The value of rapid functional assays of germline p53 status in LFS and LFL families

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Summary We have tested two rapid assays of p53 function, namely the apoptotic assay and the FASAY as means of detecting germline p53 mutations in members of Li–Fraumeni and Li–Fraumeni-like families. Results of the functional assays have been compared with direct sequencing of all 11 exons of the p53 gene. The results show good agreement between the two functional assays and between them and sequencing. No false-positives or negatives were seen with either functional assay although the apoptotic assay gave one borderline result for an individual without a mutation. As an initial screen the apoptotic assay is not only rapid but inexpensive and very simple to perform. It would be expected to detect any germline defect that leads to loss of p53 function. The apoptotic assay could be ideal as a means of prescreening large numbers of samples and identifying those that require further investigation. The FASAY detects mutations in exons 4–10, is rapid and distinguishes between functionally important and silent mutations. © 2000 Cancer Research Campaign

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Rare cancer-prone families were first recognized in the late 1960s with a very particular clinical picture involving early onset sarcomas, breast carcinoma, brain tumours, leukaemias and adrenal carcinoma (Li and Fraumeni 1969); these families are said to exhibit Li–Fraumeni syndrome (LFS). More recently, families have been recognized with similar spectra of malignancies, which do not fully meet the precise criteria of classical LFS (Birch et al, 1994; Eeles, 1995). Such families have been called Li–Fraumeni-like (LFL). In 1990, Malkin et al demonstrated a relationship between germline p53 mutations and LFS. p53 is a tumour suppressor gene and mutation and deletion of the gene are the most common genetic defects seen in sporadic clinical cancer. Recent reports indicate that up to 75% of families with classical LFS have germline mutations in the p53 gene as do 10–22% of LFL families (Eeles, 1995; Varley et al, 1997). The identification of these mutations is important clinically because carriers are at markedly increased risk of developing cancer (cumulative lifetime risk in women 90%) and may be at greater risk of radiation-induced carcinogenesis (Eeles, 1995).

Point mutations, which disrupt p53 function, were originally thought to be largely restricted to the core DNA binding region of p53. However, this central area of p53 has been more extensively investigated for mutations than either the N- or C-termini, therefore the frequency of mutations outside the core domain may be higher than is thought (Casey et al, 1996). Thus, if sequencing is to be used to detect all coding germline p53 mutations, then all exons and splice junctions should be investigated (Varley et al, 1997). The families with p53-related germline defects are rare and they often first come to light due to a high incidence of cancer. A rapid screening assay would be valuable to determine, from the much larger number of cancer-prone families, the minority due to a p53-related defect. We have tested two potential assays of p53 function on a number of LFS/LFL family members as part of a larger study. The first of these assays was developed in our laboratory and depends upon the measurement of the apoptotic response of peripheral blood lymphocytes (PBL) to radiation-induced DNA damage (Camplejohn et al, 1995). In individuals with functional p53 most PBL die within 48 h of a dose of 4 Gy radiation, whilst in individuals with an inherited defect leading to loss of p53 function PBL are resistant to the lethal effects of radiation. We believe that this assay will detect any inherited p53 defect which results in loss of p53 function. The second test, the FASAY, is a yeast-based assay which tests the ability of p53 protein to transcriptionally transactivate a target gene by binding to the RGC consensus sequence (Flaman et al, 1995; Lomax et al, 1997). Functional p53 leads to the growth of white yeast colonies, whilst mutant p53 results in red colonies. This assay is also rapid, inexpensive and links any detected defect directly to exons 4–10 of the p53 gene.

MATERIALS AND METHODS

Subjects

A minimum of 20 ml of blood was collected from 20 affected and unaffected members of LFS/LFL families and from 50 normal individuals with no history of excessive cancer occurrence in their
families. Data on all LFS/LFL family members for whom samples were received between the beginning of 1994 and the middle of 1997 are included in this study provided that results for all three assay methods are available.

**Apoptotic assay**

PBL were separated from fresh whole blood samples and cultured in standard medium (RPMI, 10% fetal calf serum, glutamine and antibiotics) for three days. At 72 h of culture, half of the cells were exposed to 4 Gy of gamma radiation. Both irradiated and control cells were returned to the incubator for a further 24 h at which point the cells were fixed in 70% ethanol at 4°C. For analysis, the samples were acid-denatured for 12 min in 0.1 M hydrochloric acid at 37°C and then stained with propidium iodide. The amount of apoptosis was assessed by the size of the sub-G1 peak on DNA profiles. In all but a few cases where there were insufficient cells, flow cytometry was performed in triplicate with good reproducibility. This flow cytometric method has been validated by comparison with other techniques, including electron microscopic counting and cell sorting of apoptotic cells (Camplejohn et al, 1995) to confirm that the sub-G1 peak did consist of apoptotic cells.

**FASAY**

mRNA extracted from phytohaemagglutinin (PHA)-stimulated PBL was subjected to the FASAY (Flaman et al, 1995; Lomax et al, 1997). In this assay, purified mRNA was subjected to reverse transcription polymerase chain reaction (RT-PCR). The unpurified p53 RT-PCR product was then co-transfected into yeast with a linearized expression vector carrying the 5′ and 3′ ends of the p53 open reading frame. This results in the constitutive expression of the human p53 protein present. Yeast that have repaired the plasmid were selected by their ability to grow in the absence of leucine and with a limiting concentration of adenine; if the p53 gene is wild-type the ADE2 gene is activated, producing adenine and white colonies are formed. Mutant p53 does not activate the ADE2 gene and the resultant colonies are red due to the accumulation of red adenosine precursor. This assay recognizes mutations from exon 4 to exon 10 of the p53 gene and the resultant colonies are red due to the accumulation of a red adenosine precursor. This assay recognizes mutations from exon 4 to exon 10 of the p53 gene as this is the extent of the PCR product that recombines with the gapped vector. Many control and repeat experiments were performed to demonstrate the reproducibility of the FASAY, some of these are described in Lomax et al (1997).

**Sequencing**

DNA was isolated from PBL by the sucrose lysis method. DNA fragments were PCR-amplified using the primer sequence below:

| Exon   | Forward                     | Reverse                      |
|--------|-----------------------------|------------------------------|
| Exon 1 | 5′-GAGAATCTCTGACTCTGCACC    | 5′-AGCCGAGCCGGTACCTCA        |
| Exons 2/3 | 5′-ATGCTGAGATCCCACCTTTTC  | 5′-AGAGCAAGTCAGAGGACCAAG   |
| Exon 4  | 5′-GACCTGTCCTCTGACGCTCT   | 5′-GCATTGAGCTCTGAGAAGAG    |
| Exon 5  | 5′-ACTTGTCCTGCGATTCTGAAACT  | 5′-CAATAGGAGGAAATGAGGACCC   |
| Exon 6  | 5′-TCAGATAGCGATGTTGCCAGAG | 5′-GCCACTGACAACCCACCTTA    |

The annealing temperature was 56°C for exons 2–3, 4, 10 and 11 and 60°C for exons 1 and 5–9. To remove the excess primers and dNTPs before sequencing, the PCR products were purified by ammonium acetate and isopropanol precipitation. Cycle-sequencing of both strands was performed using either of the two primers used in PCR with dye-terminator chemistry and AmpliTagFS (Perkin-Elmer Applied Biosystems). To remove excess dye before loading samples on to the sequencer the samples were purified by sodium acetate and ethanol precipitation. The products were analysed on an ABI 310 sequencer.

**RESULTS**

In this report the results of the apoptotic assay and the FASAY are compared with each other and with the results of sequencing. Data from 20 affected and unaffected individuals who are members of LFS/LFL families are included (Table 1). These data are compared to results from 50 normal individuals with no known inherited susceptibility to cancer. This paper includes data on LFS/LFL family members collected up to the middle of 1997 as part of an on-going larger study in which sporadic cancer patients and carriers of germline p53 mutations. A normal apoptotic response is thus defined as anything greater than 27%, whilst any value below 23% is consistent with the presence of a mutation. This leaves a borderline region of 23–27% into which occasional results fall. Such results are considered as suspicious and worthy of further investigation, initially with the FASAY.

When comparing the various assays, the results show excellent agreement between the two functional assays and between them and sequencing. Both the FASAY and the apoptotic assay successfully detected all known carriers of germline p53 defects. In the apoptotic assay these carriers had responses significantly lower than those seen in normal individuals and non-affected members of LFS/LFL families (see Figure 1). Further, in the FASAY all carriers of germline p53 mutations yielded between 44 and 60% red colonies, well above the maximum background level of 10%. In addition, no individuals from the LFS/LFL families who had homozygous wild-type p53 were classified as abnormal by either functional assay, though one such individual did yield a borderline apoptotic assay result.

**DISCUSSION**

In this study, both of the functional assays detected all known carriers of germline p53 mutations. Although it requires to be established with a larger series of mutation carriers, we would...
Table 1  Apoptotic assay results denote a mutation (Mut) if the increase in apoptotic cells after 4 Gy radiation is < 23%, borderline (B) [23–27%] and wild-type (WT) [> 27%]

| Case | Family status | Cancer diagnosed in individual | Apoptotic assay | FASAY | Sequencing of exons 1–11 |
|------|---------------|-------------------------------|----------------|-------|--------------------------|
| 4001 | LFL           | Yes                           | Mut            | Mut   | Mut codon 151           |
| 4002 | LFS           | Yes                           | Mut            | Mut   | Mut codon 248 (subject II-2 family 266, Varley et al 1997) |
| 4005 | LFL           | Yes                           | Mut            | Mut   | Mut codon 337           |
| 4006 | LFL           | Yes                           | Mut            | Mut   | Mut codon 245 (subject F. Camplejohn et al 1995) |
| 5020 | LFL           | Yes                           | Mut            | Mut   | Mut codon 273           |
| 6034 | LFS           | Yes                           | Mut            | Mut   | Mut codon 213           |
| 5004 | LFL           | No                            | WT             | WT    | WT                       |
| 5006 | LFL           | No                            | WT             | WT    | WT                       |
| 5010 | LFL           | No                            | WT             | WT    | WT                       |
| 5023 | LFS           | No                            | WT             | WT    | WT                       |
| 6024 | LFS           | No                            | WT             | WT    | WT                       |
| 6033 | LFL           | No                            | WT             | WT    | WT                       |
| 6072 | LFL           | No                            | WT             | WT    | WT                       |
| 6107 | LFL           | No                            | WT             | WT    | WT                       |
| 6137 | LFL           | No                            | B              | WT    | WT                       |
| 6141 | LFL           | No                            | WT             | WT    | WT                       |
| 6146 | LFL           | No                            | WT             | WT    | WT                       |
| 7094 | LFL           | No                            | WT             | WT    | WT                       |
| 7099 | LFS           | No                            | WT             | WT    | WT                       |

These figures are based on a study of around 300 samples. Results on 50 normal volunteers yielded a mean apoptotic response of 44% (1 s.d. 9%). The FASAY was performed as described by Flaman et al (1995) and Lomax et al (1997); 10% or less red colonies denotes a homozygous wild-type result, > 10% red colonies denotes the presence of a mutation.

Figure 1  The figure shows the distribution of values for the apoptotic response in groups of LFS/LFL individuals with and without p53 mutations and normal volunteers. The mutation group differs significantly from the LFS/LFL group lacking a mutation ($P = 3 \times 10^{-5}$) and the normal group ($P = 3 \times 10^{-7}$). The unaffected and control groups do not differ significantly ($P = 0.4$). Statistical comparison was done using Fisher’s exact test. Only one sample from the unaffected and control groups has a lower apoptotic response than any of the affected individuals. Unfortunately, this individual was no longer available for further study.
expect, in principle, that all germline defects which lead to loss of p53 function would be detected by the apoptotic assay. However, the FASAY will only detect mutations between exons 4 and 10 and will miss mutations which lead to loss of expression of one allele. In addition, we have found that a splice donor mutation in exon 4, which leads to the production of three species of aberrant mRNA was not detected by the FASAY (Varley et al, 1998). We also have evidence from other studies on sporadic breast tumours that the FASAY may fail to detect at least some stop mutations in the p53 gene (RS Camplejohn, unpublished data). However, in the current study as well as detecting all carriers of germline p53 mutations, no individuals with two wild-type alleles were wrongly identified as carriers of inherited defects; in the FASAY all such individuals had < 10% red colonies. In the apoptotic assay non-affected individuals all gave results above the maximum level seen in gene carriers, apart from one who gave a borderline result (see Table 1 legend for details). A larger study of sporadic cancer patients and members of families with other inherited cancer predispositions suggests that such results are seen occasionally for reasons that are not yet clear. In addition, a sample from one normal volunteer gave a low apoptotic response in the range seen with family members having a p53 mutation. The probability of this individual having a germline p53 mutation is very low but, unfortunately, no material was available for further study. However, the apoptotic assay would only be used as an initial screening technique to identify cases worthy of further investigation by more detailed methods such as sequencing. Thus a small number of borderline, or even false-positive, results would not be a major problem in the event of the assay being used as a clinical screening test. Therefore, we consider that both assays show real promise as a screening process to identify samples which warrant further molecular sequencing analysis and both have a potential role in identifying germline p53 mutations in members of cancer-prone families. As an initial screen the apoptotic assay does have the great advantages of simplicity and low cost.

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