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p53 Gene Mutations in Women With Breast Cancer and a Previous History of Benign Breast Disease

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Mutations of the p53 tumour suppressor gene are the most common genetic lesions in human cancers and have been reported in breast cancer as part of the Li-Fraumeni syndrome. In the present study, we determined frequencies and types of the p53 mutations in breast cancer tissues in women with a history of benign breast disease (BBD) identified in Florence, Italy, with (n = 6) or without (n = 10) a family history of breast cancer. Among the cases with a family history of breast cancer and BBD, 2 out of 6 had p53 gene mutations in cancer samples. 1 patient had a mutation at codon 248 and the other had double mutations at codons 243 and 241. In these cases, the p53 gene was also analysed in the tissue samples from previous BBD lesions; however, no mutations were observed (0 out of 6). These results suggest that the p53 mutations occur during advanced stages of tumour progression. In sporadic breast cancer cases with a history of BBD, p53 point mutations were observed in four samples (4 out of 10). Two of these mutations turned out to be silent changes and one of the samples showed triple mutations at amino acid positions 267, 277 and 296. No p53 gene mutations were found in the breast tumour tissues of 10 additional women from the same area with a family history of breast cancer, but no previous BBD (0 out of 10). Family history of breast cancer does not appear to affect the frequency of p53 mutations in women with a previous history of BBD.

Key words: breast cancer, benign breast disease, p53 tumour suppressor gene, mutations
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INTRODUCTION

OVER THE past 50 years, the incidence of breast cancer among women in the U.S.A. has nearly doubled [1]. In this decade alone, it is estimated that 1.7 million women—175 000 per year—will be diagnosed with breast cancer. In the U.S.A., one in eight women will develop breast cancer and an estimated 30% of them will ultimately die from this disease [1, 2].

Familial breast cancer has been an area of intense research because of the clinical importance of counselling and follow-up of those at elevated risk, and for its potential in identifying candidate breast cancer gene(s) [3]. In studies that compare familial and sporadic breast cancer, it has been found that a family history of breast cancer increases a woman's risk for having breast cancer by 3-fold [4]. Moreover, women with a family history of breast cancer are diagnosed at a younger age and have a higher frequency of bilateral disease [5].

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Benign breast disease (BBD) has been shown to be a risk factor for later development of breast cancer. A person with a history of atypical hyperplasia, the most risky of the BBD histological subtypes, has a relative risk of 3.5-fold [5, 6]. A person with a history of both atypical hyperplasia and familial breast cancer has an increased risk of 9-fold over the risk of either occurrence alone [6]. Collectively, these data suggest that there is at least a partial common pathway between BBD and breast cancer, and that it is possible that BBD lesions are precursor lesions in the development of the disease.

The p53 gene has been found to be the most commonly mutated gene in human cancers [7, 8]. Mutations of this gene have been reported to occur in 15–46% of human breast cancers [8]. Mutation of the p53 gene is commonly associated with loss of normal allele on the short arm of chromosome 17, where the p53 gene is located [8]. Estimates of the frequency of loss of heterozygosity (LOH) at 17p range from 56 to 69% [8]. Overexpression of p53 is commonly associated with missense mutations in the p53 gene [1]. Overexpression of the p53 protein in sporadic breast cancer ranges from 45 to 62% [9–12]. Breast cancer is a frequent component to the Li-Fraumeni inherited cancer syndrome [13, 14].

To date, the p53 gene has not been examined in benign breast disease. Identifying p53 gene mutations in the early stages of breast cancer would provide insights into the correlation between the occurrence of p53 mutations and disease status. In this

paper, we report our findings upon the frequency and type of p53 mutations in a series of breast cancer patients from a previous epidemiological study in Florence, Italy [15]. We examined cancer samples of sporadic and familial patients having a previous history of BBD, and BBD lesions of a subgroup of these patients.

MATERIALS AND METHODS

Samples

Samples were collected from Florence, Italy. Interviews, reviews of medical records and available histological specimens were used to subclassify the cases as to family history of cancer and type of benign breast disease. BBD had been histologically classified and each lesion was categorised as non-proliferative (NP), proliferative disease without atypia (PDWA) or atypical hyperplasia (AH) in a previous study [15]. The DNA samples were prepared from formalin-fixed, paraffin-embedded archival material. An additional group of 10 women with breast cancer and a family history, but no previous BBD, was identified in the same area, and tissue samples were retrieved from the archival files of the Pathology Department of the University of Florence.

DNA isolation

To isolate DNA from archival, formalin-fixed, paraffin-embedded tissue sections, areas of relatively pure tumour cells were localised in 20-μm unstained sections by comparison with neighbouring thin-stained (Haematoxilin and Eosin) sections [16]. The tumour or normal tissue was placed in 1 ml of absolute ethanol, heated to 85°C and the ethanol paraffin solution was removed. The samples were air-dried, rehydrated with 100 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4) and then 5 μl proteinase K (20 mg/ml) was added. Digestion was performed at 54°C for 24 h, and samples were boiled for 3 min to inactivate the proteinase K. One to ten microlitres of this sample were used as a template per 100 μl of polymerase chain reaction (PCR).

DNA amplification by PCR and purification of DNA fragments

The template DNA was amplified as described previously [16, 17] using 30 cycles of PCR (94° for 1 min, 56° for 1 min and 72° for 2 min) in a 100-μl reaction mixture containing approximately 100 ng of genomic DNA, 50 pmol of each primer, 5 units of Taq polymerase, 10 μl of 10 X PCR buffer (500 mM Tris–HCl, pH 9.0, 30 mM $MgCl_2$) and 16 μl of 2.5 μM dinucliotide triphosphates (dNTPs). Using an internal primer, a second PCR of 300 μl was performed using 10 μl of the first PCR mixture as template DNA, again using 30 cycles [16]. The PCR products were gel-purified by electrophoresis on a 4% NuSieve agarose gel. The appropriate DNA fragment was extracted from the gel band by electroelution, as described previously [16, 17].

Direct DNA sequencing of the p53 exon (5–8) amplified DNA fragments

Each sample was screened for p53 gene mutations by the direct DNA sequencing method using a modification of the Sanger dideoxy-chain termination procedure, described previously [16, 17]. Mutations were confirmed by performing a second PCR reaction and sequencing the opposite strand.

RESULTS

The frequency and type of p53 gene mutations were determined in a group of familial and sporadic breast cancer with a history of BBD (Table 1). The frequency of p53 mutations in the BBD lesions of 6 patients who had a family history of breast cancer was also determined.

Identification of the p53 gene mutations in women with BBD, breast cancer and a family history of breast cancer

Two out of the six cancer tissue samples (case P3 had a sister and a maternal cousin with breast cancer and case P5 had a sister) showed p53 gene mutations (Figure 1). Sample P3 had a mutation at the second base pair of codon 248 (G to A) causing an amino acid change from Arg to Gln (Table 2). Interestingly, sample P5 had two point mutations. At codon 241, the second base pair was altered from C to T causing a serine to phenylalanine amino acid change; at codon 243 the third base pair was altered from G to A causing a methionine to isoleucine change (Table 2).

In all three mutations, in samples P3 and P5, the wild-type band in the sequencing gel (Figure 1) was present at equal or greater intensity than the corresponding mutant band (Figure 1), suggesting that there was no 17p deletion of the normal p53 allele. Microscopic examination of the relative number of tumour to non-tumour nuclei in the sample from which DNA was extracted did not support the hypothesis that the wild-type band was the result of contamination with stroma or normal tissue.

To identify p53 gene mutations at an earlier stage, we also amplified DNA from BBD tissue samples of the 6 patients (2 with positive and 4 with negative cancer tissue specimens), and

Table 1. Analysis of the p53 gene mutations in breast cancer patients with a family history of BBD and breast cancer

		p53 gene mutations	
	Cases	BBD lesions	Breast tumours
Patients with breast cancer, a family history of breast cancer and a history of BBD	6	0	2
Patients with sporadic breast cancer and a history of BBD	10	ND	4*
Patients with breast cancer, a family history of breast cancer, but no BBD	10	—	0

BBD, benign breast disease; ND, not determined. *Two point mutations did not alter the amino acids.

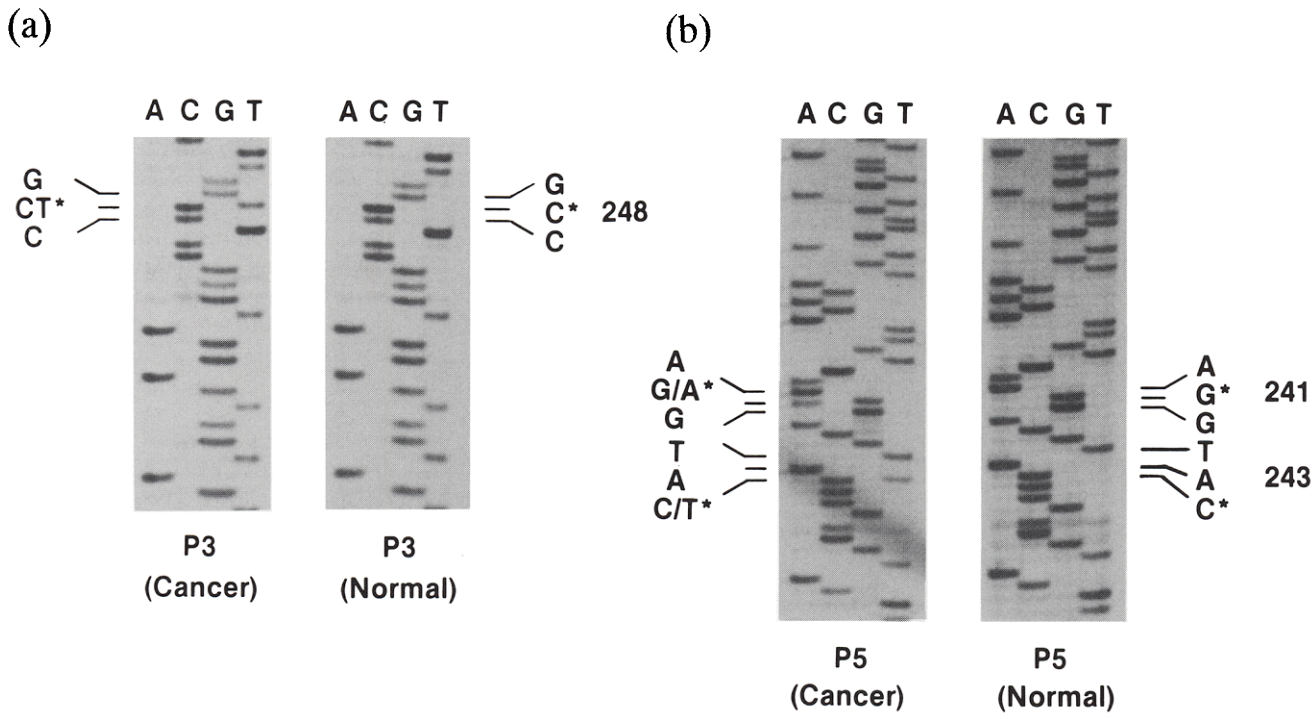

Figure 1. Sequence data for samples (a) P3 and (b) P5, both carcinomas of the breast, in individuals with both a history of benign breast disease and a family history of breast cancer. The DNA sequence of the cancer sample is paired with an example of normal sample. The figure shows an antisense sequence reading 3′ to 5′ from top to bottom. All three mutations show a wild-type band that is more intense than the mutated band, suggesting that the tumours are heterozygous for mutated and normal p53 at those codons.

Table 2. Summary of the p53 gene mutations in familial and sporadic breast cancer with a history of BBD

Tumour sample	Exon	Codon	Nucleotide substitution	Amino acid substitution
Patients with familial breast cancer* and BBD†				
P3	7	248	CGG→CA/GG	ARG→GLN
P5	7	241	TCC→TC/TC	SER→PHE
P5	7	243	ATG→ATG/A	MET→ILE
Patients with sporadic breast cancer and BBD				
21	6	213	CGA→CGG	ARG→ARG
3	8	296	CAC→CGC	HIS→ARG
3	8	277	TGT→TAT	CYS→TYR
3	8	268	AAC→CAC	ASN→HIS
8	8	292	AAA→AAG	LYS→LYS
19	7	243	ATG→ATG/A	MET→ILE

This table lists the instances in which other than a wild-type sequence was observed for exons 5, 6, 7, 8 and adjacent consensus splice sites of the p53 gene. The codon nucleotide sequence is that of the coding strand and reads 5′ to 3′. In cases where the wild-type band was of equal or greater intensity to the mutant band, both bases are entered with a / separating them to indicate a probable heterozygote. In two cases, sample 21 and sample 8, the mutations are silent and probably represent polymorphisms, as have been reported at these sites.

*At least a first degree relative had breast carcinoma and, at the time of diagnosis, the age of patients and their affected relatives ranged between 29 and 77 years. †An additional group of 10 familial breast cancer patients without a history of BBD did not show p53 gene mutations.

subjected them to DNA sequence analysis. Surprisingly, none of these six samples showed point mutations in the BBD lesions (three had been originally classified as non-proliferative, three as atypical hyperplasias), suggesting that p53 mutations in breast cancer occur at an advanced stage of the disease.

Mutational spectrum of the p53 gene in sporadic breast cancer with a history of BBD

A total of 10 sporadic breast cancers in women with a history of BBD were analysed for mutations in p53. Four of the 10 tumours contained mutated versions of the wild-type sequences (Table 2). However, two of those four (sample numbers 21 and 8) turned out to be silent changes at the sites of known DNA polymorphisms [18]. Tumour sample number 21 revealed an A:T to G:C transversion in exon six at codon 213. This nucleotide alteration does not change the amino acid residue, thus the arginine at codon 213 is maintained. Similarly, in sample 8, the A:T to G:C transversion in exon 8 at codon 292 does not change the existing lysine.

Tumour sample number 3 contained three mutations, all of which occurred in exon 8 (Figure 2). The nucleotide sequence corresponding to codon 268 reveals an A:T to C:G transversion which leads to histidine replacing asparagine. Codon 277 contains a G:C to A:T transversion which causes tyrosine to replace cysteine and there is an A:T to G:C transition occurring in codon 296, which changes histidine to arginine.

In tumour sample number 19, the ATG (methionine) at codon 243 is altered to ATG/A (methionine and isoleucine). The presence of both the wild-type and mutated base in this sample (data not shown) and the observation that the wild-type G band

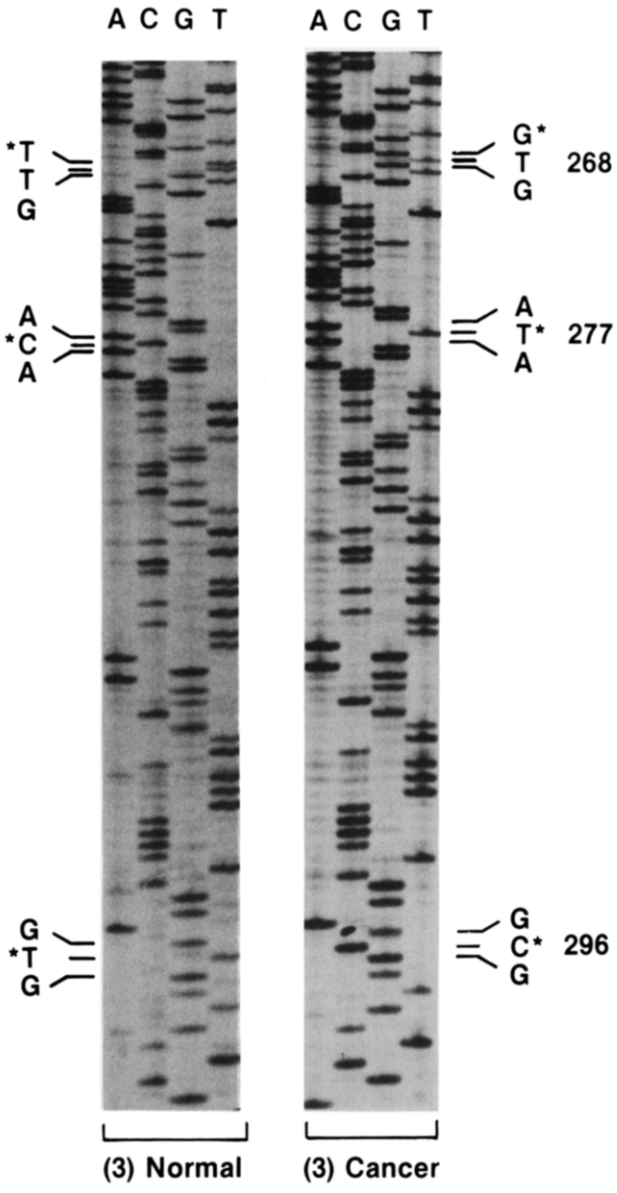

Figure 2. Sequence data for sample 3, a carcinoma of the breast in an individual with a history of benign breast disease for a section of exon 8 of the p53 gene. The figure shows an antisense sequence reading 3′ to 5′ from the top. Three mutations were observed at codon positions 268, 277 and 296. Only the mutant band was observed, suggesting that the tumour was hemizygous for the p53 locus, probably secondary to a deletion.

is more intense than the mutated A band suggest that the tumour is heterozygous.

Mutational spectrum of the p53 gene in breast cancers from individuals with a family history of breast cancer but without a history of BBD

Tumour samples from 10 patients with a family history of breast cancer were analysed for p53 gene mutations. No p53 mutations were found in exons 5–8. Similarly, it has been reported recently that the p53 gene is not mutated in the germline of individuals with a history of familial breast cancer [19]. Thus, mutation in the p53 gene does not appear to be an informative marker for familial breast cancer.

DISCUSSION

In this study, we report the frequency of p53 gene mutations in a group of 16 breast cancer patients with a history of benign breast disease.

Our observation that the frequency of p53 mutations occurring in this series of breast cancers is 37.5% (six out of 16) is consistent with previous reports of 13–46% [8, 9, 11, 12]. It should be noted, however, that our cases differed from those reported elsewhere in that they had a history of histologically-confirmed benign breast disease [15]. Our results show that the family history of breast cancer does not appear to affect the frequency of occurrence of p53 mutations among these patients, at least within the limits of the current number of samples analysed.

We observed two out of six (33.3%) p53 mutations in cancers from women with a family history of breast cancer and BBD. None of the p53 mutations, seen in these individuals with a family history of breast cancer, were found in the germline. Warren and colleagues similarly found no p53 mutations (0/25) in the germline for 25 breast cancer families examined [19]. No p53 mutations were detected in exons 5–8 in BBD lesions obtained from the same patients. In contrast, no p53 mutation was found (0/10) in the cancer tissue samples of an additional group of 10 patients with a positive family history, but no previous BBD.

These data suggest that women with a personal history of BBD are at increased risk of developing a p53 mutation-associated form of breast cancer. Such breast cancers are associated with the more aggressive type, oestrogen receptor-negative malignancies [20].

Overall, four out of nine (44%) mutations we observed were G:C to A:T transversions (Table 3). This compares with 50% for all tumours, 48% of solid tumours and 40% for breast tumours reported previously [7]. In both studies, the G:C to A:T transition is the most common mutational change in breast cancer. Interestingly, we found a single instance (one out of eight) which has an A:T to T:A transition; this alteration was not reported previously (Table 3) [7].

We observed two cases with multiple mutations, i.e. sample P5 with mutations at positions 241 and 243 and sample number 3 at codons 268, 277 and 296 (Table 2). Three of these mutations (codons 243, 268 and 296) have not been reported previously [7]. Mutation at codon number 243 was observed twice, leading

Table 3. Nature of p53 gene mutations

Cancer	Mutations at G:C →A:T	→T:A	→C:G	Mutations at A:T →T:A	→C:G	→G:C
P3	F					
P5	F					
P5			F			
19				S		
3	S					
3						S
3					S	
21						S*
8						S*
Breast (this study)	33%	—	11%	11%	11%	33%
Breast (previous study)	40%	23%	20%	0%	10%	6%
All solid tumours	48%	24%	10%	4%	9%	4%

S, sporadic; F, familial. *Silent change.

to concern about cross-contamination. However, the mutation at 243 in P5 is coupled with a 241 mutation to exon 7. The 241 mutation was not present in sample 19, thereby excluding possible cross-contamination.

Codons 268 and 296 are located outside of the evolutionary conserved regions and do not show great phylogenetic conservation [21]. It is possible that these mutations represent weakly inactive p53 mutations, which lead to clonal expansion, but may be superseded or added to by a more deleterious subsequent mutation at codon 277 that is located in a conserved region.

We observe that in three of our six positive samples there is evidence for relative differing band intensities, suggesting that the tumour is heterozygous for mutated and wild-type p53 genes. This result is similar to the one reported by Davidoff and colleagues where three of seven breast cancers were heterozygous [22]. Both of our reported cases of p53 mutation-positive breast cancer with breast cancer family and BBD history were instances of heterozygous p53-mutant tumours. In the case of P5, both mutations showed heterozygosity; it is unclear why breast cancers should incur a higher frequency of heterozygosity than other types of tumours.

The lack of p53 mutations in the cases of BBD lesions examined supports the hypothesis that p53 mutations are a late event in breast carcinogenesis. In this respect, p53 mutations in breast cancer would seem to be similar to adenocarcinomas of the colon [22].

1. Harris R, Lippman ME, Veronesi U, Willett W. Breast cancer. *N Engl J Med* 1992, **327**, 319–328, 390–398, 473–480.
2. Kessler LG, Feuer EJ, Brown ML. Projections of breast cancer burden to US women: 1990–2000. *Prev Med* 1991, **20**, 170.
3. King M-C. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990, **250**, 1684–1688.
4. King M-C. Breast cancer genes: How many, where, and who are they? *Nature Genet* 1992, **2**, 89–90.
5. Carter CL, Corle DK, Micozzi MS, Schatzkin A, Taylor PR. A prospective study of the development of breast cancer in 16,692 women with benign breast disease. *Am J Epidemiol* 1988, **128**, 467–477.
6. Henderson IC, Harris JR, Kinne DW, Hellman S. Cancer of the breast. In DeVita VT Jr, Hellman S, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology*. Philadelphia, J.B. Lippincott, 1989, 1197–1268.
7. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991, **253**, 49–53.
8. Tominaga O, Hamelin R, Remvikos Y, Salmon RJ, Thomas G. p53 from basic research to clinical applications. *Crit Rev Oncog* 1992, **3**, 257–282.
9. Osborne RJ, Merlo GR, Mitsudomi T, *et al.* Mutations in the p53 gene in primary human breast cancers. *Cancer Res* 1991, **51**, 6194.
10. Davidoff AM, Humphrey PA, Iglehart JD, Marks JR. Genetic basis for p53 overexpression in human breast cancer. *Proc Natl Acad Sci USA* 1991, **88**, 5006–5010.
11. Kovach JS, McGovern RM, Cassady JD, *et al.* Direct sequencing from touch preparations of human carcinomas: analysis of p53 mutations in breast carcinomas. *J Natl Cancer Inst* 1991, **83**, 1004–1009.
12. Runnebaum IB, Nagarajan M, Bowman M, Soto D, Sukumar S. Mutations in p53 as potential molecular markers for human breast cancer. *Proc Natl Acad Sci USA* 1991, **88**, 10657.
13. Malkin D, Li FP, Strong LL, *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 1990, **250**, 1233–1238.
14. Srivastava S, Zov Z, Pirollo K, Blattner W, Chang E. Germline transmission of a mutation p53 gene in a cancer-prince family with Li-Fraumeni syndrome. *Nature* 1990, **348**, 747–749.
15. Palli D, Rosselli Del Turco M, Simoncini R, Bianchi S. Benign breast disease and breast cancer: a case–control study in a cohort in Italy. *Int J Cancer* 1991, **47**, 703–706.
16. Seth A, Mariano J, Metcalf R, *et al.* Relative paucity of p53 gene mutations in male breast carcinomas. *Int J Oncol* 1993, **2**, 739–744.
17. Metcalf RA, Welsh JA, Bennett WP, *et al.* p53 and Kirsten-*ras* mutations in human mesothelioma cell lines. *Cancer Res* 1992, **52**, 2610–2615.
18. Mazars G-R, Jeanteur P, Lynch HT, Lenoir G, Theillet C. Nucleotide sequence polymorphism in a hotspot mutation region of the p53 gene. *Oncogene* 1992, **7**, 781–782.
19. Warren W, Eeles RA, Ponder BAJ, *et al.* No evidence for germline mutations in exons 5–9 of the p53 gene in 25 breast cancer families. *Oncogene* 1992, **7**, 1043–1046.
20. Mazars R, Spinardi L, BenCheikh M, Simony-Lafontaine J, Jeanteur P, Theillet C. p53 mutations occur in aggressive breast cancer. *Cancer Res* 1992, **52**, 3918–3923.
21. Soussi T, Caron de Fromentel C, May P. Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* 1990, **5**, 945–951.
22. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990, **61**, 759–767.