blocks (17). For each DNA template, two independent PCR reactions were performed. The sense and antisense primers were 181F, 5′-CCGGA ATTCC GGTGC CCCCA CCATG-3′, and 181R, 5′-CGGGA TCCCG CAATC AGTGA GGAAT C-3′, respectively. The primers 181F and 181R have synthetic *Eco*RI and *Bam*HI sites, respectively, at their 5′ ends to facilitate cloning. The PCR reactions were performed as described (3). The PCR products were submitted to *Hae* II analysis or digested with *Bam*HI and *Eco*RI and fractionated by electrophoresis on low-melt agarose. Fragments were ligated into the Bluescript vector "pBSK" (Stratagene) and, after bacterial transformation, individual clones were analyzed with the enzyme *Hae* II and sequenced using Universal primer or T3 primer (Pharmacia).

RESULTS

Effect of the Germ-line Mutants on the Growth of Malignant Cells. To determine if germ-line p53 mutations had inactivated the wild-type protein, constructs expressing germ-line mutant proteins were transfected into Saos-2 osteosarcoma cells. Both p53 alleles are deleted in this cell line. Previous studies have shown that the expression of transfected wild-type p53 blocks the progression of the cell cycle prior to S phase and that expression of the wild-type protein is not tolerated in stably transfected Saos-2 cells (10). Transfection of the plasmids expressing the germ-line mutants, except the mutant at codon 181, resulted in ≈8-fold more colonies than occurred when the plasmid pC53-SN3 [expressing wild-type p53 (7)] was transfected (Table 1, Fig. 1*A*). Analysis of p53 expression in stable transfectants revealed that most of the colonies derived from the transfections with the plasmids encoding the mutants at codons 245, 248, 252, 258, and 282 expressed high levels of p53 protein (Table 1, Fig. 1*B*). These results demonstrate that these five mutant proteins [like the mutant protein at codon 273 (10)] were unable to inhibit the growth of the Saos-2 cells. In contrast, none of the colonies derived from the transfection with the plasmid expressing the mutant protein at codon 181 (Fig. 1*B*) or the wild-type protein was found to express p53. This result indicates that the expression of the mutant protein at codon 181, like the expression of the wild-type p53, was incompatible with the growth of Saos-2 cells.

Structural Properties of the Germ-line Mutants. Next, we tested whether germ-line p53 mutants shared certain unique structural properties. Many inactivating mutations, which have been detected in sporadic tumors, have been shown to produce structural modifications of p53: (*i*) most of the mutant proteins are recognized by the antibody PAb240, which is specific for a mutant conformation (1, 18); (*ii*) frequently, the mutant proteins complex with hsc70, a constitutively expressed member of the heat shock protein family (19); (*iii*) all of the mutations observed in sporadic tumors and analyzed so far have been shown to disrupt the binding of p53 to SV40 T antigen (1).

As shown in Fig. 1*C*, immunoprecipitations performed with the antibody PAb240 revealed that only the germ-line mutant proteins at codons 245, 252, 258, and 282 were detected by PAb240. In contrast, the mutants at codons 181 and 248, like the mutation at codon 273 (1), were not recognized by this antibody (Fig. 1*C*).

Immunoprecipitation of the p53 proteins (Fig. 1 *B* and *C*) and Western analysis performed on the immunoprecipitated

Table 1. Comparison of germ-line p53 mutants to the wild-type protein

Mutant	Amino acid switch	Conserved region	Diagnosis	Saos-2 cell growth			PAb240	hsc70 binding	T binding
				Inhibition	No.*	Ratio†			
181	Arg → His	III	BC (30)	+	344 ± 77	0/7	−	−	+
245	Gly → Cys	IV	LFS (3)	−	1371 ± 131	4/4	+	±	+
248‡	Arg → Trp	IV	LFS (3), SMN (29)	−	1141 ± 36	4/8	−	−	−
252	Leu → Pro	IV	LFS (3)	−	1481 ± 44	7/8	++	++	−
258	Glu → Lys	IV	LFS (3)	−	1248 ± 120	5/5	++	++	+
273‡	Arg → His	V	SMN (29)	−	Ref. 10		−	−	−
282‡	Arg → Trp	V	SMN (29)	−	1132 ± 130	5/6	+	±	+
Wild type				+	174 ± 60	0/4	−	−	+

BC, breast carcinoma; LFS, Li–Fraumeni syndrome; SMN, second malignant neoplasm.
*Number of stable colonies in two or more independent experiments.
†Ratio of number of colonies positive for p53 expression/number of colonies analyzed.
‡The same mutation has been previously reported in sporadic tumors (14).

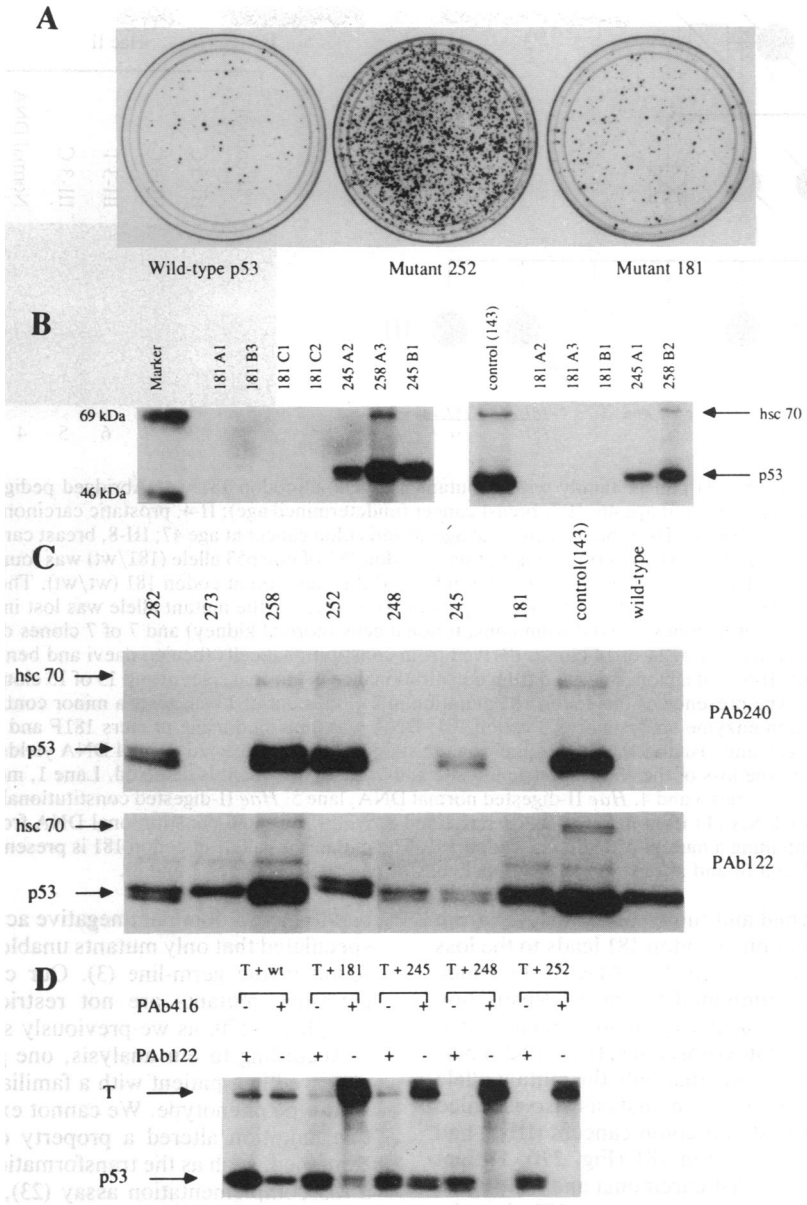

FIG. 1. Biological properties of germ-line p53 mutants. (*A*) Effect of germ-line p53 mutants on the growth of Saos-2 cells. Representative plates of Saos-2 cells stably transfected with constructs expressing the wild-type protein, the mutant protein at codon 252, and the mutant protein at codon 181 are shown. (*B*) p53 levels in stably transfected Saos-2 cells. Lysates from [^{35}S]methionine-labeled cells were immunoprecipitated with the monoclonal antibody PAb122. Transfected Saos-2 cells, expressing a mutant protein at codon 143, were used as a positive control for p53 and hsc70 binding (10). (*C*) Immunoprecipitation of germ-line p53 mutants with the antibody PAb240. Forty-eight hours after transient transfection of p53 plasmids, Saos-2 cells were [^{35}S]methionine labeled and lysates were immunoprecipitated with antibody PAb240 (top) or with antibody PAb122 (bottom). Cells expressing a mutant protein at codon 143 were used as a positive control for PAb240 (10). (*D*) Binding of germ-line mutants to SV40 large T antigen. Saos-2 cells were cotransfected with p53 plasmids and the plasmid pSVlac0 (16). Forty-eight hours after the transfection, lysates from [^{35}S]methionine-labeled cells were immunoprecipitated with either antibody PAb122 (p53) or PAb416 (large T antigen). p53 proteins that bind large T antigen are coprecipitated by both antibodies.

proteins (data not shown) showed that the mutant proteins at codon 252 and 258 were able to complex hsc70. A much weaker binding to hsc70 was occasionally detectable in cells stably transfected with constructs expressing the mutants at codon 245 and 282 (data not shown). In contrast, the mutant proteins at codons 181 and 248 as well as the mutant protein at codon 273 (1, 15) were unable to bind hsc70.

Cotransfection experiments of the plasmids expressing the germ-line mutants with a plasmid coding for SV40 large T antigen (16) demonstrated that only the mutant proteins at codons 248, 252, and 273 failed to bind large T antigen (Table 1, Fig. 1*D*). As shown in Table 1, the structural properties of the germ-line p53 mutants show a high degree of heterogeneity. However, with the exception of the mutant at codon 181, none of the germ-line mutants retained all of the structural features of the wild-type protein.

Genetic Analysis of the Mutation at Codon 181. The functional and structural studies of the germ-line mutants suggested that the mutant at codon 181 (Arg → His) was identical to the wild-type p53 (Table 1). Therefore, we speculated that this germ-line mutation might not provide an increased risk for cancer. To test this hypothesis, we performed genetic analysis on the family in which this germ-line p53 mutation has been observed. This mutation was detected in a patient (III-8) with a familial breast carcinoma (Fig. 2*A*) (30). This family is suggestive of Li–Fraumeni syndrome but does not fit the classic definition because the only sarcoma occurred after age 45 (20, 21). Using the PCR, we analyzed the codon

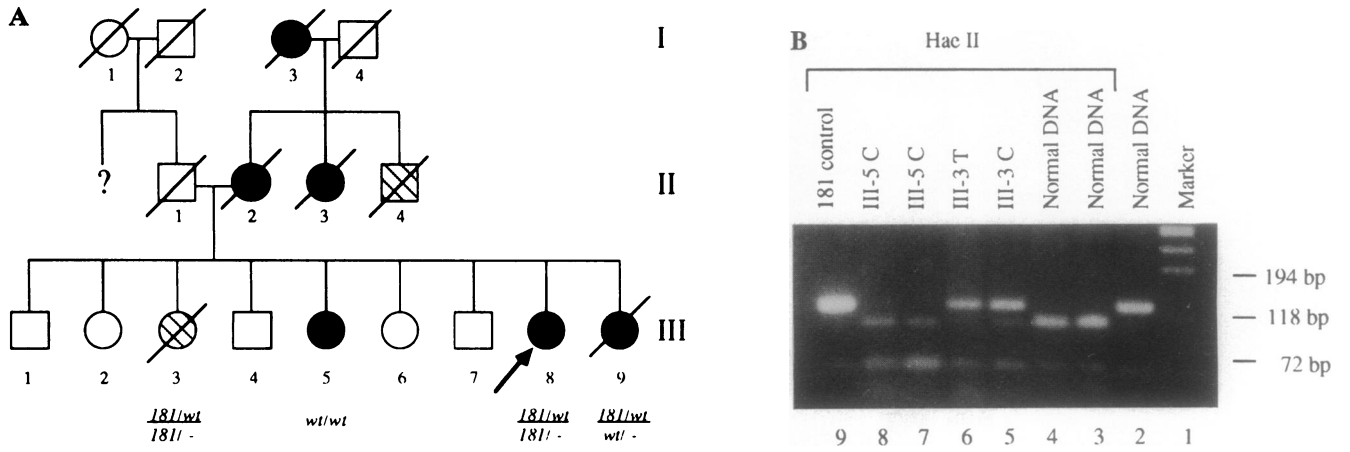

FIG. 2. Genetic analysis of a breast cancer family with a mutant p53 gene at codon 181. (*A*) Abridged pedigree of the family; the arrow indicates the proband: II-2, breast cancer at age 46; II-3, breast cancer (undetermined age); II-4, prostatic carcinoma (undetermined age); III-3, retroperitoneal leiomyosarcoma at age 49; III-5, breast cancer at age 40 and colon cancer at age 47; III-8, breast cancer at age 41; III-9, Hodgkin disease at age 22 and breast cancer at age 31. A germ-line mutation at codon 181 of one p53 allele (181/wt) was found in three affected members (III-3, III-8, III-9), whereas one affected member (III-5) has not inherited the mutation at codon 181 (wt/wt). The wild-type allele was lost in the leiomyosarcoma of patient III-3 and in the breast cancer of patient III-8 (181/−); the mutant allele was lost in the breast cancer of patient III-9 (wt/−). For patient III-3, 2 of 6 clones derived from constitutional cells (normal kidney) and 7 of 7 clones derived from tumor cells had the codon 181 mutation. For patient III-5, 14 of 14 clones derived from constitutional cells (benign naevi and benign colonic polyp) were wild type at codon 181. For patient III-9, 3 of 6 clones derived from constitutional cells (normal spleen) and 13 of 15 clones derived from tumor block were wild type at codon 181. The presence of the codon 181 mutation in 2 clones out of 15 suggests a minor contamination by normal cells in the tumor block. (*B*) Restriction enzyme analysis of p53 codon 181. DNA was amplified using primers 181F and 181R, digested with *Hae* II, separated on a 1.8% agarose gel, and visualized by ethidium bromide staining. *Hae* II-digested normal DNA yields 99-bp and 30-bp fragments. Mutation at codon 181 leads to the loss of the *Hae* II restriction site and a 129-bp fragment is detected. Lane 1, molecular weight marker; lane 2, normal DNA before digestion; lanes 3 and 4, *Hae* II-digested normal DNA; lane 5, *Hae* II-digested constitutional DNA (C) from patient III-3; lane 6, *Hae* II-digested tumor DNA (T) from patient III-3; lanes 7 and 8, *Hae* II-digested constitutional DNA from patient III-5; lane 9, *Hae* II-digested plasmid DNA containing a mutant p53 allele at codon 181. The mutant p53 allele at codon 181 is present in constitutional and tumor cells of patient III-3 (lanes 5 and 6) and absent in constitutional cells of patient III-5 (lanes 7 and 8).

181 of the p53 gene in normal and tumor tissue derived from three affected sisters. Mutation at codon 181 leads to the loss of the *Hae* II restriction site (AGCGCT → AGCACT), which is normally present in the amplified fragment. Restriction analysis (Fig. 2*B*) and nucleic acid sequencing indicated that one affected sister with a leiomyosarcoma (III-3) had inherited the mutation at codon 181 and that only the mutant allele was retained in the tumor. Restriction analysis also revealed that another sister with breast and colon cancers (III-5) had not inherited the mutation at codon 181 (Fig. 2*B*). Patient III-9, who had developed a breast carcinoma and a Hodgkin disease, has inherited the mutation at codon 181, but the mutant allele was lost somatically in the breast tumor (Fig. 2*A*).

DISCUSSION

The ability of the wild-type p53 protein to block the growth of malignant cells in culture is one of the main findings that has suggested that the p53 gene is a tumor suppressor gene (1, 7–13). Our results demonstrate that germ-line mutations observed in patients with Li–Fraumeni syndrome or with second malignancies disrupt this function of the wild-type p53 protein. These data indicate that one p53 allele has been inactivated in these patients. The inactivation of the growth inhibitory properties of p53 can explain why patients with these germ-line mutations have an increased risk of cancer. The biological significance of these germ-line mutations is confirmed by the fact that none of the germ-line mutants observed in patients with Li–Fraumeni syndrome or with second malignancies that we have analyzed have the same structural properties as the wild-type protein. Among these structural properties, it is important to point out the ability of some germ-line mutants to bind hsc70. The hsc70 binding property of some mutant p53 proteins observed in tumors has been linked to their ability to inactivate the wild-type protein and to have a dominant negative activity (15, 22). It has been speculated that only mutants unable to complex hsc70 will be found in the germ-line (3). Our current results show that germ-line mutants are not restricted to those unable to complex hsc70, as we previously suggested (3).

According to our analysis, one germ-line mutant protein observed in a patient with a familial breast carcinoma had a wild-type phenotype. We cannot exclude the possibility that this mutation altered a property of p53 that we have not examined, such as the transformation-suppressing activity in a *ras* complementation assay (23), transactivation (24, 25), and/or DNA binding activity (26). Nevertheless, genetic analysis of the cancer-prone family in which this mutation at codon 181 was observed provided two pieces of data that suggest that the mutation at codon 181 was not associated with the development of cancer in all members of this family. This mutation was not present in the germ line of a family member who developed two cancers. More important, the mutant allele at codon 181 was somatically lost during the development of a cancer in another relative.

We conclude from this study that six germ-line missense mutations reported in patients with Li–Fraumeni syndrome and in patients with second malignancies have inactivated the wild-type p53 protein. Nevertheless, our analysis of the mutation at codon 181 indicates that germ-line mutations, which change the amino acid sequence in the conserved domains of p53 (27), will not always be associated with an increased risk for early cancer. Therefore, genetic or biological analysis of the germ-line mutations in tumor suppressor genes should be a prerequisite before any counseling about cancer risk is provided to patients.

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