Two Li–Fraumeni syndrome families with novel germline p53 mutations: loss of the wild-type p53 allele in only 50% of tumours

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Summary We describe two Li–Fraumeni syndrome families. Family A was remarkable for two early childhood cases of adrenocortical tumours, family B for a high incidence of many characteristic cancers, including a childhood case of choroid plexus tumour. Using direct sequencing, we analysed exons 5–9 of the p53 gene in constitutional DNA of individuals from both families and found two novel germline mutations in exon 5. In family A, we detected a point substitution in codon 138 (GCC to CCC), which resulted in the replacement of the alanine by a proline residue. Family B harboured a single-base pair deletion in codon 178 (CAC to –AC), resulting in a frameshift and premature chain termination. Three out of six tumours examined from both families, a renal cell carcinoma, a rhabdomyosarcoma and a breast cancer, showed loss of heterozygosity and contained only the mutant p53 allele. The remaining three neoplasms, both adrenocortical tumours and the choroid plexus tumour retained heterozygosity. Immunohistochemistry with anti-p53 antibody confirmed accumulation of p53 protein in tumours with loss of heterozygosity, while the remaining tumours were p53 negative. These results support the view that complete loss of activity of the wild-type p53 need not be the initial event in the formation of all tumours in Li–Fraumeni individuals.

Keywords: Li–Fraumeni syndrome; germline p53 mutation; loss of heterozygosity; p53 immunohistochemistry

The Li–Fraumeni syndrome (LFS), first described in 1969 (Li and Fraumeni, 1969), is a rare dominantly inherited condition that confers an increased susceptibility to cancer to family members. These individuals are at a high risk of developing a large spectrum of cancers, often with a very early onset. Multiple primary tumours are also common. The prevalent types of cancer are soft-tissue sarcomas, osteosarcomas, brain tumours, leukaemias, adrenocortical carcinomas and premenopausal breast cancer, but other cancers have also been reported (Li et al, 1988). A family with a high incidence of cancer is diagnosed as LFS if there is a proband with a sarcoma under 45 years, one first-degree relative with cancer under 45 years and another first- or second-degree relative with cancer under 45 years or a sarcoma at any age (Li et al, 1988).

In 1990, germline mutations in the p53 tumour-suppressor gene, which is located on chromosome 17p13, were identified as a genetic basis of LFS (Malkin et al, 1990; Srinivastava et al, 1990). Since then, a variety of germline mutations have been identified in approximately 50% of LFS families analysed (Birch et al, 1994a; reviewed in Malkin, 1994; Eeles, 1995; Frebourg et al, 1995). In addition, germline p53 mutations have been reported in settings not conforming to the criteria of classical LFS, for example as in isolated sarcoma patients (Toguchida et al, 1992) or in individuals with multiple tumours (Malkin et al, 1992). Similar to somatic p53 mutations frequently found in sporadic tumours, most of the germline mutations are missense mutations, clustering in several conserved domains of the p53 gene (Malkin, 1994; Eeles, 1995). At present it is not clear whether there is a heterogeneity in LFS with respect to presence or absence of germline p53 mutations (Wang et al, 1996). There are cases of LFS pedigrees, however, in which the linkage between the cancer susceptibility and the p53 locus has been excluded (Birch et al, 1994b).

In accordance with the concept of p53 being a tumour-suppressor gene, the tumours in LFS patients are expected to lose the functional (by the germline mutation unaffected) p53 allele. There are several single-case reports and one recent larger study indicating, however, that loss of heterozygosity is not observed in a significant fraction of these tumours (Varley et al, 1997).

Although genetic diagnosis of germline p53 mutations is possible in many LFS families, the syndrome is associated with medical, counselling, psychological and ethical problems (Li et al, 1992). Up to now, no correlation between the type and location of the germline mutation within the p53 gene and clinical features has been identified. The exact penetrance and age-, sex- and site-specific cancer risk figures for carriers of diverse mutations are not known. The heterogeneity of the malignancies does not allow a simple and effective preventive screening, and the treatment of aggressive tumours associated with LFS is often complicated. Further studies are therefore needed to generate additional background knowledge for pilot intervention efforts in carriers of germline p53 mutations.

To obtain further data on the nature and implications of specific p53 germline mutations and on the behaviour of the p53 gene and...
protein in tumours of carriers of these mutations, we analysed in detail two LFS families and identified two yet unreported germline mutations in exon 5 of the p53 gene. We also observed interesting patterns of loss of heterozygosity at the p53 locus and of p53 protein accumulation in tumours in these families.

MATERIALS AND METHODS

Family studies

Families A and B (Figure 1) were referred from the Department of Medical Genetics, Charles University Hospital, Prague-Motol, and the Second Radiotherapeutic Clinic, Paskov, respectively, based on the unusual clustering of cancer cases. Both families were offered counselling and were asked for consent before sampling and testing. The diagnoses of tumours were confirmed through hospital and pathology reports and death certificates. Independent confirmation of histology was possible for the tumours of A-II.1, A-IV.1 (2×), A-IV.2, B-III.2 and B-IV.1.

PCR

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods (Baas et al., 1984). Exons 5 and 6 of the p53 gene were amplified by primers e56F (TGCCCTGACTTTTCAACCTGTGTC) and e56B (CCACTGACAACCCCTTAAACC), exon 7 by primers e7F (AAGGCGCAGCCTGCTCATCTTTG) and e7B (CCAGTGTGCAAGGTTGCAAGTG) and exons 8 and 9 by primers e89F (GACCTGATTCTCTTACTGCTTCTC) and e89B (CCACCTGATAAGGTCAGGCAAG). The 50-µl polymerase chain reaction (PCR) contained 30 pmol of each primer, 250 µM of each dNTP, 50–100 ng of genomic DNA and 0.2 units of Super Taq DNA polymerase (Stehelin & Cie) in a buffer recommended by the supplier. The reaction mixture was subjected to 35 cycles of denaturation at 93°C for 60 s, annealing at 65°C for 45 s and extension at 75°C for 90 s. The annealing and extension temperatures for primers e89F and e89B were lowered to 59°C and 72°C respectively. DNA from archival paraffin-embedded tissues was isolated using the microwave method (Banerjee et al., 1995). Shorter PCR products spanning the mutation sites were amplified using primers e56F and e138B (TGTCCTGACTGGTCTGAGG) in samples from family A and e56F and e5B (TCAGTGGAGATTAGGCCTTG) in samples from family B. In these cases, the number of PCR cycles was increased to 45.

Sequencing

The PCR products were purified from agarose gels using QIAquick Gel Extraction Kit (QIAGEN). Cycle-sequencing of both strands (Carothers et al., 1989) was performed using either of the two primers used in PCR with dye terminator chemistry and AmpliTaq FS (Perkin-Elmer). The products were analysed on an ABI 373A sequencer.
Table 1. Summary of results of molecular and immunohistochemical studies in families A and B

| Family | Codon | Nucleotide change | Amino acid change | Subject | Blood | Type | Loss of wt p53 Allele | p53 Staining positive cells (%) |
|--------|-------|-------------------|-------------------|---------|-------|------|-----------------------|--------------------------------|
| A      | 138   | GCC > CCC         | Ala > Pro         | A-II.1  | –     | Renal cell carcinoma | Yes                   | 35–47                            |
|        |       |                   |                   | A-III.2 | wt/mut| Adrenocortical carcinoma | No | 0                             |
|        |       |                   |                   | A-IV.1  | wt/mut| Rhabdomyosarcoma       | Yes            | 28–36                           |
|        |       |                   |                   | A-IV.2  | wt/mut| Adrenocortical adenoma | No             | Rare cells                     |
| B      | 178   | CAC > -AC         | Frameshift        | B-III.2 | wt/mut| Breast cancer          | Yes            | 90–95                           |
|        |       |                   |                   | B-IV.1  | –     | Choroid plexus tumour  | No             | 0                              |

wt, Wild type allele; mut, mutant allele. Loss of the wt allele was deduced from sequencing gels.

**p53 Staining**

Immunohistochemistry using the p53 antibody DO-7 (Dako) diluted 1:50 was performed on paraffin-embedded tumour material using a high-performance detection system APAAP (BioGenex). The primary serum was substituted by non-immune serum for negative controls. Positive controls were provided with each set of antibodies. The slides were counterstained with Mayer’s haematoxylin.

**RESULTS**

**Identification of germline mutations**

The pedigrees of families A and B showed clustering of cancer cases, with a high proportion of tumours being characteristic for LFS and often occurring at a young age (Figure 1). Both families conformed to the criteria of classical LFS (Li et al, 1988). Peripheral blood lymphocyte DNA samples from individuals A-III.2, A-IV.1, A-IV.2 and B-III.2 were available. The results of the search for germline p53 mutations in these samples are summarized in Table 1.

In family A, the direct sequencing of PCR products generated from exons 5–9 of the p53 gene from lymphocyte DNA of individuals A-III.2, A-IV.1 and A-IV.2 revealed heterozygosity for a G to C transition in the first position of codon 138. The codon change from GCC to CCC resulted in an amino acid change from alanine to proline (Figure 2).

The sequence analysis of constitutional DNA of individual B-III.2, the only living affected member of family B, revealed heterozygous deletion of one C nucleotide in the first position of codon 178 of the p53 gene. The frameshift caused by this deletion results, if translated, in the incorporation of 68 illegitimate amino acid residues and premature chain termination (Figure 2).

**Analysis of tumours**

Archival tumour samples from the renal cell carcinoma of A-II.1, adrenocortical tumours of A-IV.1 and A-IV.2, rhabdomyosarcoma of A-IV.1, breast cancer of B-III.2 and brain tumour of B-IV.1 were available for independent confirmation of the diagnoses as well as for DNA analysis and immunohistochemistry. The results of these studies are summarized in Table 1.

The renal cell carcinoma of patient A-II.1 had a typical clear-cell cytology and displayed a solid and tubular growth pattern. Sequencing of the tumour DNA isolated from archival samples showed loss of the wild-type p53 allele at the site of germline mutation in codon 138 of the p53 gene. Immunohistochemical detection of the p53 protein with antibody DO-7 in this tumour revealed a strong to moderate staining in a high proportion of tumour cells (Figure 3A). Similar to all other tumours examined in family A, the immunohistochemical p53 positivity was confined to tumour cell nuclei. Stromal cells, such as vascular endothelium, were negative in all tumours examined.

The adrenocortical tumour of patient A-IV.1 was grossly encapsulated, measuring 45×50×20 mm. It was composed of large polygonal cells with marked nuclear irregularities, growing in diffuse sheets and showing numerous mitotic figures, including multipolar mitoses (mitotic count averaged 8/10 high-power fields (HPF), using objective 40x and eyepiece 12.5x). There were microscopic foci of capsular invasion by the tumour into the surrounding adipose tissue. Vascular invasion was not observed. The tumour was hormonally active causing virilization. It was diagnosed as an adrenocortical carcinoma according to the criteria outlined by Weiss et al (1984) and van Slooten et al (1985). The tumour retained heterozygosity in codon 138 of the p53 gene and p53 staining was negative.

The soft-tissue tumour of the above patient was a predominantly spindle-cell sarcoma with occasional tadpole and round rhabdomyoblasts positive for desmin, sarcomeric actin and myoglobin. It was classified as an embryonal rhabdomyosarcoma. It showed loss of the wild-type allele in codon 138 (Figure 2) and strong nuclear staining with the anti-p53 antibody (Figure 3B).

The adrenocortical tumour of patient A-IV.2 was encapsulated, measuring 50×50×40 mm. Light microscopy revealed solid trabecular and solid alveolar patterns. The tumour cells showed moderate nuclear atypia. The mitotic count was low (<1/10 HPF). No capsular or vascular invasion was found. The tumour was hormonally active with virilizing effects and was classified as an adrenocortical adenoma. This tumour did not show loss of heterozygosity in codon 138. It was generally p53 negative, although rare scattered foci of tumour cells with positive nuclear p53 staining were observed (Figure 3C).

The breast cancer of the patient B-III.2 from family B was classified as ductal infiltrating carcinoma. It showed loss of heterozygosity in codon 178 of the p53 gene and displayed only the mutant allele (Figure 2). The p53 immunohistochemistry revealed a strong staining of most tumour cells (Figure 3D). Non-neoplastic epithelial cells of breast ducts and lobules as well as stromal cells were negative.

The choroid plexus carcinoma of patient B-IV.1 was characterized by marked cellular atypias with foci of solid growth pattern besides more typical papillary areas. The tumour DNA retained
heterozygosity in codon 178, and the immunohistochemistry with anti-p53 antibody yielded a negative result.

DISCUSSION

Both families A and B conform to the criteria of classical LFS and show further typical features of this syndrome (Li et al, 1988). Our analysis identified germline mutations in the p53 gene in both families studied.

Family A is remarkable because of the occurrence of two cases of early childhood adrenocortical tumours in two sisters (A-IV.1 and A-IV.2) and a childhood sarcoma in the first child. The renal cell carcinoma of the grandfather of the two children (A-II.1) is a rare, but already reported cancer in LFS (Li et al, 1988). The presence of rhabdomyosarcoma and adrenocortical tumours in young children point to a high risk of carrying a germline p53 mutation (Birch et al, 1994a). Indeed, a germline mutation in codon 138 was identified in both girls and their mother (A-III.2). The mother is now 30 and cancer free, but is at a very high risk of developing breast cancer. The outbreak of early childhood cancer in children from a 30-year-old asymptomatic carrier whose ancestors developed cancer between 37 and 45 years of age may possibly have been influenced in the last generation by a change in the genetic background on which the germline p53 mutation exists in this pedigree.

The functional consequences of the missense mutation in codon 138 remain to be determined, but several lines of evidence support its causal role in the LFS in family A. Codon 138 is located in the DNA binding domain of the p53 protein (Cho et al, 1994). The alanine residue in position 138 is located in the conserved domain II. of p53 and shows absolute evolutionary conservation in p53 sequences of all vertebrate and one mollusc species reported so far (Soussi and May, 1996). Owing to the cyclic nature of proline, the replacement of alanine by proline may have profound consequences on the protein structure. No polymorphisms have been described for codon 138 (De Vries et al, 1996). A different germline mutation of this codon, GCC to TCC, replacing alanine by serine, was described in a family with gliosarcoma and non-Hodgkin's lymphoma (Kyritsis et al, 1994). The databases of somatic p53 mutations in tumours list 13 tumours with missense codon 138 mutations, five of which are identical with the mutation seen in family A (De Vries et al, 1996; Hollstein et al, 1996). In family A itself, the mutation segregates with cancer susceptibility, and two tumours analysed show loss of the wild-type allele while retaining the mutant allele.

Family B represents a textbook example of LFS. The occurrence of choroid plexus tumour in B-IV.1 supports the view that this tumour may also be frequent in LFS (Li and Fraumeni, 1994). The frameshift germline p53 mutation identified in this family very likely abolishes the function of the mutant allele. The deletion in codon 178 occurs in a contiguous stretch of five C nucleotides, which represents one of the hot spots for somatic insertion–deletion mutations in p53 (Greenblatt et al, 1996). An identical mutation also plays a role in sporadic cancer and was reported as a
somatic mutation in ten different tumours (De Vries et al, 1996; Hollstein et al, 1996). Translation of the mutant allele can give rise to a truncated protein. There are several reports showing that mRNA corresponding to p53 alleles with premature stop codons is absent from cells harbouring these mutations in a heterozygous state (Felix et al, 1993; Horio et al, 1994; Stolzenberg et al, 1994).

Frameshift mutations caused by insertion–deletion events or splicing mutations represent approximately only 10% of germline p53 mutations reported so far (Malkin, 1994; Eeles, 1995). Individuals or families carrying these mutations do not show any particular features compared with those harbouring diverse missense mutations. Although it is tempting to speculate that the mutation in family B has a very high penetrance and that the cancer onset is relatively early, more data are needed to establish possible genotype–phenotype correlations in LFS.

Our studies of patterns of loss of heterozygosity at the p53 locus and of protein p53 accumulation yielded interesting results. Two out of four tumours analysed in family A and one of two in family B showed loss of the wild-type p53 allele at the site of the germline mutation, while the remaining tumours retained heterozygosity. This pattern is in agreement with published data, in which the fraction of tumours with loss of heterozygosity ranges from 44% to 69% (Varley et al, 1997). Our results are further supported by immunohistochemistry with the anti-p53 antibody, which indicated the accumulation of the p53 protein in tumours that have lost the wild-type allele. With the exception of rare foci of p53-positive cells in the adrenocortical tumour of A-IV.2, no accumulation of p53 was detected in tumours that retained heterozygosity at the site of germline mutation. Although a second independent somatic mutation disabling the wild-type p53 allele in these tumours cannot be ruled out, the above results suggest that wild-type p53 is not inactivated here.

It is interesting that the tumour types in which neither loss of heterozygosity nor positive p53 staining were observed belong to early childhood tumours characteristic of LFS. While the sporadic adrenocortical tumours are most common between the fifth and seventh decade of life, a substantial fraction of childhood cases of this tumour belong to LFS families. Similarly, choroid plexus tumour may be another specific early manifestation of LFS. It may be possible that the initial event in the formation of these tumours in LFS individuals is not the complete loss of activity of the wild-type p53, but that they may be initiated in heterozygous cells by a p53 dosage effect during a period that is critical for the particular tissue or cell type. The post-natal regression of the fetal adrenal cortex and a progressive remodelling of the fetal zone into the definitive zones fasciculata and reticularis (Symington, 1982) may be one such critical point.

The identification of germline p53 mutations is at present accompanied by counselling and medical problems (Li et al, 1992). The situation is slightly different for those members of cancer-prone families who are already affected by cancer (A-IV.1, A-IV.2, B-III.2) or who are aware of their obligate carriership.

Figure 3. Immunohistochemical detection of the p53 protein showing nuclear positivity in tumour cells. The cell nuclei are counterstained with haematoxylin. (A) Renal cell carcinoma of patient A-II.1. The intensity of staining varies, being generally strong to moderate. Magnification x350. (B) Embryonal rhabdomyosarcoma of A-IV.1. A spindle-cell area with positive nuclear staining. Magnification x350. (C) Adrenocortical adenoma of A-IV.2. One of the rare foci of tumour cells positive for the p53 protein (arrow). The staining is homogenous and obscures the chromatin pattern seen in negative tumour cell nuclei counterstained by haematoxylin. Magnification x350. (D) Infiltrating ductal breast carcinoma of patient B-III.2. Strong positivity for p53 in most cell nuclei. Magnification x225.
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