

p53 PROTEIN ACCUMULATION AND MUTATIONS IN NORMAL AND BENIGN BREAST TISSUE

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Mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer, and there are several reports suggesting that changes in p53 may contribute to the pathogenesis of this disease. In a previous case-control study, we demonstrated that p53 protein accumulation detected by immunohistochemistry in normal or benign breast tissue was associated with a 2.5-fold increase in the risk of subsequent breast cancer. In this study, we investigated whether p53 gene mutations were present in the 29 p53 immunopositive normal or benign breast tissue samples and in 15 p53 immunonegative normal or benign breast tissue samples selected randomly from the original study. DNA was extracted from paraffin sections and underwent PCR-SSCP analysis for exons 4 to 10. PCR products that showed abnormal mobility were excised and sequenced. Sixteen (59.2%) of the 27 immunopositive breast tissue samples and 4 (26.7%) of the 15 immunonegative samples had p53 sequence changes. There was no obvious association between the occurrence of these alterations and any specific histopathologic features. Ten cases showed p53 mutations, and they were all missense base substitutions of the transition type. Thirteen other gene changes occurred in 11 breast tissue samples and consisted of 8 silent (no amino acid change), 4 intronic alterations, and 1 indeterminate alteration. One individual had both a mutation and a silent change. In summary, p53 gene alterations can occur in normal or benign breast tissue, but resolution of their role in the pathogenesis of breast cancer will require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations as well as functional assays to determine their significance. *Int. J. Cancer* 87:73–78, 2000.

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Carcinogenesis is a complex multistep process that arises from the accumulation of critical genetic changes (Shackney and Shankey, 1997). The molecular changes leading to the development of breast cancer are not well characterized. However, mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer (Phillips *et al.*, 1999) and several clinical and experimental studies have suggested that changes in p53 may contribute to the pathogenesis of this disease.

In experimental studies, p53 mutations occur in the preneoplastic stage of mouse mammary tumour development (Jerry *et al.*, 1993). It has been shown that transgenic mice expressing a mutant p53 172^{R-H} minigene that had been targeted to the mammary gland developed chemically induced breast cancer with shorter latency periods and greater tumour burden than did their nontransgenic littermates (Li *et al.*, 1998). Gao *et al.* (1996) have shown that ablation of p53 function by a dominant negative p53 mutant can result in immortalization of normal human mammary epithelial cells. However, not all dominant negative mutants induce immortalization (Gao *et al.*, 1997), suggesting that the contribution of mutant p53 to the development of cancer is complex.

In clinical studies, p53 mutations and/or p53 protein accumulation have been detected in intraductal carcinomas (Done *et al.*, 1998; Lisboa *et al.*, 1998; Phillips *et al.*, 1999). p53 protein accumulation has also been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome (Thor *et al.*, 1992) and in benign tissue adjacent to breast cancer in women with a cancer syndrome distinct from Li-Frau-

meni syndrome (Barnes *et al.*, 1992). Several reports have also shown p53 mutations and/or positive immunostaining for p53 in sporadic forms of benign breast disease (Millikan *et al.*, 1995; Schmitt *et al.*, 1995; Younes *et al.*, 1995; Lisboa *et al.*, 1997; Rohan *et al.*, 1998). Collectively, these findings suggest that p53 changes can occur prior to the development of breast cancer. This is in keeping with observations by others that p53 alterations can occur in putative precursor lesions of other cancers and in normal tissues. For example, p53 mutations have been detected in Barrett's esophagus (Campomenosi *et al.*, 1996), and mutations in codons 247 and 248 have been detected in normal skin and have been shown to be associated with increased risk of developing basal cell carcinoma (Ouhitit *et al.*, 1998).

In a previous study (Rohan *et al.*, 1998) in which histological sections of normal or benign breast tissue were stained immunohistochemically for p53 (using the DO-7 antibody), we identified 29 subjects who showed p53 protein accumulation. One explanation for the p53 immunopositivity is that the tissue had an underlying p53 mutation. It is also possible that some of the 330 immunonegative subjects in that study had p53 mutations, since immunoreactivity can depend on the antibody used, on the type and duration of tissue fixation, or on the type of mutation, given that some mutations may not alter the protein in such a way that it can be detected immunohistochemically (Phillips *et al.*, 1999). In relation to the latter point, one study showed that approximately 33% of breast cancers with p53 gene mutations identified by complementary DNA sequencing did not show positive immunostaining in tissue sections using the Cl 1801 antibody (Sjögren *et al.*, 1996). In this study, we investigated whether the 29 p53 immunopositive breast tissue samples and 15 randomly selected p53 immunonegative breast tissue samples had p53 gene mutations.

MATERIAL AND METHODS

Clinical history and histopathology review

Breast tissue specimens from 44 women whose biopsies showed either no histopathological change or benign breast disease were analyzed. The women selected for the study had their biopsies performed between 1980 and 1987. For each patient a representative paraffin block containing tissue from the breast biopsy was obtained. Five- μ m sections were cut, stained with hematoxylin and eosin, examined by light microscopy, and classified according to the criteria developed by Page and Anderson (1987).

p53 immunostaining

As described previously (Rohan *et al.*, 1998), 5- μ m sections were cut from the paraffin blocks, mounted on aminopropyltri-

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ethoxysilane (2%, Sigma, St. Louis, MO) coated slides and deparaffinized, and underwent antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0, for 15 min at a medium-high setting). Endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with goat serum (20 µl/ml, Vector, Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, UK). The sections were incubated overnight at 4°C with antibody reactive with p53 (DO-7, dilution 1:40, Novocastra Laboratories, Newcastle Upon Tyne, UK). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (dilution 1:200, Vector) for 30 min at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector). Immunoreactivity was visualized with 3',3'-diaminobenzidine tetrahydrochloride (Vector) and the sections counterstained briefly with hematoxylin. The positive controls were sections from a paraffin-embedded breast cancer that was known to have a p53 mutation associated with p53 protein accumulation. The negative control consisted of replacing the primary antibody either with PBS or with mouse nonimmune serum. The presence of nuclear staining in any number of cells seen at 100× magnification was considered a positive reaction. Cytoplasmic staining was considered nonspecific and interpreted as negative.

p53 molecular analysis

Five-µm sections were cut from the paraffin blocks and stored for up to 3 years. Prior to microdissection, the sections were dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that had shown p53 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections that showed no p53 protein accumulation immunohistochemically underwent random microdissection of epithelium. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hr at 55°C. The proteinase K was inactivated by heating at 95°C for 15 min.

An aliquot of the digest was amplified using PCR, [α -³²P]-dATP and exon-specific primers (see Table I). An aliquot of the reaction product was separated on an 8% nondenaturing polyacrylamide gel, and the gel was processed for autoradiography. Potential mutations were detected by shifts in band mobility. If no band shifts were detected in these samples, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. If the two PCR-SSCP analyses generated different band shifts, another section was cut, microdissected, and processed for PCR-SSCP analysis as described above. Negative controls including cells that contained no mutation and a blank water control were included in each analysis. In addition, positive controls for exons 5 to 9 (exon 5: SKBr 3; exon 6: T47D; exon 7: colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines used as positive

controls had been embedded in agar, fixed in 10% formalin, and were paraffin-embedded to simulate the processing conditions of the breast tissue.

The abnormally shifted band was excised from SSCP gels, and the DNA was eluted into water. The DNA was reamplified by PCR using the same primers, and the product was run on a 2% agarose gel. The band was extracted using QIAquick gel extraction kit (Qiagen, Mississauga, ON). The purified DNA was sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Gene alterations were compared with those listed for breast cancer in a p53 database (<http://www.iarc.fr/p53>).

RESULTS

For two of the immunopositive cases, we were unable to extract DNA, and these cases were eliminated from the study. Of the 42 cases from which we could extract DNA, 22 showed fibrocystic change, 8 showed adenosis with or without fibrocystic change or fibrosis, 8 had hyperplasia (mild, moderate, or florid), 2 had fibroadenomas, and 2 showed no histopathological change.

Exons 4 to 10 were analyzed for mutations and a representative SSCP gel and its corresponding sequencing gel are shown in Figure 1. For all cases except one, the SSCP changes were reproducible. In the one case (case 24) where the SSCP change was not reproducible, the repeat analysis had been done on DNA extracted from a different section and only wild-type DNA sequences were seen on the second analysis. In all, 23 sequence alterations were detected in 20 individuals, and they were all base substitutions of the transition type (Tables II and III). Ten of these changes were missense mutations resulting in an amino acid change. Two of these mutations occurred at CpG dinucleotide sequences (cases 2 and 24), and 2 occurred at known hot spots on the p53 gene, one at codon 175 and the other at codon 245. The missense mutations were distributed amongst exons 4, 5, 7, and 9.

The other 13 gene changes (13/23) consisted of 8 silent (no amino acid change) base substitutions, 4 intronic base substitutions, and 1 uninterpretable change. The latter gene change occurred in case 5, for which an abnormal pattern for exon 9 was detected in the SSCP gel, while all the other exons showed wild-type patterns. Although the sequencing pattern could not be interpreted because of the presence of numerous extra bands, this sample was still considered to have a gene alteration. The 8 silent changes were detected in exons 4, 6, and 7, and the intronic changes were in introns 6, 7, and 9. Three individuals had 2 sequence changes each; in 2 of them both changes were silent and

TABLE I—P53 PRIMER SEQUENCES AND PCR CONDITIONS

Primers	Sequences	Product size (bp)	PCR conditions
Exon 4 ¹	5'-ATCTACAGTCCCCCTTGCCG-3' 5'-GCAACTGACCGTGCAAGTCA-3'	296 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 35 cycles
Exon 5 ²	5'-GCTGCCGTGTTCCAGTTGCT-3' 5'-CCAGCCCTGTCGTCTCTCCA-3'	294 bp	95°C, 50 sec; 58°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 6 ²	5'-GGCCTCTGATTCCTCACTGA-3' 5'-GCCACTGACAACCCTTA-3'	199 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 7 ²	5'-TGCCACAGGTCTCCCAAGG-3' 5'-AGTGTGCAGGGTGGCAAGTG-3'	196 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 8 ²	5'-CCTTACTGCCTCTTGCTTCT-3' 5'-ATAACTGCACCCTTGGTCTC-3'	225 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 9 ³	5'-GCCTCAGATTCACCTTTATCAC-3' 5'-CTTCCACTTGATAAGAGGTCCC-3'	152 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 10 ¹	5'-TGTTGCTGCAGATCCGTGGG-3' 5'-GAGGTCACCTACCTGGAGTG-3'	130 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 33 cycles

¹Reference (Mashiyama *et al.*, 1991).—²Reference (Millikan *et al.*, 1995).—³Reference (Mazars *et al.*, 1992).

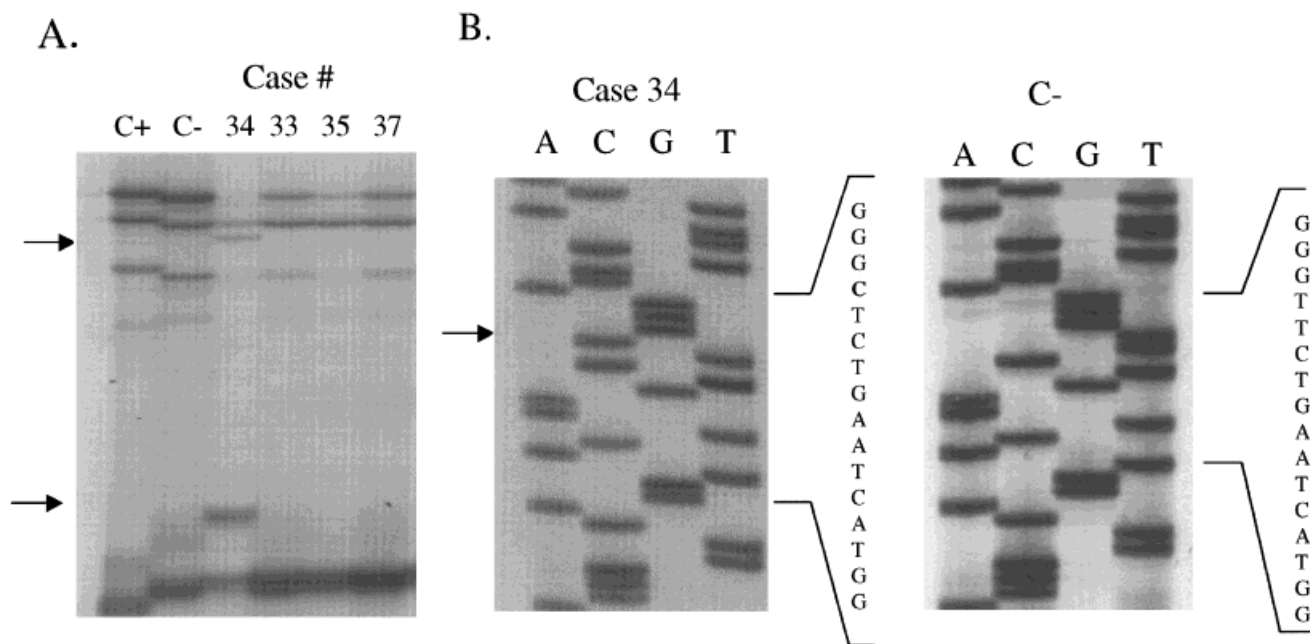


FIGURE 1 – (a) Representative SSCP gel of exon 9 PCR product from 4 cases. Case 34 shows a band shift as indicated by the arrow. The negative control that had wild-type p53 (C–) and positive (C+) control (cell line SW480) are included. (b) The corresponding sequencing gel of case 34 shows a base substitution (t→c) as indicated by the arrow. The sequencing pattern for the negative control (C–) in the same region is also shown.

TABLE II – P53 MUTATIONS IN BREAST TISSUE

Case number	p53 ICH ¹	Location	Codon	Sequence change	Amino acid
24	+	Exon 4	72	CGC → CGT	Arg → Arg
		Exon 4	110	CGT → TGT	Arg → Cys
46	+	Exon 4	76	GCA → ACA	Ala → Thr
2	+	Exon 5	175	CGC → CAC	Arg → His
4	+	Exon 5	135	TGC → TAC	Cys → Tyr
28	+	Exon 5	133	ATG → GTG	Met → Val
36	–	Exon 5	178	CAC → CGC	His → Arg
9	+	Exon 7	227	TCT → TTT	Ser → Phe
40	+	Exon 7	244	GGC → GAC	Gly → Asp
3	+	Exon 7	245	GGC → GAC	Gly → Asp
16	+	Exon 9	325	GGA → GAA	Gly → Glu

¹ICH, immunohistochemical staining; +, present, –, absent.

in the third, 1 of the 2 resulted in an amino acid change. Of the 4 intronic alterations, 2 were in the same location (nucleotide residue 14766) in intron 9 and showed the same change (t→c). The others were at nucleotides 13466 in intron 6 and 14114 in intron 7. None of the intronic mutations occurred at a splice site or created a new splice site. In addition to the results shown in Tables II and III, the known p53 polymorphism in codon 72 (CGC→CCC) was detected in 2 cases.

All 10 missense mutations occurred in codons identified in the p53 breast cancer database as having mutations. Seven of them showed the same base and amino acid change as has been identified in breast cancer. Of the 8 silent changes, 3 showed the same base change as has been identified in breast cancer and 5 showed a different alteration in the same codon. A similar comparison could not be done for the intronic mutations because the nucleotide residues of the intronic mutations are not provided in the database. Similar to those reported in the p53 database, most base substitutions in this study were G:A and C:T (IARC p53 mutations database <http://www.iarc.fr/p53>).

For all individuals with a p53 gene alteration, the adjacent stromal tissue underwent microdissection and extraction of the

DNA. The exon that had been identified as abnormal in the epithelial cells was analyzed by PCR-SSCP. In 18 of 20 samples/subjects, wild-type p53 banding patterns were observed (Fig. 2). In the other two (sample/subject 29 and 34), the same gene alteration was present in the stromal cells as in the epithelial cells.

Of the 27 breast tissue samples with p53 immunopositivity, 16 (59.2%) had p53 sequence changes. Nine of these 16 had mutations. One breast tissue with a p53 mutation was immunonegative (Table II). Seven of the 10 breast tissues with sequence alterations (silent or intronic) showed p53 immunoreactivity (Table III). Four of the 15 women (26.7%) whose biopsies were immunonegative showed sequence changes. One had a mutation (Table II) and 3 showed sequence alterations (Table III). There was no obvious association between the occurrence of gene alterations and any specific histopathologic features (Table IV). A representative photomicrograph of a section stained for p53 is shown in Figure 3.

DISCUSSION

p53 is involved in regulating cell proliferation and DNA repair, inducing apoptosis, and promoting chromosomal stability (Levine,

TABLE III – P53 SEQUENCE CHANGES THAT DO NOT CAUSE AMINO ACID CHANGES

Case number	p53 ICH ¹	Location	Site	Sequence	Amino acid
Silent change					
32	–	Exon 4	codon 74	GCC → GCT	Ala → Ala
		Exon 4	codon 111	CTG → CTA	Leu → Leu
44	+	Exon 4	codon 111	CTG → CTA	Leu → Leu
18	+	Exon 6	codon 217	GTG → GTA	Val → Val
27	+	Exon 7	codon 231	ACC → ACT	Thr → Thr
		Exon 7	codon 239	AAC → AAT	Asn → Asn
48	+	Exon 7	codon 226	GGC → GGT	Gly → Gly
Intronic change					
26	+	Intron 6	nr ² 13466	g → a	
17	+	Intron 7	nr 14114	g → a	
29	–	Intron 9	nr 14766	t → c	
34	–	Intron 9	nr 14766	t → c	
Noninterpretable change					
5	+	Exon 9	–	–	

¹IHC, immunohistochemical staining; +, present, –, absent. ²nr, nucleotide residue.

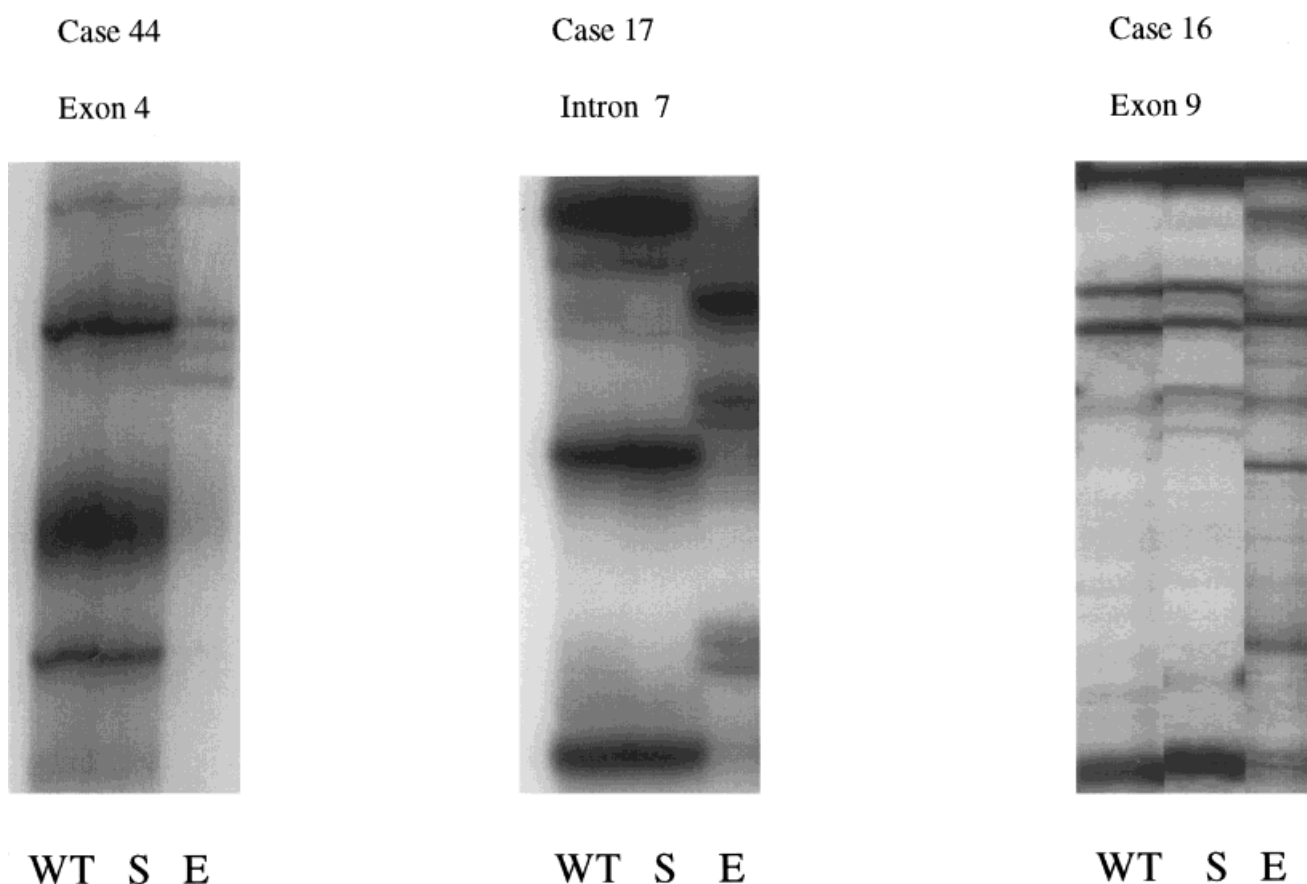


FIGURE 2 – Representative SSCP gels of exon 4 (case 44), intron 7 (case 17), and exon 9 (case 16) showing DNA that had been extracted from epithelial cells (E), from corresponding stromal cells (S), and the appropriate negative control (WT). The DNA from the epithelial cells shows a different band pattern than the corresponding stromal and negative control DNA.

1997). Changes in p53 might contribute to carcinogenesis by conferring a proliferative advantage to cells with or without abnormal DNA and/or by facilitating the accumulation of additional genetic changes, for example by allowing aneuploidy and genetic instability to occur (Shackney and Shankey, 1997). To date, it is not known at which stage in the carcinogenic process p53 abnormalities develop (Phillips *et al.*, 1999).

Our results demonstrate that p53 gene alterations can be detected in breast tissue that is either normal or shows changes of

benign breast disease. p53 changes were found more commonly in tissue that showed p53 protein accumulation (positive immunostaining) than in tissue that did not. All of the changes detected were of the transition type. This is in keeping with experimental data showing that DNA proofreading corrects transversions more efficiently than transitions (Schaaper, 1993).

There have been 3 other reports of p53 gene analysis in normal or benign breast tissue. Millikan *et al.* (1995) detected p53 point mutations in 5 of 60 paraffin-embedded breast samples. Two of the

TABLE IV – SUMMARY OF ANALYSES OF P53 IMMUNOHISTOCHEMICALLY DETECTED PROTEIN ACCUMULATION AND GENE CHANGES ACCORDING TO HISTOLOGICAL FEATURES

Histology	Number of samples	Number of samples		Number of samples		Number of samples	
		I ⁺	M ⁺ ²	I ⁺	M ⁻	I ⁻	M ⁺
Normal	2	2		0		0	
FCC ³	22	9		5		2	6
Adenosis ⁴	8	1		4		1	2
Hyperplasia ⁵	8	3		2		1	2
Fibroadenoma	2	1		0		0	1
Total	42	16		11		4	11

¹I+, immunopositive; I-, immunonegative. ²M+, gene change present; M-, gene change absent. ³FCC, fibrocystic change. ⁴Adenosis, adenosis ± FCC ± fibrosis. ⁵Hyperplasia, either mild, moderate, or florid.

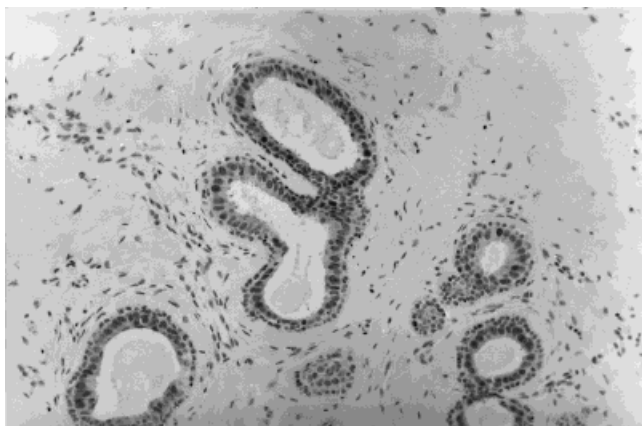


FIGURE 3 – Photomicrograph of normal breast ducts showing p53 immunopositivity (immunoperoxidase with hematoxylin counterstain, magnification 160×).

mutations occurred within the 14 cases that were immunopositive for p53 and the other 3 occurred in the 46 immunonegative cases. All of the mutations were transition types, and 3 resulted in an amino acid change. In contrast to our study, their analysis involved only exons 4 to 8, which may provide a partial explanation for the lower frequency of mutations in their study. Lisboa *et al.* (1997) detected a p53 mutation in one of 13 cases of normal or benign breast tissue examined. However, they examined only exons 5 to 8 inclusive and did not perform microdissection and so may have missed mutation(s) present in only a small number of cells. Done *et al.* (1998) identified 7 cases of breast cancer from which they were able to microdissect 41 foci of surrounding normal epithelium or epithelium showing changes of benign breast disease. The p53 gene analysis was performed on DNA extracted from paraffin-embedded tissue. They did not detect any mutations, but their study was based on a small number of cases and only exons 4 to 8 were studied.

In this study, sequence changes occurred overall in 59.2% (16/27) of p53 immunopositive samples. p53 mutations occurred in 33% (9/27) of immunopositive cases. Although this value may appear low, it is in keeping with the findings of several studies of breast cancer, which have examined the correlation between immunostaining and the presence of mutations detected by sequencing. In those studies, 20% (Dunn *et al.*, 1993) to 70% (Visscher *et al.*, 1996) of immunopositive breast cancers showed mutations. The relatively low value that we observed may in part reflect the fact that we considered the presence of any p53 immunopositivity to represent a positive case, whereas it has been suggested by others that only cases showing immunopositivity in greater than 5% of cells should be considered to have p53 protein accumulation (Clausen, 1998). It is also possible that more mutations may have been identified if the entire coding region (exons 2 to 11) and not

just exons 4 to 10 had been sequenced. Alternatively, the p53 protein accumulation may be due to mechanisms other than p53 mutation. Four (26.7%) of our p53 immunonegative cases showed gene alterations, one of which was a mutation. It is not surprising that p53 changes were detected in the absence of positive immunostaining as it is well accepted that not all p53 mutations will result in immunohistochemically detectable p53 protein (Sjögren *et al.*, 1996; Visscher *et al.*, 1996).

Several features of our study suggest that the mutations that were detected were real and were not artifacts of the methodology used to detect them. It has been shown that PCR-induced sequence changes can be minimized if the proteinase digestion time of the tissue is sufficiently prolonged (at least 48 hr), the products generated by PCR are relatively small (Shiao *et al.*, 1997), and enough DNA template is used (Krawczak *et al.*, 1989). In our study, the tissue was digested for at least 48 hr and the products were all less than 300 bp in size. Although we were unable to quantify the amount of DNA in each analysis, a fixed cycle number was used in the PCR for each exon of all samples and it was not increased if the product was undetectable. Secondly, repeat PCR-SSCP analysis showed that the band shifts were reproducible. In the one case where it was not reproducible, the analysis had been done on DNA extracted from a different section and it is likely that the area with the p53 change was no longer present. The DNA in the abnormal SSCP band was sequenced in both directions to ensure that the sequence change was not a PCR-induced artifact and in each case the same mutation(s) was detected. Thirdly, DNA from stromal tissue showed wild-type p53 sequences in 18 of 20 cases. The other 2 cases (29 and 34) had the same mutation in both epithelial and stromal DNA. For these samples, the changes might represent inadvertent microdissection of some epithelial cells with the stromal tissue or a true germline mutation or a polymorphism. We consider it more likely that the change detected in these two is a polymorphism because it has been detected in approximately 4% of tumours in a breast cancer tumour bank (data not shown). Fourthly, we were able to detect a known polymorphism in 2 other cases. Finally, other studies, such as that of Nadji *et al.* (1996), have shown that DNA extracted from paraffin-embedded tissue will show p53 gene changes identical to those detected in frozen tissue, suggesting that paraffin-embedding does not induce gene mutations and that tissue processed in this way is suitable for DNA analysis. Eight (34.8%) of the 23 gene changes detected were silent. Strauss (1997) predicted that approximately 25% of mutations in a dataset will be silent if mutagenesis is random and if the silent mutation does not provide a selective advantage. Although the significance of silent mutations is not known, it is possible that they could have effects on DNA.

In conclusion, the results of this study suggest that p53 mutations can be detected in normal epithelium and benign breast tissue. This observation is in keeping with the findings of other studies demonstrating genetic changes such as loss of heterozygosity (LOH) and microsatellite instability in normal and benign breast tissue (Deng *et al.*, 1996; Lakhani *et al.*, 1996; O'Connell *et al.*, 1998; Larson *et al.*, 1998). However, the significance of p53

mutations and other sequence changes in these tissue types is unknown. For example, a study showed that genetic changes such as LOH and microsatellite instability may not correlate with the development of breast cancer (Kasami *et al.*, 1997). For skin, however, it has been suggested that p53 mutations may provide information about subsequent risk of developing nonmelanoma skin cancer (Ouhitit *et al.*, 1998). Resolution of the role of p53 gene alterations in the pathogenesis of breast cancer may require long-

term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations and assessment of the functional significance of the mutations.

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