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RESEARCH ARTICLE

Three Germline Mutations in the *TP53* Gene

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Three germline mutations in the *TP53* tumor-suppressor gene are reported, two of which are not reported previously. A missense mutation at codon 265 of *TP53* was found in three patients of a family that complied with the definition of the Li-Fraumeni syndrome. A nonsense mutation in codon 306 was found in a woman who had had a rhabdomyosarcoma at age 4 and a subsequent breast cancer at age 22. She was part of a Li-Fraumeni-like family, but the parental origin of the mutation could not be traced. Finally, while screening for somatic alterations in *TP53* in a series of 141 sporadic breast tumors, we detected a constitutional missense mutation in codon 235 in a woman diagnosed with breast cancer at age 26 and a recurrence 4 years later. The recurrence, but not the primary tumor, showed an additional missense mutation at codon 245 as well as loss of the wild-type allele. This suggests that the 245 mutation was particularly important for tumor progression and that there might exist heterogeneity in terms of cancer predisposition potential among the various germline *TP53* mutations. *Hum Mutat* 9:157-163, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: *TP53*, Li-Fraumeni-like family, missense mutation, tumor progression

INTRODUCTION

Somatically acquired mutations in evolutionary conserved domains of the *TP53* gene have been found in many tumor types (Levine et al., 1991; Caron de Fromental and Soussi, 1992). In a number of malignancies, including colon and breast cancer, these mutations are strongly associated with loss of the wild-type allele (Hollstein et al., 1991). In addition, germline mutations have been detected in families with the Li-Fraumeni syndrome (LFS) (Malkin et al., 1990; Srivastasa et al., 1990). These data are genetic evidence that *TP53* is a tumor suppressor gene (Levine et al., 1994).

Families affected by LFS are characterized by a proband diagnosed with sarcoma before the age of 45 years, a first degree relative with cancer before the age of 45 years, and another first- or second-degree relative with either a sarcoma diagnosed at any age or any cancer diagnosed under the age of 45 years (Li et al., 1988). The spectrum of cancers associated with this syndrome is described as including mainly soft tissue sarcoma, osteosarcoma, brain tumors, leukemia, adrenocortical tumors and premeno-

pausal breast cancer (Li et al., 1988). When patients with early-onset breast cancer (Børresen et al., 1992; Sidransky et al., 1992) or multiple malignant neoplasms (Malkin et al., 1992; Eeles et al., 1993) are selected, germline mutations are detected in low frequencies (1-7%). These patients often derive from familial aggregations of various forms of cancer that diverge from the initial description of the Li-Fraumeni syndrome. Recently, the definition of the LFS has been extended to include families with multiple neoplasms and early-onset sarcomas as well. The cancers that arise in these families contain the mutant allele and commonly a reduction to homozygosity at this locus so that no wild-type alleles are present in the cancer (Levine et al., 1991).

During our ongoing studies of hereditary breast cancer (Cornelis et al., 1995) and somatic genetic changes at *TP53* in sporadic breast tumors (Cornelis

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et al., 1994), we detected three *TP53* germline mutations, two of which have not been reported before. These were found in a classic Li-Fraumeni family, in a patient with a rhabdomyosarcoma at age 4 as well as breast cancer at age 22, and in a breast cancer patient at age 26.

MATERIALS AND METHODS

Patients

A Li-Fraumeni family was brought to our attention by the Foundation for the Detection of Hereditary Tumors and a patient with multiple tumors by the Department of Pediatric Oncology of the University of Amsterdam. The 141 unselected breast cancer patients were consecutively collected from three hospitals in the Leiden area between 1983 and 1991 (Cornelis et al., 1994).

DNA Isolation

Constitutive genomic DNA was isolated from freshly collected peripheral blood leukocytes according to standard protocols (Miller et al., 1988). DNA from frozen tumor tissue and paraffin embedded tumor tissue was isolated as previously described (Gruis et al., 1993).

Mutation Screening

For two families, four DNA fragments covering the exons 5–8 of the *TP53* gene respectively, were amplified by polymerase chain reaction (PCR) and analysed separately by denaturing gradient gel electrophoresis (DGGE) (Abrams et al., 1990). Primers, PCR conditions, gradient concentrations, and running conditions for each amplified DNA fragment were as described (Top et al., 1994). The homoduplex mutant bands, as observed in DGGE, were gel-purified and used as template for sequence analysis. Sequence analysis was performed using a cycle sequencing procedure (Cycle sequencing Kit, Perkin Elmer Cetus, Gouda, the Netherlands) according to a method described earlier (Top et al., 1994). The germline mutation that was screened out from the 141 unselected cases was sequenced directly from the PCR-product obtained from constitutional DNA as described (Cornelis et al., 1994). Mutations were annotated according to Beaudet and Tsui (1993).

DNA Polymorphism and LOH

The microsatellite polymorphism detected by PCR was a (CA)_n polymorphism close to the *TP53* gene (Jones et al., 1992). Quantification of the radioactive signals was performed with a Phosphor Imager (Mol. Dynamics, Sunnyvale, CA). Any

imbalance in the ratio of allele intensities in the tumor samples relative to that in lymphocyte DNA was scored positive if it was roughly consonant with the percentage of tumor cells (Devilee et al., 1991).

Immunohistochemistry on p53

Protein Expression

For immunohistochemical detection of p53 protein, an avidin-biotinyl peroxidase technique was used on formalin-fixed, paraffin-embedded tumor tissue as previously described (Cornelis et al., 1994). The antibody used was DO7 (Dako, Glostrup, Denmark), dilution 1:200.

Isolation of Tumor Cells by Flow Cytometry

The isolation of tumor cells by flow sorting on the basis of their nuclear DNA content was done as described by Abeln et al. (1994). Nuclei were isolated from sections cut from paraffin embedded tissue blocks and stained with propidium iodide after RNase treatment. Sorting was performed on a FACStar flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a argon-ion laser (Spectral Physics, Mountain View, CA).

RESULTS

A family with two brothers, each of whom had rhabdomyosarcoma at the age of 2 years, and a father with a brain tumor at age 40, was classified by definition as having the Li-Fraumeni syndrome (Fig. 1). The paternal grandmother (I-2) is 77 years old and is still free of cancer. Her brother had a gastric cancer at age 65 and her husband, who died at age 56 from an unknown cause, had a brother who died at a young age from cancer of an unknown site. In this family DGGE analysis of the constitutional DNA of the father and his one available affected child showed a mobility shift of a PCR product containing exon 8 of the *TP53* gene. DNA sequencing confirmed that both individuals carried a T→C transition leading to a Leu265Pro mutation (Fig. 2A). Genomic DNA from lymphocytes of all available individuals as well as DNA isolated from paraffin-embedded tumor material of both sarcoma patients were typed for a polymorphic marker in the *TP53* region (*TP53*). This showed that both affected children share the paternal allele which co-segregates with the mutation (Fig. 1). This is further supported by comparison of the constitutional DNA and the tumor DNA in person III-3, which showed loss of the maternal allele in the tumor (Fig. 1). The genotype of individual III-1 derived only from tumor tissue and might therefore reflect a hemizygous "2"

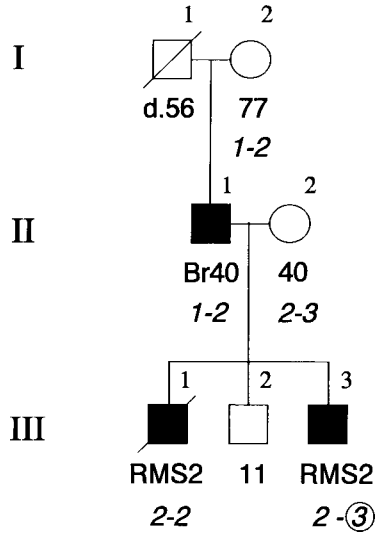


FIGURE 1. Pedigree of a Li-Fraumeni family with a constitutional p53 mutation. Circles represent females and squares represent males. Filled symbols are affecteds and crossed out individuals are deceased. For affected subjects, diagnosis is given with ages at onset and for unaffected members the current age or age at death is given. The genotypes at the polymorphic marker *TP53* are given below each typed individual. Individuals II-1, II-2, and III-3 were screened by DGGE. The results of DNA-analysis of III-2 have been omitted from the figure for reasons of confidentiality. d, deceased; Br, brain tumor; RMS, rhabdomyosarcoma; O, allele lost in the tumor.

of unknown parental origin. Yet no residual signal from allele "1" or "3" was detected, making this unlikely. Because DNA of person I-1 was not available and the *TP53* polymorphism was not informative, the grandparental origin of the mutation remained unresolved. A clear positive nuclear staining with the antibody DO7 was found in both rhabdomyosarcomas, but not in the normal tissue surrounding these tumors (data not shown).

Another patient, who had a rhabdomyosarcoma at age 4 and breast cancer at age 22, was also referred to our laboratory for analysis of *TP53*. The mother of this patient had developed breast cancer at an unknown age and her father a prostate cancer at age 45. Further family history was unretrievable. The constitutional DNA of this patient showed an additional band compared to the wild-type controls in the DGGE analysis of exon 8 of the *TP53* gene. DNA sequencing revealed a nonsense mutation Arg306End (CGA→TGA) (Fig. 2B). In the DNA isolated from the breast tumor tissue of this patient, we detected LOH with the *TP53* marker. As no DNA was available from the parents of this patient, it was not possible to determine the origin of the mutation nor of the

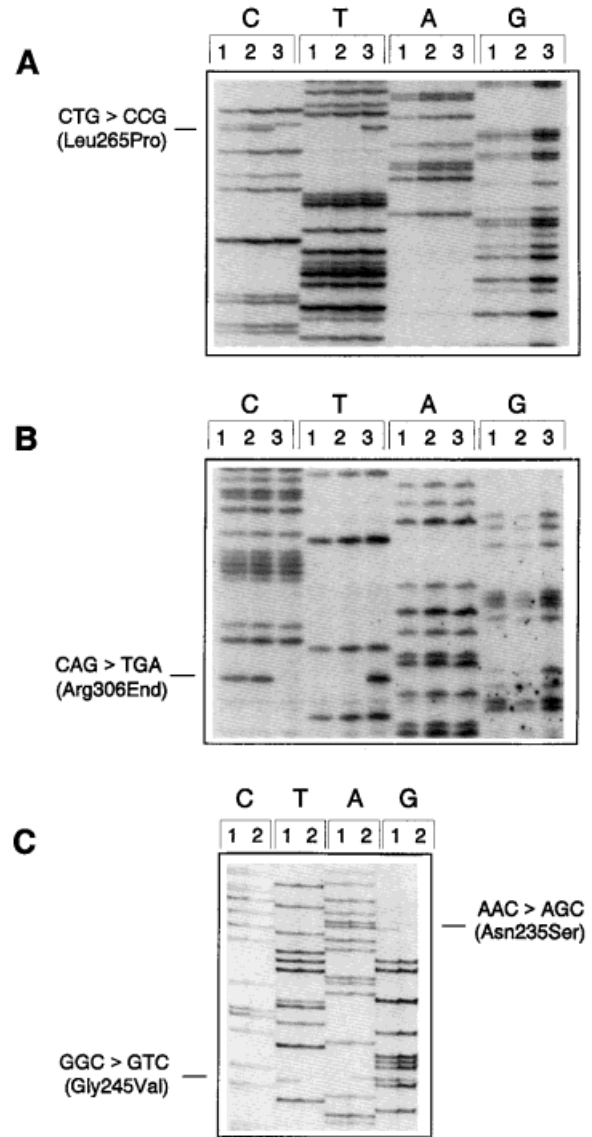


FIGURE 2. **A,B:** Sequence of exon 8 of the *TP53* gene. 1, lymphocyte DNA of individual III-3 in Figure 1; 2, lymphocyte DNA of individual II-1 in Figure 1; 3, lymphocyte DNA of the patient with a rhabdomyosarcoma and breast cancer. **C:** Sequence of exon 7 of *TP53*; 1, DNA of the recurrence of patient BT381 (Table 1); 2, DNA of an unrelated individual.

lost allele. No staining was found with the p53-antibody DO7 in the breast tumor.

While screening for somatic alterations in the *TP53* gene by CDGE and DGGE in a consecutive unselected series of 141 breast tumors (Cornelis et al., 1994), one case BT381 was found to carry a germline mutation. In fact, we detected two *TP53* mutations in the tumor of BT381, one was Asn235Ser (AAC→AGC), and the other was Gly245Val (GGC→GTC) (Fig. 2C). The G245V

TABLE 1. Schematic Representation of Results Obtained for Case BT381^a

	Lymphocytes	Primary tumor	Recurrence	Sorted fractions
DNA Index	1.00	1.13 1.84	1.13 1.84 2.23	1.13 2.23
A235S	+	+	+	?
G245V	-	-	+	+
LOH at <i>TP53</i>		-	+ ^b	+
Staining with DO7	-	+	+	

^aThe tumor cells of the recurrence with a DNA index of 1.13 and 2.23 was sorted by flow cytometry.

^bPartial loss. LOH, loss of heterozygosity; ?, not tested

mutation was screened out by DGGE on DNA from the tumor, but not from the lymphocytes. Only after direct sequencing of the same fragment, the A235S mutation was observed both in tumor and in lymphocyte DNA. Thus, the A235S mutation has been germline transmitted, while the G245V mutation has been acquired during tumor progression. After retrieving the pathology record of this patient, she appeared to have had a primary breast tumor at age 26 and a recurrence 4 years later, of which she died shortly thereafter. The tumor we examined for *TP53* mutation by DGGE actually was this recurrence. Anamnestically, she had a mother with ovarian cancer and a sister with breast cancer both diagnosed at unknown ages. Paraffin material of the primary breast tumor was analyzed by DGGE and sequencing and found to contain only the germline A235S mutation (Table 1). The primary tumor retained heterozygosity at *TP53*. In the recurrence however, a partial loss of one allele was found. Flow cytometry indicated multiple aneuploid stemlines in the primary tumor as well as in the recurrence. The primary tumor showed DNA-indices of 1.13 and 1.84. In addition to these, a cell population with a DNA index of 2.23 was detected in the recurrence, suggesting a tetraploidization of the 1.13 stemline had occurred. We isolated the tumor cell populations from the recurrence with DNA indices of 1.13 and 2.23 by flow sorting. In both populations, DGGE analysis evidenced the G245V mutation. In addition both populations showed an almost complete loss of one allele at *TP53*, indicating that the allele bearing the G245V mutation was retained. Unfortunately, the number of sorted cells was insufficient to allow DNA sequencing analysis, so we were unable to determine whether or not the remaining allele also contained the germline A235S mutation. Positive staining with the antibody DO7 was found in the paraffin

material of the primary tumor and in the recurrence but not in the normal tissue.

DISCUSSION

To date, approximately 70 germline mutations have been reported in *TP53*, predominantly of the missense type and affecting codons 141–282 (Malkin, 1994). The mutation spectrum does not exclude the possibility that some mutations are not tolerated during gametogenesis and hence cannot be transmitted through the germline. We describe three germline mutations and the characterization of the associated tumors for protein staining and allele loss, further exemplifying the complexity of p53 involvement in inherited cancer.

The *TP53* gene encodes a 53-kD nuclear phosphoprotein with tumor suppressor activity (Levine et al., 1994). In cells with DNA damage, p53 protein levels are increased either to arrest the cell cycle in G1 to allow DNA repair or to trigger cell death by apoptosis (Lane, 1992). Biochemically, p53 is characterized by its ability to bind to DNA and to transactivate gene transcription, for which it must oligomerize to form a quaternary structure. The somatic mutations found in *TP53* are most often point missense mutations in the central region of the protein (Hollstein et al., 1991), which constitutes its DNA binding pocket. Indeed, amino acid residues directly involved in DNA binding are the most frequently altered in cancer (Cho et al., 1994). Most examined mutants are defective in DNA binding and/or transactivation and inactivation of p53 function is therefore an important step in the development of many different malignancies. Although dominant-negative effects for some mutants have been observed (Harvey et al., 1995), in many tumors the mutation is accompanied by a loss of the wild-type allele. The p53 protein is present at very low levels in normal tissues and usually cannot be detected using conventional immunohistochemical methods. However, a very strong correlation has been observed between high-level expression of the proteins in tumors and point missense mutations (Lane, 1994), suggesting that these mutations confer increased stability to the protein.

We found a missense mutation in codon 265 in a family with the classical Li-Fraumeni syndrome (Li et al., 1988). To date, germline mutations in the *TP53* gene have been reported in approximately one-half of all investigated families with this syndrome (Birch et al., 1994; Frebourg et al., 1995). Both rhabdomyosarcoma patients inherited the mutation from their father, who had had a

brain tumor at age 40. One rhabdomyosarcoma was proven to have lost the wild-type TP53 allele. This is fully consistent with Knudson's two-hit model for the inactivation of a tumor suppressor gene and argues strongly against the mutation being a neutral polymorphism. Interestingly, the tumor cells, but not the adjacent normal tissue of both rhabdomyosarcomas in this family showed positive nuclear staining with the p53-antibody DO7. Clearly, the mutation itself, while present heterozygously in normal cells of both patients, is not sufficient to induce protein stability, other, tumor-specific, events are needed to achieve this. It has been proposed (Lane, 1994) that p53 in tumor cells is stable because the cell is in a permanent state of damage, perhaps related to chromosome damage, similar to the induction of wild-type p53 stability in cells exposed to DNA-damaging agents (Kuerbitz et al., 1992).

A nonsense germline mutation at codon 306 was found in a patient with multiple primary tumors and a LFS-like family history, consisting of a father with prostate cancer and a mother with breast cancer. Mothers of children with a soft tissue sarcoma have been found to be at increased risk for breast cancer (Strong et al., 1987), but we were unable to determine whether or not the mutation was inherited and, if so, from which parent. Germline mutations in TP53 have been found in up to 7% of patients with a soft tissue sarcoma and a subsequent other malignancy (Malkin et al., 1992; Toguchida et al., 1992; Eeles et al., 1993). Nonsense germline TP53 mutations are rare and are not very likely to be a polymorphism. In the breast tumor of this patient, we detected LOH, suggesting that the remaining wild-type allele was eliminated. As expected for a nonsense mutation, no immunostaining was found in the breast tumor, perhaps because the resulting protein is unstable due to its loss of the oligomerization domain (Levine et al., 1994).

The third germline mutation was A235S and was present in a patient diagnosed with breast cancer at age 26 and a recurrence 4 years later. Again, we were unable to ascertain if her mother, who had ovarian cancer, and her sister with breast cancer, were carrying the same mutation. While most breast-ovarian cancer families are explained by mutations in BRCA1 on 17q12-q21 (Narod et al., 1995), a splice-site mutation in TP53 in one breast-ovarian cancer family (Jolly et al., 1994) suggests that p53 might be involved in some of them. Possibly, upon further extending the family history of these families, other malignancies, consistent with LFS, might be encountered.

No LOH was observed in the primary tumor of our patient. Her recurrence did show LOH, but also acquired a second mutation in TP53, a G245V. Peculiarly, the tumor cells of both the primary tumor and the recurrence specifically stained positive with the p53 antibody DO7. The absence of LOH in the primary tumor might indicate that the germline mutation conferred no selective growth advantage and that this tumor developed through other genetic pathways. In that case, the observed immunostaining would reflect p53 stabilized by the continuous presence of DNA damage. Codon 235 is not directly involved in DNA binding (Cho et al., 1994) and is located in between two domains of the protein, which interact extensively to provide DNA contacts. Interestingly, Diller et al. (1995) have found the same germline mutation in codon 235 in a sporadic 2-year-old rhabdomyosarcoma patient, age 21 at the time of analysis, without having had any other malignancies. The mutation might therefore indeed be less significant in terms of cancer predisposition potential. Alternatively, the A235S germline mutation could have had a dominant-negative effect (Harvey et al., 1995) or a gain-of-function (Bhatia et al., 1993) as a result, in which case loss of the wild-type allele would not necessarily have been selected for.

The concomitant appearance of both LOH and an additional G245V mutation in the recurrence of this patient suggests that the two events are related and provided a selective growth advantage. Although we were unable to determine whether or not the A235S germline mutation was located on the same allele as the G245V, the fact that in the unfractionated recurrence both partial LOH and the A235S were observed, suggests that they were. The somatic mutation could have been induced by radiation therapy given as a treatment to the patient after surgery of the primary tumor. The glycine at codon 245 makes direct contact with the DNA (Cho et al., 1994) and its mutation into valine, which has been found recurrently in many malignancies (Hollstein et al., 1991), will have dramatic impact on p53 function. The available data thus suggest that the G245V mutation was particularly important for tumor progression, while the tumorigenic potential of the A235S is currently undetermined. However, they do not fully exclude the possibility that the A235S confers some gain-of-function effect on the p53 protein. It is intriguing in this regard that this mutation has not yet been reported in US or LFS-like families (Malkin, 1994). Clearly, functional studies of the A235S are needed to validate its relevance in terms of cancer predisposition.

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