INVITED REVIEW

p53 functional assays: detecting p53 mutations in both the germline and in sporadic tumours

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Abstract. The tumour suppressor gene *p53* is the gene most often reported to be mutated in clinical cancers with something like half of all tumours harbouring mutations. Further, many studies have suggested that *p53* mutations have prognostic importance and sometimes are a significant factor in determining the response of tumours to therapy. The value of knowing the *p53* status of individual tumours will increase if currently researched strategies aimed at developing *p53*-based treatment protocols come to fruition. There are quite a number of techniques used to detect *p53* defects in both tumours and in the germline of cancer-prone families, although some of these methods are indirect and each has certain drawbacks. In this brief review we will discuss the value of two assays of *p53* function as a means of detecting and partly characterizing *p53* mutations. The two assays are the apoptotic assay, which measures the response of peripheral blood lymphocytes to radiation-induced DNA damage and the FASAY, a yeast based assay which assesses the ability of a given *p53* protein to transactivate *p53* target genes. Both of these assays are rapid, yielding results within 5 days. Further, they not only offer the possibility of detecting *p53* mutations but also of characterizing a given mutation in terms of two of *p53*'s most important functions, namely the induction of apoptosis and the transactivation of target genes.

OVERVIEW OF THE ROLE OF *P53* AS A TUMOUR SUPPRESSOR GENE

The general area of *p53* research has resulted in over 13 000 publications with more than 10 000 individual mutations of the gene being reported in this extensive literature (Hainaut & Hollstein 2000). Included amongst these publications are some excellent general *p53* reviews, including that of Hainaut & Hollstein (2000) as well as more detailed reviews of particular aspects of *p53* research. A few of these will be detailed in the following text. No attempt will be made to duplicate such wide-ranging reviews here but rather, this article will be restricted to the discussion of one small, specific aspect of *p53* research, namely the value of assays of *p53* function as a means of

Figure 1. The top panel illustrates the spectrum of all reported mutations in the *p53* gene in terms of amino acid positions in the corresponding protein (data from Soussi *et al.* 2000). The lower panel shows a diagrammatic representation of the *p53* protein showing conserved regions (grey boxes I–V), functional domains and binding sites for cellular and viral proteins.

detecting *p53* mutations. However, a brief overview of the central role of *p53* in tumour biology is included to place the central topic of this article in context.

p53 protein was discovered in 1979 by its property of binding to large T-antigen, the oncogene product of SV40 (Lane & Crawford 1979). The human *p53* gene encompasses approximately 20 kb of genomic DNA and consists of 11 exons. It is located on the short arm of chromosome 17 (17p13) and shows a high level of conservation through species of vertebrates. The product of the *p53* gene is a 393 amino acid nuclear phospho-protein found in very low levels in normal, undamaged cells. The low level of *p53* protein in normal cells is due mainly to the short half-life of the protein (6–20 min). *p53* protein can be divided into at least four functional domains (Fig. 1).

At the amino-terminus of the protein is a transcriptional transactivation domain involved in up-regulation of other genes such as the DNA damage-response gene *GADD45*, the *p21* cyclin-kinase inhibitor gene and the *MDM2* gene. This region of p53 is exposed in the wild type protein and is very immunogenic, containing the epitopes to many anti-p53 antibodies. The central core domain of p53 (residues 102–292) is essential for the tumour suppressor function of p53. Much of this region of the protein is hydrophobic and tightly bound explaining the scarcity of antibodies directed against this region produced by early studies. The core domain of p53 is involved in sequence-specific binding of p53 to DNA. p53 binds to DNA as a tetramer and the region through which p53 monomers interact with each other is called the oligomerization domain (residues 323–355). The C-terminus of the protein contains a number of important regions including a negative regulatory domain.

The *p53* gene originally was regarded as a dominant oncogene but it rapidly became clear that the *p53* genes involved in these early studies were mutated. Subsequent studies have clearly demonstrated that wild-type *p53* acts as a tumour suppressor gene. Much work has been done looking at the role of p53 in the cellular response to DNA damage, particularly ionizing radiation. This work has shown that p53 plays a complex but central role in maintaining the genetic integrity of the cell and this function involves preventing cells with damaged DNA from proliferating further. Wild-type p53 is involved in triggering cell cycle arrest of DNA-damaged cells, most importantly at the G₁–S boundary (Kastan *et al.* 1991) and ensuring their removal by the process of apoptosis (Clarke *et al.* 1993; Lowe *et al.* 1993). However, p53 can be induced by stimuli other than DNA damage, for example, by expression of mitogenic oncogenes (Lowe 1999). Quite a number of upstream signalling molecules have been implicated in activating p53 function including the ATM protein, mutations in which give rise to the radiation sensitizing condition Ataxia Telangectasia. Also of particular interest are recent studies on the *hChk2* gene, which suggest a role in stabilizing p53 after DNA damage (Chehab *et al.* 2000; Shieh *et al.* 2000). Mutations in this gene have recently been implicated in the clinical condition Li Fraumeni Syndrome (described below), which is normally associated with germline *p53* mutations (Bell *et al.* 1999). p53 mediated G₁ arrest is caused largely by transactivation of the cyclin-dependent kinase inhibitor *p21*, which inhibits CDK-dependent phosphorylation of the retinoblastoma protein (Rb). This in turn prevents release of the transcription factor E2F1 and leads to cell cycle arrest. Transactivation of target genes is a major function of p53 and this ability is involved in one of the functional assays discussed in detail in this article, the FASAY. However, in the case of apoptosis induction both transactivation and other mechanisms seem to be involved, one of which may be transrepression of a different set of target genes (see Bates & Vousden 1999 for a detailed discussion of p53 mediated apoptosis). Apoptosis induction is the basis of the other functional assay discussed in detail later.

Given the central role of p53 in maintaining genetic stability, it is not surprising that *p53* mutation and deletion are the most common genetic defects seen in clinical cancer. Overall, something like 50% of all sporadic clinical cancers harbour *p53* mutations (Levine 1997). In addition, over the past 30 years a syndrome has been recognized in which families exhibit a very precise pattern of cancer susceptibility (Li & Fraumeni 1969; Li *et al.* 1988) involving early onset sarcomas, breast carcinoma, brain tumours, lymphoma and adrenal carcinoma; these families are said to exhibit Li–Fraumeni syndrome (LFS). Malkin *et al.* (1990) demonstrated a relationship between *p53* mutations and LFS. LFS is extremely rare with fewer than 100 families being recognized world wide. However, recently it has become apparent that the situation is more complex as families with similar spectra of malignancies have been recognized, which do not fully meet the precise criteria of classical LFS. Such families have been called Li-Fraumeni-like (LFL) (Birch *et al.* 1994). Germline *p53* mutations account for no more than about 75% of cases

of LFS and for LFL the figure is lower at about 10–20%. As mentioned above, other genes such as *hChk2* may also cause LFS/LFL.

p53 mutation spectrum and methods for detecting mutations

A cursory perusal of the literature would suggest that *p53* mutations occur almost exclusively in the core DNA binding region of the protein with over 90% of reported mutations in tumours being found between exons 5 and 8. Certain 'hotspots' have been identified at which mutation is particularly common, such as codons 175, 248 and 273 (Fig. 1). However, the degree to which mutations are restricted to this region of the gene has been exaggerated by the fact that most studies only look at this central region (Hernandez-Boussard *et al.* 1999). A number of studies such as that of Casey *et al.* (1996) have suggested that a significant minority of mutations are outside exons 5–8. Results in our laboratory on a series of 48 breast carcinomas (Duddy *et al.* 2000) confirmed the results of Hartmann *et al.* (1995) in that about one in five or six mutations in breast tumours are outside this region. Thus mutation detection assays that look at as much of the gene as possible and ideally all of it, are recommended. In a recent meta-analysis, Pharoah *et al.* (1999) gathered together results from all of the published studies that had investigated the association between somatic mutations in the *p53* gene and prognosis in breast cancer. Surprisingly, none of the studies listed had carried out complete sequencing of all 11 exons of the *p53* gene.

A variety of methods currently are used to assess the *p53* status of individual tumours. The most commonly applied is immunohistochemical detection of stabilized *p53* protein. However, this method does not directly assess mutational status of tumours and, although many tumours demonstrating high levels of protein do possess a mutation, the correlation is far from perfect (Barnes & Camplejohn 1996). In a recent study in our laboratory, Duddy *et al.* (2000) found that 10/24 mutations in breast tumours were not accompanied by raised *p53* protein levels. However, Duddy *et al.* (2000) did not detect any tumours with raised *p53* protein levels which lacked a *p53* mutation but others have reported such tumours (Sjogren *et al.* 1996). In this latter study 30% of tumours with stabilized protein were reported to lack a mutation. The discrepancy between these two studies is probably at least partly due to technical problems with either the immunohistochemical staining technique (Barnes & Camplejohn 1996) or to the fact, as discussed below, that automated sequencing fails to detect a significant minority of mutations. Other methods used to detect *p53* mutations include direct DNA sequencing, which can be performed manually or using automated sequencers. Manual sequencing is generally an accurate method of detecting mutations but is very slow and labour intensive. Automated sequencing of tumour material, whilst being more rapid, has been shown by a number of studies to miss around 20% of mutations in tumour material (Ahrendt *et al.* 1999; Duddy *et al.* 2000). Other reasonably rapid PCR based methods of mutation detection, such as single strand conformation polymorphisms (SSCP) and denaturing gel electrophoresis (DGGE) have been applied to *p53* (Hernandez-Boussard *et al.* 1999, Rines *et al.* 1998). Each method has its advantages and disadvantages but most methods, apart from manual sequencing, miss a significant percentage of mutations from tumour material. For example, Tolbert *et al.* (1999) reported that SSCP failed to detect 38% of *p53* mutations in a series of gastric cancers. New methods are being developed and one of the most exciting of these involves the use of oligonucleotide probe arrays such as the GeneChip. This technology is developing rapidly and may in the future be a reliable and rapid means of detecting *p53* mutations in clinical material. However, like all new technology it needs to be carefully evaluated and a recent study by Ahrendt *et al.* (1999) suggests it still requires some improvement. In this study the GeneChip assay was found to miss 19% of mutations present in a series of lung cancers.

Figure 2. The top two histograms (a and b) illustrate typical DNA histograms from PBL from a *p53* germline mutation carrier. Histogram (a) shows the apoptotic fraction for unirradiated cells and (b) for cells irradiated with 4Gy. In this case the radiation-induced increase in apoptotic cells is 18%. The bottom two panels illustrate typical data for PBL from a normal individual; in this case the radiation-induced apoptotic increase is 54%.

APOPTOTIC ASSAY

Principles

As described earlier, a major function of p53 is to activate the apoptotic pathway in cells that have sustained DNA damage. Studies on *p53* knockout mice showed not only an almost complete abolition of radiation-induced apoptosis in thymocytes from *p53*^{-/-} mice, but also a marked reduction in this response in heterozygous knockouts (Clarke *et al.* 1993; Lowe *et al.* 1993). Members of cancer prone families with a functionally disabling heterozygous *p53* mutation are analogous to such *p53*^{+/-} mice. Camplejohn *et al.* (1995) demonstrated that peripheral blood lymphocytes (PBL) from patients with a heterozygous *p53* mutation also had a defective apoptotic response to ionizing radiation. These results were confirmed in a more detailed but still relatively small study published recently (Camplejohn *et al.* 2000). This defective apoptotic response to DNA damage can be used as the basis of a simple and rapid assay to detect those members of cancer prone families who carry a germline *p53* mutation (Fig. 2).

Method

The protocol below describes in detail how the assay is performed in our laboratory (Camplejohn *et al.* 1995).

Separation and culture of PBL

Twenty millilitres of whole blood is collected in a heparinized tube and taken as rapidly as possible at room temperature to the laboratory for separation of mononuclear cells. After removal of plasma, PBL are separated by centrifugation on 10 ml Histopaque (Sigma, Gillingham, UK), are collected, washed and resuspended in 10 ml RPMI 1640 medium (Gibco, Paisley, UK) containing 10% serum plus antibiotics. Cell concentrations are determined using an automated cell counter such as the Casy Counter (Schärfe System, Reutlingen, Germany) and the concentration adjusted by addition of medium to achieve a concentration of approximately 10^6 PBL/ml. Then 10 ml of this suspension is added to a series of Falcon T25 tissue culture flasks (Becton Dickinson, Oxfordshire, UK), the flasks are stood on their ends and cells are cultured for 70 h. Cells are irradiated or mock-treated at this time and cultured for a further 24 h, at which time they are split into three aliquots and fixed in 70% ethanol.

Irradiation procedure

Irradiation is carried out using a cell irradiator such as the Gammacell 1000 Elite (Nordion International Inc, Buckinghamshire, UK), which contains a caesium 137 source and typically has a dose rate of around 858 cGy per minute. Cells are subjected to a dose of 4 Gy. Control (unirradiated) flasks of cells are treated in an otherwise identical manner to those being irradiated.

Analytical flow cytometry

After removal of ethanol from fixed PBL, 2×10^6 cell aliquots are subjected to treatment with 0.1 M HCl at 37 °C for 12 min. This procedure was found to efficiently extract low molecular weight DNA from PBL with minimal denaturation of intact DNA. After washes in PBS, cells are stained for DNA content by addition of propidium iodide (PI, Sigma) at a final concentration of 50 µg/ml in a volume of 1 ml. Cells are stained for a minimum of 20 min prior to measurement of red fluorescence (PI), forward and 90° light scatter on a Becton Dickinson, FACSCalibur flow cytometer. At least 10 000 cells per sample are scanned and data are stored in list mode prior to analysis using CellQuest software. Doublet discrimination using pulse area/width analysis on the PI signal is used to remove cell clumps from the analysis, which is restricted to lymphocytes using forward and side scatter.

Measurement of apoptosis

Measurement of the extent of apoptosis is performed by assessment of cells appearing in a sub-G1 peak on DNA profiles (Fig. 2). The apoptotic response to radiation is defined as the increase in apoptosis seen when comparing the irradiated with the unirradiated sample (% apoptosis after 4 Gy – % apoptosis after 0 Gy). The final result is the average of the three replicates. This flow cytometric method has been validated in many publications and by comparison in our laboratory with a number of other techniques including electron microscopic counting of apoptotic cells and cell sorting of apoptotic cells.

Summary of experimental findings and conclusions

We are unaware of any published data from the apoptotic assay produced in laboratories other than ours. A recent publication (Camplejohn *et al.* 2000) reported on a study of 22 members of cancer prone families whose p53 status was assessed by three different techniques, namely

automated sequencing of the whole gene (exons 1–11), the apoptotic assay and the FASAY. The apoptotic assay results were compared to those from 50 normal individuals. Only six family members proved to have a germline mutation and all six were detected by all three assays. The apoptotic assay did not yield any false positive or negative results in this series of individuals. However, in a larger data set currently under analysis, fairly rare false positives are seen if the assay is performed on sporadic cancer patients but only one false positive has been seen in an apparently normal individual, who unfortunately was not available for further study. A few false positives are not a major problem for this assay as it would only be used as a rapid screening technique to identify individuals worthy of more detailed study by the FASAY and/or sequencing. False negatives are more of a problem, however, and we have had two such results. One is from an individual with an unusual insertion mutation, which also seems apoptotically functional in an experimental model (Rutherford *et al.*, manuscript in preparation). The other false negative result is from an individual, who is so far free of cancer, with a codon 245 mutation who consistently gives a normal apoptotic response. This is despite another family member carrying the same mutation, but who has had malignancies, showing the expected abnormal response. Currently, we have no explanation for this latter finding but it is possible that other genetic factors involved in the apoptotic response of PBL to DNA damage are preventing the appearance of the expected p53 related defect. Overall, preliminary analysis of our complete database suggests that 17 assays performed on separate blood samples from seven individuals carrying p53 germline mutations show abnormal apoptotic responses. Four assays on the two individuals described above gave normal apoptotic responses. Clearly, the number of mutation carriers studied so far is small due to the difficulty of obtaining fresh blood from these rare individuals. Thus caution must be applied in drawing conclusions. However, an apoptotic defect would be expected from all types of p53 mutation which lead to a functional defect. In one patient the assay was repeated on six occasions over a period of 4 years and consistently gave a markedly reduced apoptotic response.

As the assay is applicable only to PBL, its application is restricted to the study of germline mutations, which are themselves rare. Thus the question might be asked as to how valuable a simple screening test for germline p53 mutations would be. However, despite the rarity of such mutations a much larger number of families, which have a high incidence of cancer, are screened to rule out the involvement of the p53 gene. Further, the apoptotic assay has proved useful in our laboratory as part of a process to functionally characterize unusual germline p53 defects. The assay is rapid, yielding a result in 5 days, is very inexpensive and can be carried out by someone with basic laboratory skills after one days training. We thus feel that the assay is worthy of further study on a wider range of p53 mutations.

FASAY (FUNCTIONAL ASSAY FOR THE SEPARATION OF ALLELES IN YEAST)

Principles

The FASAY was first described by Ishioka *et al.* (1993) and an improved and simplified technique was described 2 years later (Flaman *et al.* 1995). It is the method described by Flaman *et al.* that has been applied, with only minor modifications in our laboratory and in most published studies. We described earlier in this article, that an important function of p53 is to act as a transcriptional transactivator of a large number of target genes (Hainaut & Hollstein 2000). It is this property that is tested by the FASAY. Thus mRNA is extracted from the cells of interest,

Figure 3. The top panel (a) is a schematic representation of expression vector plasmid pRD122. The circular plasmid is cut with Hind III and Stu I to release a linearized plasmid with one end of the *p53* open reading frame at each end of the plasmid, thus allowing homologous recombination to occur with *p53* PCR products (redrawn from Flaman *et al.* 1995). Shown in the bottom panel (b) is a schematic representation of expression vector plasmid pLS210, which contains three copies of the ribosomal gene cluster derived *p53* binding site (redrawn from Flaman *et al.* 1995). Only functional *p53* can bind to this consensus sequence and thereby transactivate the *Ade2* gene and allow the yeast to grow as normal white colonies in the FASAY.

converted into DNA by means of RT-PCR and the PCR product of exons 4–10 (codons 68–346) of *p53* is cotransformed with linearized pRD122 plasmid (Fig. 3a) into yeast strain yIG397. The pRD122 plasmid contains a leucine selection marker and the 3' and 5' ends of *p53*. A recombination event occurs between the ends of the *p53* gene in the pRD122 plasmid and the *p53* PCR product and this allows the yeast to grow in the absence of leucine. The yeast contain a second plasmid (pLS210; Fig. 3b) which contains the selection marker *Ade2* driven by the minimal promoter CYC1. Immediately upstream of CYC1 in the original method described by Flaman

et al. (1995) were three copies of the p53 consensus binding sequence from the ribosomal gene cluster (RGC) (Kern *et al.* 1991) and this is the yeast strain used in the method described in detail below. However, yeast have been engineered with other p53 binding sites based on those found in the *p21*, *bax* and *PIG3* promoters (Flaman *et al.* 1998). These yeast allow differential effects on various p53 target genes to be studied. In all cases the FASAY tests the ability of the p53 protein of interest to transactivate the *Ade2* gene. If the p53 is functional the yeast are able to synthesize adenine and grow normally as white colonies. If the p53 is nonfunctional the *Ade2* gene is not transactivated and the yeast grow as smaller red colonies due to the accumulation of a red coloured intermediate of the adenine biosynthetic pathway.

Standard FASAY method as performed in our laboratory

mRNA extraction and purification

This part of the technique is performed using the Quick Prep™ Micro kit (Amersham Pharmacia Biotech Inc, Little Chalfont, UK) according to the manufacturers' instructions.

RT

Reverse transcription is performed using the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech Inc) according to the manufacturers' instructions.

PCR

The polymerase chain reaction is performed using the proof reading polymerase *Pfu*. Each tube contains the following reagents: 2 µl 10x concentrated Pfu buffer (Stratagene), 2 µl DMSO, 0.4 µl 10 mM dNTP (dATP, dGTP, dCTP, dTTP), 9.4 µl dH₂O, 2 µl 50 µg/ml primer P3 (ATTT-GATGCTGTCCCCGACGATATTGAA(S)C), 2 µl 50 µg/ml primer P4 (ACCCTTTTGGAC-TTCAGGTGGCTGGAGT(S)C), 0.5 µl Pfu polymerase (Stratagene).

It is important that all reagents are mixed carefully. The tubes are then placed in the thermal cycler for 5 min at 95 °C. Then 35 cycles are run as follows: 94 °C for 30 s, 65 °C for 60 s, 78 °C for 80 s.

The sequence of primer P3 represents the sequence of codons 42–51 of the *p53* gene and P4 codons 365–374 and it is these two regions which recombine with the ends of the *p53* gene present in plasmid pRDI22.

Transformation of yeast

Yeast strain yIG397 are grown overnight at 30 °C, diluted to an optical density of 0.2 (OD₆₀₀), grown on until the optical density is 0.8–1.0. and then washed in distilled water and an LiAc/TE buffer made by diluting 1 ml of the two 10x concentrated stock solutions detailed below with 8 ml distilled water:

10x TE (filter sterilize): 100 ml 0.1 M Tris (1.21 g), 0.01 M EDTA (.372 g) pH 7.5

10x LiAc (filter sterilize): 100 ml 1 M LiAc (10.2 g) pH 7.5 (with acetic acid)

The yeast are re-suspended in 250 µl LiAc/TE and 50 µl of this yeast suspension is added to each tube containing 50 ng gapped pRDI22 vector (see Fig. 3a), 5 µl PCR product and 5 µl 10 mg/ml boiled, sonicated salmon sperm DNA (Sigma). 300 µl PEG(polyethelene glycol)/LiAc/TE is added to all tubes which are then incubated at 30 °C for 30 min in a shaking incubator and then heat-shocked at 42 °C for 15 min in a stationary incubator. The yeast are then spun, washed in water and plated at appropriate concentrations (depends on plating efficiency) on plates lacking leucine and with a limiting concentration of adenine. The yeast are then grown for 3 days, placed at 4 °C overnight to develop the colour of the colonies and then the numbers of white and red colonies are counted.

Summary of experimental findings and conclusions

An advantage of the FASAY is that mRNA can be extracted from most cell types and used successfully in this technique. In our laboratory the FASAY has been applied to plasmids, cell lines, PHA-stimulated PBL and clinical tumours. Flaman *et al.* (1995) reported FASAY results from seven cell lines and subsequently a number of large studies of the p53 status of cell lines have also been published (Friend *et al.* 1994; Jia *et al.* 1997). Together these two studies looked at over 200 cell lines.

Detection of germline mutations

The use of PBL allows easy testing of potential carriers of germline p53 mutations. Six such individuals were investigated in the original study by Ishioka *et al.* (1993) and one individual was included in the report from Flaman *et al.* (1995). We have used the FASAY extensively to detect and partially characterize germline p53 mutations in cancer prone families (Lomax *et al.* 1997, 1998; Camplejohn *et al.* 1995, 2000). The studies by Lomax *et al.* (1997, 1998) led to the discovery and characterization of a particularly interesting mutation in the oligomerization domain of p53 at codon 337. This mutation (R337C) leads to the replacement of a cysteine with arginine. The mutation was originally missed in a study using sequencing of genomic DNA (Barnes *et al.* 1992) but was subsequently detected in the FASAY. This mutation did not, however, result in red colonies but rather in pink ones and Lomax *et al.* (1998) confirmed that this mutation did retain partial function in a range of assays of transactivation, apoptosis induction and suppression of colony formation in Saos2 cells. This mutation has been found subsequently in at least four other families. The finding of pink colonies in this case raises the question as to whether the FASAY is semiquantitative in terms of assessing the transcriptional transactivation function of mutant p53 proteins. By combining data from our own laboratory and that of Patrick Chene (Rollenhagen & Chene 1998) Fig. 4 was produced. This figure compares the colour of colonies produced in the FASAY with transcriptional transactivation ability in mammalian cell systems using luciferase reporter constructs. The results suggest that the FASAY is, indeed, semiquantitative.

Mutation detection in sporadic tumours

As discussed earlier, automated sequencing is particularly prone to miss point mutations in material from tumours, due partly at least to dilution of any mutant tumour-derived p53 allele by wild type p53 present in contaminating normal cells. The FASAY in contrast seems biased in favour of detecting mutations as tumour cells often have higher mRNA levels than normal cells (Duddy *et al.* 2000). The study by Duddy *et al.* (2000) is the only one we are aware of in which the FASAY was compared, not only with immunohistochemical staining, but also sequencing of all 11 exons of the p53 gene in a series of clinical cancers. In this study, the FASAY detected all 24 mutations found in the series of 48 tumours, whilst initial automated sequencing of genomic DNA detected 18/24 mutations. A second round of automated sequencing carried out using an independent source of genomic DNA detected mutations in three of the six tumours, which originally appeared to lack a mutation in genomic DNA. All but one of the mutations originally missed by sequencing of genomic DNA were point mutations. Five mutations in this series (21%) were outside the commonly investigated exons 5–8, reinforcing the need to extend sequencing beyond this region. All 14 tumours with strong immunohistochemical staining had p53 mutations; the majority of mutations missed by immunohistochemistry produced a truncated protein. Strong staining was never seen in tumours lacking a p53 mutation. In this study, the FASAY proved to be a rapid, reliable and effective method for identifying those breast tumours harbouring p53 mutations. In a recently published study by Chappuis *et al.* (1999) the FASAY was employed to look at the prognostic significance of p53 mutations in 180 patients with breast

Figure 4. This histogram compares the colour of colonies (represented by the colour of the columns) produced by a number of oligomerization domain mutants of p53 in the FASAY with the ability of the same mutants to transactivate reporter constructs in mammalian cells. These results suggest that the FASAY is semiquantitative in that fully functional p53 proteins yield white colonies, partially functional mutants yield pink colonies of various hue and nonfunctional mutants result in red colonies.

cancer. They showed that the FASAY worked well and that half of the mutations identified were nonmissense. However, they did not find that p53 status added independent prognostic power when nodal status and tumour size were combined with histological grade. In a small study Smardova *et al.* (1999) demonstrated a good, but not perfect, correlation between results from the FASAY and immunohistochemical staining.

In the study by Duddy *et al.* (2000) the FASAY detected all mutations present in the series of breast tumours. However, we know that the FASAY does fail to detect some p53 mutations. Firstly this method can only detect mutations between codons 68 and 346 (exons 4–10), which is the span of the PCR product inserted into the gapped vector (see Fig. 3) during recombination in the yeast. However, despite the bias towards the central exons in most published studies of the p53 mutation spectrum, the evidence does suggest that few mutations occur outside exons 4–10. Some mutations within this region are also missed by the FASAY, though once again our experience suggests these are rare. The FASAY may fail to detect mutations in the splice sites of the p53 gene; we failed to pick up one such mutation in our laboratory (Varley *et al.* 1998). The FASAY will also not detect mutations which lead to lack of mRNA from the mutant allele and again we have found one such mutation (Rutherford *et al.* manuscript in preparation). Forced expression of this mutant allele in an experimental system did lead to the expected red colonies in the FASAY. Despite these failures to detect certain mutations using the FASAY, our experience suggests that the technique does detect the vast majority of such defects (>95%).

Another advantage of the FASAY is that because the pRD122 plasmid is centromeric, individual yeast colonies are derived from a single recombination event and hence a single allele of the *p53* gene. This separation of alleles means that extraction of DNA from single red yeast colonies yields clean material from the mutant allele, lacking contaminating material from normal cells, for subsequent sequencing. This results in the production of excellent quality sequences which, in our experience, allow easy identification of all types of mutation (Duddy *et al.* 2000). The FASAY also has the advantage of directly distinguishing between dysfunctional *p53* mutations and silent mutations or polymorphisms. In this context it is interesting to note that about 4% of mutations in the databases may be silent mutations with no functional significance (Hainaut & Hollstein 2000).

In addition to its use in detecting *p53* mutations, the FASAY can be used for more experimental studies. We have followed the lead of Flaman *et al.* (1998) in using the FASAY to partially characterize the transactivational transcription activity of mutants against the RGC, p21, bax and PIG3 promoters. In addition, Inga *et al.* (1997a) used the FASAY to compare naturally occurring mutation spectra with experimentally induced spectra. The same authors (Inga *et al.* 1997b) used a modified FASAY to investigate whether specific mutant *p53* proteins were recessive or dominant against wild type *p53*.

SUMMARY

In terms of its use as a mutation detection method, our experience with the FASAY is extremely good and we find the method easy to perform and rapid. We would be in agreement with the view expressed by Soussi *et al.* (2000) that the FASAY be considered as 'the method of choice for routine clinical analysis of *p53* mutations.' Clearly, improvements in other technologies such as those using oligonucleotide probe arrays (chips) may change this situation in the future but even then chips will not give functional information about novel mutations.

The apoptotic assay, in contrast with the FASAY, is only applicable to the detection of germline *p53* mutations. Further, the apoptotic assay does require application to a wider range of mutations to allow the possibility of false negative results to be further investigated. Nevertheless, we also feel that the apoptotic assay may have a role as a very simple, rapid and inexpensive prescreening method to detect individuals likely to harbour germline *p53* related defects. In combination with the FASAY, it can not only detect a wide range of mutations but also allow their partial characterization in terms of two of *p53*'s most important functions, namely transcriptional transactivation and apoptosis.

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