Improving the Detection of *p53* Mutations in Breast Cancer by Use of the FASAY, a Functional Assay

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The aim of this investigation was to examine the ability of the yeast-based functional assay, the functional analysis for the separation of alleles in yeast (FASAY), to detect *p53* mutations in breast cancers when compared with immunohistochemistry and automated sequencing of the whole *p53* gene (exons 1–11). To achieve this, all three methods were carried out on a cohort of aggressive breast tumors. In those tumors, in which the FASAY analysis indicated the presence of a mutation, cDNA was extracted from red yeast colonies and was sequenced to identify the base change in the *p53* gene. The FASAY detected all 24 mutations found in the series of 48 tumors, whereas 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 3 of the 6 tumors that originally appeared to lack a mutation in genomic DNA. All but 1 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. Of 24 tumors, 14 had strong immunohistochemical staining, and all 14 had *p53* mutations; the majority of mutations missed by immunohistochemistry produced a truncated protein. Strong staining was not seen in tumors lacking a *p53* mutation. The FASAY proved to be a rapid, reliable, and effective method for identifying those breast tumors harboring *p53* mutations. (*J Mol Diag* 2000, 2:139–144)

A *p53* gene mutation is the most common genetic defect seen in human cancers, with a quarter of breast cancers having a mutation in the core DNA-binding domain of *p53*.¹ The gene, which consists of 11 exons, is an important tumor suppressor involved in maintaining genetic stability, and one of its functions is to act as a transcription factor, targeting genes such as *mdm2*, *Gadd45*, *p21*, and *Bcl2*.² There is evidence that the *p53* status of individual tumors can have prognostic and therapeutic significance.³ Current methods

of determining *p53* status include immunohistochemistry and automated sequencing of the *p53* gene. Immunohistochemistry assesses *p53* protein levels using *p53*-specific antibodies but there is not a perfect correlation between stabilized *p53* protein and the presence of a mutation.² Automated sequencing of DNA from tumor material, generally considered the gold standard, can also lead to a significant minority of mutations being missed.⁴ In many studies, sequencing of *p53* is restricted to exons 5 to 8, neglecting the remainder of the gene, where a significant minority of mutations occur. An ideal method of detecting *p53* mutations in tumor tissue would need to be rapid, reliable, and able to detect the vast majority of functionally significant mutations.

Few studies have compared sequencing of the whole *p53* gene with other methods and, as far as we are aware, none has compared sequencing with a functional assay such as the functional analysis for the separation of alleles in yeast (FASAY). The FASAY examines the transcriptional transactivation ability of the *p53* protein and detects mutations between exons 4 and 10 in the *p53* gene.⁵ In the current study three methods were used to determine the *p53* status in a series of primary mammary carcinomas: immunohistochemistry, automated sequencing, and the FASAY.

Materials and Methods

Specimens

Samples of primary mammary carcinomas from 48 women patients were obtained from the tissue bank at The Hedley Atkins/ICRF Breast Pathology Unit at Guy's Hospital. Samples were selected from aggressive tumors (63% were infiltrating ductal grade III carcinomas) on the assumption that there was an increased likelihood that they possessed *p53* mutations. All samples were identified by number only.

FASAY

The frozen specimens were dismembrated in the presence of frozen extraction buffer containing guanidinium thiocyanate (Amersham Pharmacia Biotech, Little Chal-

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font, UK) using a Mikro-dismembrator (Braun, Melsingen, Germany). The resulting powder of tissue and buffer was used to obtain mRNA. The extraction of mRNA was carried out using the QuickPrep *Micro* mRNA Purification Kit (Amersham Pharmacia Biotech). The mRNA obtained from each specimen was subsequently used in a yeast-based assay, the FASAY.⁵⁻⁷ mRNA was reverse transcribed to produce cDNA (First-Strand cDNA Synthesis Kit, Amersham Pharmacia Biotech) and this was amplified by polymerase chain reaction (PCR) using *Pfu* polymerase (Stratagene, La Jolla, CA). The PCR product was cotransfected with linearized expression vector into yeast. Colonies were selected by their ability to grow on medium lacking leucine and with a limiting concentration of adenine. Yeast cells expressing wild-type p53 protein form white colonies, and cells expressing mutant p53 protein form red colonies. The percentage of red colonies was calculated for each sample. Up to 10% red colonies was considered wild-type and those >10% were considered to have a p53 abnormality. This assay can detect mutations between exons 4 and 10 of the *p53* gene. For a more detailed method, see Lomax et al⁶ and Flaman et al.⁵

Immunohistochemical Analysis

Immunohistochemical measurement of p53 protein levels and the determination of the degree of staining were carried out as described by Dublin et al.⁸ Thin sections of formalin-fixed, paraffin-embedded tissue were stained with the p53-specific monoclonal antibody DO-1 (p53 [Ab-6], Oncogene Research Products, Cambridge, UK) and a second layer of biotinylated rabbit anti-mouse immunoglobulins followed by peroxidase-conjugated streptavidin biotin (Dako, High Wycombe, UK). The degree of staining was scored on a scale of 0 to 7. Sections with 0 staining were classified as nonstaining, and a score between 2 and 4 was classified as weak staining. Sections with a score of 5 and above were classified as showing moderate to strong staining and were considered to be positive for p53 protein.

Automated DNA Sequencing

All sequencing was carried out using ABI Prism 310 and 377 DNA Sequencers (PE Applied Biosystems, Warrington, UK). The plasmid containing *p53* cDNA from the tumor tissue was recovered from single red yeast colonies⁶ and the cDNA was sequenced using the following oligonucleotide primers: exons 4 to 6, CGTTCTGTC-CCCCTTGC; exons 6 to 8, AACTGGCCAAGACCTGC; exons 9 and 10, GCCCATCCTCACCATCA. These primers allowed sequencing in the 5'-to-3' direction. The cycle sequencing reactions were carried out using Big Dye Terminator chemistry (PE Applied Biosystems) according to the manufacturer's protocol.

Genomic DNA from tumor tissue was obtained by isoamyl alcohol/chloroform extraction and ethanol precipitation. The exons of the *p53* gene were amplified

using the genomic DNA and the following oligonucleotide primers, all written 5' to 3'.

Exon 1, forward: GAGAATCCTGACTCTGCACC, reverse: AGCCGAGAGCCCGTGACTCA; exon 2/3, forward: CCAGGGTTGGAAGCGTCTCATGC, reverse: GAGCAGTCAGAG-GACCAGGTCC; exon 4, forward: GACCTGGTCCTCTGAC-TGCT, reverse: GCATTGAAGTCTCATGGAAG; exon 5, forward: ACTTGTGCCCTGACTTTCAACT, reverse: CAATC-AGTGAGGAATCAGAGGC; exon 6, forward: TCAGATAGC-GATGGTGAGCAG, reverse: GCCACTGACAACCACCC-TTA; exon 7, forward: AGGCGCACTGGCCTCATCTT, reverse: GAAATCGGTAAGAGGTGGGC; exon 8, forward: GGAGTAGATGGAGCCTGGTTT, reverse: GGTGATAAAAG-TGAATCTGAGGC; exon 9, forward: GGAGACCAAGGGTG-CAGTTAT, reverse: GTTAGTTAGCTACAACCAGGAGCC; exon 10, forward: CAATTGTAACCTGAACCATC, reverse: AT-GAGAATGGAATCCTATGG; exon 11, forward: GCACAGAC-CCTCTCACTCATGT, reverse: CAAGGGTTCAAAGAC-CCAAAAC.

Primers for exons 1, 4, 10, and 11 have an annealing temperature (T_m) of 56°C, exons 5 to 9 a T_m of 61°C, and exon 2/3 a T_m of 64°C. For each exon, a polymerase chain reaction was prepared with 200 ng of genomic DNA, 1× PCR Buffer II (PE Applied Biosystems), 100 μmol/L dNTPs (Promega, Madison, WI), 125 ng of forward primer, 125 ng of reverse primer, 1 mmol/L $MgCl_2$ and 2 units of AmpliTaq DNA Polymerase (PE Applied Biosystems). The reaction was subjected to 35 cycles of denaturation at 94°C for 45 seconds, annealing for 45 seconds, and extension at 72°C for 45 seconds. PCR products were purified using Microcon-100 centrifugal filter devices (Millipore Ltd., Watford, UK) according to the manufacturer's protocol. All cycle sequencing extension products were purified by ethanol precipitation, using commercially prepared 95% ethanol. Electropherograms were checked manually for the presence of any mutations.

Results

FASAY

The FASAY was carried out twice on each of the 48 tumors and the average percentage of red colonies obtained for each specimen was determined. Twenty-four (50%) had >10% red colonies, suggesting that they had a *p53* mutation (Table 1). The remainder all had 10% or less red colonies in both replicate assays, suggesting the expression of only functional p53 protein.

Sequencing of cDNA from Samples with >10% Red Colonies in the FASAY

To identify the location and type of mutation in each specimen with a positive FASAY result, cDNA was recovered

Table 1. *p53* Gene Mutation Status Detected Using the Three Methods Described

Sample	Average % red colonies in FASAY (results of duplicate assays)	Sequencing from cDNA		Mutation detected in initial genomic DNA sample	Strong immunohistochemical staining (staining score scale 0–7)
		Base change	Exon		
799/89	82 (83, 80)	258, GAA → CAA Glu → Gln	7	Yes	Yes (6)
503/90	63 (64, 62)	285, GAG → AAG Glu → Lys	8	No*	Yes (7)
554/90	87 (90, 84)	220, TAT → TCT Tyr → Ser	6	Yes	Yes (7)
127/91	75 (72, 78)	258, GAA → AAA Glu → Lys	7	Yes	Yes (7)
396/91	60 (60, 60)	330, CTT → CGT Leu → Arg	9	Yes	Yes (7)
355/92	53 (56, 50)	285, GAG → AAG Glu → Lys	8	No	Yes (6)
591/92	40 (41, 39)	134, TTT → CTT Phe → Leu	5	Yes	No (3)
990/92	90 (89, 90)	282, CGG → TGG Arg → Trp	8	Yes	Yes (6)
1052/92	54 (53, 55)	306, CGA → TGA Arg → stop	8	No	Yes (5)
1144/92	50 (50, 49)	273, CGT → CAT Arg → His	8	Yes	Yes (7)
194/93	16 (17, 15)	144, deletion of 14bp	5	Yes	No (0)
287/93	80 (79, 80)	126, TAC → GAC Tyr → Asp	5	Yes	No (4)
0378/93	86 (87, 85)	132, AAG → GAG Lys → Glu	5	Yes	Yes (6)
0434/93	87 (90, 84)	125, ACG → AGG Thr → Arg	4	Yes	Yes (7)
0467/93	21 (27, 14)	83, deletion of 4bp	4	Yes	No (0)
509/93	70 (71, 69)	248, CGG → CAG Arg → Gln	7	Yes	No (4)
546/93	49 (48, 49)	177, CCC → CGC Pro → Arg	5	Yes	Yes (5)
642/93	21 (25, 17)	103, deletion of 16bp	4	No*	No (0)
702/93	27 (29, 25)	258, deletion of 3rd base	7	Yes	No (0)
82/94	41 (43, 39)	342, deletion of 1st base	10	Yes	No (4)
160/94	68 (65, 71)	250, CCC → CTC Pro → Leu	7	No	No (0)
308/94	40 (40, 40)	175, insertion of 1bp	5	Yes	No (0)
521/94	93 (95, 91)	235, Insertion of 3bp (TAC)	7	Yes	Yes (7)
623/94	18 (20, 15)	175, CGC → CAC Arg → His	5	No*	Yes (7)

*These cases had their mutation confirmed by a second round of automated sequencing, performed using an independent source of genomic DNA.

from at least 3 yeast colonies of each sample and sequenced as described. Point mutations were found in 17 of the 24 tumors with positive FASAY results. All but one resulted in the change of a single amino acid; one had a non-sense mutation giving a premature stop codon. Frame-shift mutations resulting in the introduction of a premature stop codon were found in 6 tumors. One sample had an in-frame 3-bp insertion (Table 1). Nineteen of the mutations were located in the core DNA-binding domain of *p53*, which is covered by exons 5 to 8 of the gene. The remaining 5 were located in exons 4, 9, and 10.

Confirmation of Mutations Detected by FASAY Using Genomic DNA

Genomic DNA was isolated from tumor tissue for all samples. In each case the appropriate exons of the *p53* gene were amplified by PCR and sequenced as described. Eighteen of the mutations detected by the FASAY were confirmed in the initial sequencing of genomic DNA. Six mutations could not be confirmed in these particular samples of genomic DNA. However, mutations were confirmed in a further 3 of these 6 tumors in separate DNA

samples extracted from cytosols originally stored for steroid receptor measurements. In total, 21 of the 24 mutations originally detected by the FASAY were also found by direct sequencing. (Table 1).

Sequencing of Genomic DNA from Tumors with a Negative FASAY Result

The FASAY detects mutations located between exons 4 and 10 of the *p53* gene. Twenty-four samples in this study had <10% red colonies, suggesting that no mutations were present in this region. To check for mutations in regions not covered by the FASAY, exons 1, 2, 3, and 11 of each tumor were sequenced using genomic DNA as described, but no further mutations were detected. To determine whether any mutations were missed by the FASAY in exons 4 to 10, these exons were also sequenced in the samples of genomic DNA; again, no additional mutations were detected. Sequencing these further exons ensured that the whole of the *p53* gene had been examined for the presence of mutations.

Immunohistochemical Analysis of *p53* Protein Levels

To identify elevated levels of *p53* protein expression, thin sections of tumor were stained with the *p53*-specific antibody DO-1 as described. The degree of staining was scored according to the intensity and proportion of positive staining nuclei and categorized as stated in the Materials and Methods. Twenty-six of the 48 samples scored 0 and were nonstaining. Eight had scores of between 2 and 4 and classified as weak staining. Fourteen samples were scored as ≥ 5 and classified as having moderate to strong staining and were thus taken as positive. These 14 cases all had a mutation that was detected by the FASAY. However, an additional 4 cases with weak staining and 6 cases with no staining for *p53* protein also had a positive FASAY result (Table 1). All of the tumors in which the FASAY detected no *p53* abnormality had negative or weak staining.

Comparison of Results for the Three Methods Used to Detect *p53* Status

Twenty-four of the 48 cases had a mutation in the *p53* gene as detected by the FASAY and 18 of the 24 were confirmed by the initial sequencing of genomic DNA isolated from the tumor tissue. Sequencing failed to detect 6/24 (25%) of the mutations; 3 of these were confirmed by using an alternative source of genomic DNA. In previous clinical studies, immunohistochemistry has been the most commonly used method for examining *p53* status but it does not have a high degree of accuracy. In the present study, the 24 cases shown by the FASAY not to have a mutation were also negative for *p53* staining by immunohistochemistry, whereas the 14 tumors showing strong *p53* staining all had a mutation (1 frameshift and 13 point mutations). The remaining 10 tumors with a

Table 2. Relationship between Immunohistochemical Staining and Type of Mutation

Mutation	Immunohistochemistry	
	Negative	Positive
Point	4	13
Frameshift	6	1

mutation showed either weak or negative staining; 6 of them contained frameshift mutations leading to a premature stop codon, and 4 of them had point mutations.

Statistical Analysis

Fisher's exact test was used to test the association between the type of mutation and the presence of stabilized *p53* protein. It showed a significant correlation, $P = 0.009$ (Table 2), with immunohistochemistry detecting 76% of point mutations but only 14% of frameshift mutations.

Discussion

The FASAY detected 24 mutations in the 48 tumors investigated (a mutation rate of 50%), and subsequent DNA sequencing showed that all mutations present in this series were detected by the FASAY. Sequencing of the cDNA extracted from red yeast colonies revealed that 70% were point mutations, although one of them did introduce a premature stop codon. A quarter of the total mutations were frameshift mutations, whereby insertions or deletions altered the reading frame to introduce a premature stop codon downstream from the mutation site. One important aspect of the FASAY is the ability of the method to separate the alleles of the gene. Thus the sequencing carried out on DNA from plasmids recovered from yeast colonies represents a single allele. Moreover, contamination from normal cells present in the tumor tissue was eliminated. Electropherograms produced from cDNA sequencing gave clear traces that permitted easy identification of the mutation and, where appropriate, the length and nature of the insertions and deletions.

The FASAY is able to detect mutations between codons 67 and 347 of the *p53* gene, a fifth of the total mutations found in this study were located in exons 4, 9, and 10. Many clinical studies looking at *p53* status have concentrated on the highly conserved region of the *p53* gene between exons 5 and 8. It is probable that more mutations would have been detected had these studies looked beyond exons 5 to 8.^{9,10} A meta-analysis by Pharoah et al⁹ gathered results from all of the published studies that have 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.

In the current study, DNA isolated from each tumor specimen was sequenced to confirm the mutations at the genomic level. Initial genomic sequencing failed to detect 25% of the mutations found by the FASAY, despite

the fact that the site of each mutation was already known from sequencing of cDNA from the yeast colonies. It is interesting to note that all but one of the mutations missed by this method were point mutations. In general, electropherogram traces from genomic DNA were more difficult to read than those obtained after separation of the alleles in the FASAY. This is partly due to the dilution of the mutant DNA by the wild-type DNA from normal cells present in the tumor. A recent study on lung tumors by Ahrendt and coworkers⁴ reported similar problems when using automated DNA sequencing to examine the *p53* gene. They found that 24% of mutations were missed. Ahrendt et al⁴ also showed that a gene chip assay for *p53* missed 19% of mutations present. Indeed, in this 1999 study, manual DNA sequencing was found to be the only method that detected all mutations, but it is extremely labor-intensive and time-consuming. Another possible problem with automated DNA sequencing has been highlighted in two recent reports that have suggested that wild-type sequences in genomic DNA may be amplified preferentially by PCR when wild-type and mutated material are mixed together.^{11,12} In the current study, to confirm at the genomic level the presence of all mutations found by the FASAY, DNA from 6 of the cases originally stored for steroid receptor analysis was made available and sequenced. Mutations originally detected by the FASAY were confirmed in 3 additional specimens using this alternative source of genomic DNA. Three mutations remained unconfirmed even after this second genomic DNA sequencing. Thus, a combined total of 21 of the 24 mutations detected by the FASAY were subsequently confirmed by sequencing genomic DNA.

We believe that the FASAY results are accurate for the following reasons. First, 3 of the 6 mutations initially missed by genomic sequencing were subsequently identified in the alternative source of genomic DNA, indicating that the FASAY results were genuine in these cases. Second, to avoid apparent mutations due, for example, to PCR errors, the mutations detected by the FASAY were identified in a minimum of three consecutive colonies. A possible reason for the increased sensitivity of the FASAY at detecting *p53* mutations compared with automated sequencing is that the FASAY starts with mRNA, and mRNA levels in tumor cells are increased compared with those in normal cells.

Sequencing of exons 1, 2, 3, and 11 of the *p53* gene in the tumors where the FASAY result was negative did not detect any additional mutations. These exons were sequenced to ensure that all of the exons were examined for the presence of mutations, as the yeast assay detects *p53* abnormalities only between exons 4 and 10. To date, few mutations have been detected in these additional exons; however, it is difficult to ascertain how far this is due to the fact that very few clinical studies look at the whole *p53* gene. Sequencing of exons 4 to 10 in our samples did not detect any mutations that had been missed by the FASAY. We know from other studies in our laboratory that rare mutations, such as those in splice sites, may be missed by the FASAY.¹³ However, results in the current study suggest that such mutations are not common in sporadic breast tumors.

Thin sections of the mammary carcinoma specimens were examined by immunohistochemistry. All 14 of the cases with strong *p53* staining had mutations. There were a further 10 cases with a mutation which had either weak ($n = 4$) or negative staining ($n = 6$) although 6 of these were out-of-frame mutations leading to premature stop codons. Mutations of this type would be expected to produce no protein or a truncated protein. No staining was found in any of the tumors without a mutation. In recent years, studies have shown that immunohistochemistry can yield false negative results caused by frameshift mutations and stop codons.^{14,15} In the current study similar results were obtained, with immunohistochemistry failing to detect mainly the frameshift mutations. Statistical analysis showed a significant association ($P = 0.009$) between immunohistochemical staining and the type of mutation detected. It is of interest that in our study, in which an exhaustive search for mutations has been carried out, strong staining was seen only in tumors with a *p53* mutation.

In a recently published study by Chappuis et al,¹⁶ the FASAY was used to look at the prognostic significance of *p53* mutations in breast cancer. They showed that the FASAY worked well and that half of the mutations identified were non-missense. They did not, however, test the efficiency of the FASAY at identifying tumors with *p53* mutations compared with the efficiency of automated sequencing. In their study, the exons outside the region covered by the FASAY were not checked for the presence of a mutation. As far as we are aware, the current study is the only one in which a functional assay has been compared with immunohistochemistry and sequencing of the whole *p53* gene (exons 1 to 11).

In conclusion, the FASAY is a rapid, reliable method that can distinguish between polymorphisms and functionally important mutations. Results are obtained quickly, making the method a suitable screening tool. The method is also suitable for studies in which the *p53* status of tumors is to be investigated as part of a study of basic tumor biology.

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