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p53 Expression in Cytologic Specimens from Benign and Malignant Breast Lesions

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Abstract. *This study was undertaken to determine the expression of p53 gene in cytologic specimens from benign and malignant breast lesions. To detect p53 an immunocytochemical assay with p53 (pAb421) monoclonal antibody was used. Abnormalities in p53 expression were found in 19 out of 40 Fine Needle Aspiration (FNA) smears with infiltrating ductal breast carcinomas. Benign epithelial breast cells obtained from fibroadenomas, fibrocystic disease and smears from nipple discharge reacted negatively for p53 in 38 out of 39 cases. Moderate positive reaction, confined to a few clusters of epithelial cells, was observed in one smear of fibroadenoma with cellularity. The results recorded in this study show that no significant association was found between p53 staining and stage of disease, tumor size or nodal status and that the immunocytochemical assay represents a simple method for the detection of p53 associated proteins in breast lesions.*

Breast cancer is a common disease in women in developed countries. Expression of several oncogenes including c-*ras* and c-*myc* is abnormal in breast cancer (1, 2, 3) and allele loss at chromosome 17p region has frequently also been seen (4). In the effort to understand oncogenic mechanisms, interest has recently been focused on the role of recessive oncogenes (also called tumor or onco-suppressor genes) which control the growth of normal cells and whose absence or neutralization leads to cell transformation (5). The human p53 gene lies in the 17p chromosome region, a site of allele loss in many human cancers (6, 7), suggesting that this region is the site of an important onco-suppressor gene. p53 is a nuclear 375-amino-acid phosphoprotein which was first discovered in 1979 because it formed a tight complex with the major transforming protein of the SV40 virus, the large T-antigen (8, 9).

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Normal p53 acts as an onco-suppressor gene in murine model systems (10) but various point mutations within the coding region of the murine gene can convert it from a recessive to a dominant oncogene (11). The mechanism of mutant p53 action may involve changing aminoacid residues which have been highly conserved in evolution (12), binding to and neutralizing the normal p53 (13), and may depend on the level of expression of the remaining normal p53 gene (14). Several methods have been used to assess p53 mutations in human tumors but assessing p53 at a protein level would be the best approach, since mutations in p53 often increase the half life of the mutant protein which can be detected by immunoprecipitation or immunocytochemical methods (15).

In the present study an immunocytochemical assay was carried out using p53 pAb421, a mouse monoclonal antibody reactive with mammalian p53 oncogene encoded proteins, to evaluate expression of mutant p53 in FNA cytologic specimens from human breast carcinomas and other non-malignant breast lesions and to correlate p53 protein abnormalities with histologic type, tumor and nodal stage of breast carcinoma cases.

Materials and Methods

Routine diagnostic cytologic specimens from Fine Needle Aspirations (FNA) of 40 breast carcinomas, 12 fibroadenomas, 8 cases with fibrocystic disease and 19 smears from nipple discharge were used in this study. Carcinoma cells were observed in the 40 FNA smears from breast carcinomas (malignant smears). The smears from fibroadenomas, fibrocystic disease and nipple discharge were reported by cytologic studies to be negative for cancer cells (benign smears).

All cytologic diagnoses made in FNA cytologic preparations, except for 19 smears of nipple discharges, were confirmed by histologic studies.

The tumors were classified according to the WHO classification of breast tumors (16). Thirty-seven of the carcinomas were of ductal origin and 3 lobular.

The smears were originally fixed in 90% ethanol and stained with Papanicolaou and Giemsa stain (air dried smears) for routine cytological evaluation and diagnoses (benign or malignant). The performance of immunocytochemistry in cytologic specimens for the study of oncogene expression has been previously described (17). Prior to immunocytochemical staining, the smears were immersed overnight in xylene, rehydrated

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Table I. *Summary of Results of Immunocytochemical Assay with p53 (pAb421) Monoclonal Antibody in 60 Breast FNAs and 19 Nipple Discharge Smears.*

Histology	No. of cases	Staining Intensity (-)	(+)	(++)	(+++)
Carcinomas	40				
Ductal		18	11	7	1
Lobular		3	–	–	–
Fibroadenomas	12				
Simple		9	–	–	–
Hyperplastic		2	1	–	–
Cystic Disease	8				
Simple		7	–	–	–
Complex		1	–	–	–
Nipple Discharge	19	19	–	–	–
Total	79				

Table II. *Correlations between p53 staining and the histopathology of the fine needle aspirations of breast cancer patients.*

Histopathology	Total No.	p53 staining Negative No.	Positive No.	
Carcinomas	40	21	19	*p=0.01 SD
Fibroadenomas	12	11	1	
Cystis Disease	8	8	0	
Nipple Discharge	19	19	0	

*Fisher exact T test.

and washed in 0.1 mol/l of Tris-HCl, pH 7.6. For immunostaining, the slides were treated with the mouse anti-p53 monoclonal antibody pAb421 (from Cambridge Bioscience) dissolved in 5 volumes of PBS buffer and 5 volumes of bovine serum albumin 1% in DDH_2O for 1h at 37°C in a humidified atmosphere. The slides were treated sequentially as follows: they were washed twice with PBS for 5 min each, treated with rabbit anti-mouse IgG conjugated with peroxidase (p260 from DAKO) 1:10 in 5 volumes PBS and 5 volumes normal human serum, incubated for 30 min at 37°C in a humidified atmosphere, washed twice with PBS for 5 min each, treated with swine anti-rabbit IgG conjugated with peroxidase (p217 from DAKO) 1:10 in 5 volumes PBS and 5 volumes normal human serum for 30 min at 37°C in a humidified atmosphere and washed twice with PBS for 5 min each. For localization of the primary antibody 1 mg/ml of 3,3'-Diaminobenzidine tetrahydrochloride solution was used. Smears were developed for 10 minutes at room temperature and then counter-stained with Harris Hematoxylin.

Two cell lines were used as control for immunostaining: the spontaneously immortalized rat 208F cells were used as negative controls for p53 expression and their transfected derivative RFV53HO6-3 cells, which carry the mutant mouse p53 gene carrying valine instead of alanine at amino acid 135, were used as positive controls. RFV53HO6-3 cells were derived after co-transfecting with the plasmid LTRp53cG-val containing the mutant p53 gene (10) and Homer-6 (18).

Two investigators evaluated the epithelial cell staining of approximately 300 cells (ten optic fields) in each smear. Each lesion was classified according to the proportion of the cells stained; (-) no antigen detected; +, ++, +++ when the median proportion of positive cells was 5-25%, 26-50% and 51-100% respectively.

Results

The results are summarized in Table I. The mouse monoclonal antibody p53 (pAb421) was used to stain 79 cytologic smears derived from benign and malignant breast lesions. Negative and positive cell line controls for p53 staining are shown in Figures 1 and 2. Benign smears did not stain for p53. In one case of hyperplastic fibroadenoma with cellularity, a few clusters of benign epithelial cells showed a moderate, diffuse positive cytoplasmic reaction (Figures 3, 4). In the 40 specimens of breast carcinoma 19 (48%) were positive (either +, ++ or +++) for p53 expression (Figures 5, 6, 7). Although p53 is located in the nucleus of the cell, the positive staining reaction was seen as a diffuse cytoplasmic stain.

The intensity of reaction demonstrated considerable heterogeneity from weak to intense staining within the same smear. p53 Negative staining cancer cells are shown in Figure 8. Cellular debris present in the background of malignant smears exhibited a weak positive reaction. Macrophages and foam cells in the cases of cystic disease and nipple discharge occasionally showed diffuse positive cytoplasmic stain. We found that a significantly higher number of carcinomas (19 out of 40) had positive p53 staining compared with fibroadenomas (1 out of 12), $P<0.05$ (Table II).

Positive immunostaining reaction was found in all histologic types of infiltrating duct carcinomas but no positive reaction was seen in the 3 lobular carcinomas (Table III).

Correlations between increased p53 protein expression and clinical features (19, 20) of the 40 breast carcinomas were sought (Table IV). The median age of the patients with negative p53 staining was 56 (n=21, range 43-73) and median age of patients with positive p53 staining was 50 (n=19, range 25-75). No significant association was found between p53 staining and stage of disease, tumor size or nodal status (Table V).

Figure 1. *Negative control rat 208F cells for p53 protein (× 480).*

Figure 2. *Positive control RFV53HO6-3 cells for p53 protein (× 480).*

Figure 3. *Benign fibroadenoma cells with negative immunostaining for p53 protein (× 480).*

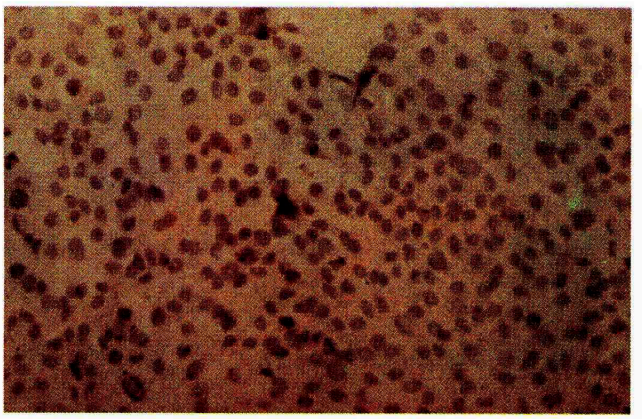
Figure 4. *Benign fibroadenoma cells with positive (+) immunostaining for p53 protein (× 480).*

Figure 5. *Positive (+) immunostaining in breast carcinoma cells for p53 protein (× 480).*

Figure 6. *Moderate (++) staining of the p53 protein in breast carcinoma cells (× 480).*

Figure 7. *Intense (+++) staining of the p53 protein in breast carcinoma cells (× 480).*

Figure 8. *Negative (-/+) immunostaining in breast carcinoma cells for p53 protein (× 480).*



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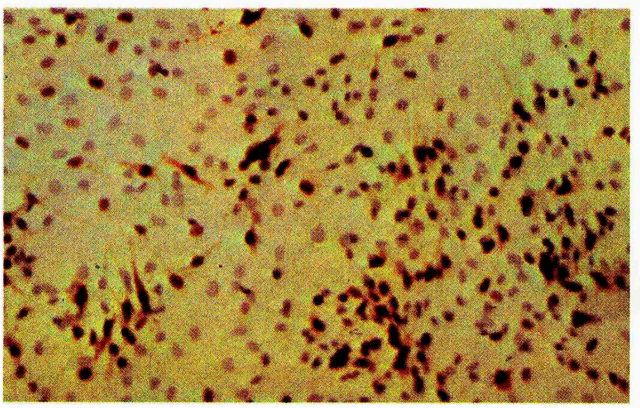

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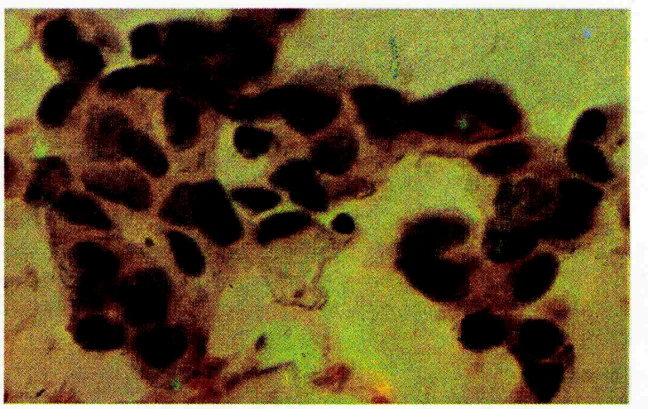

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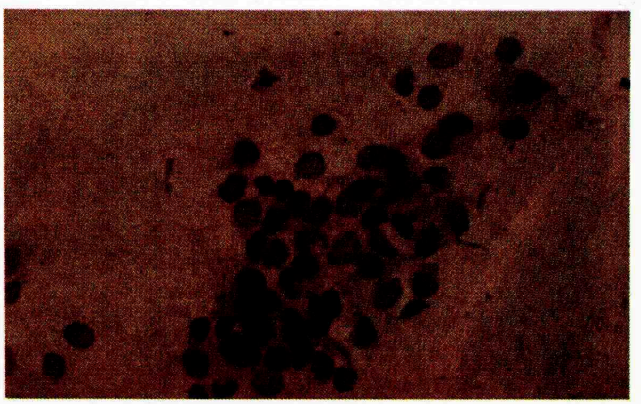

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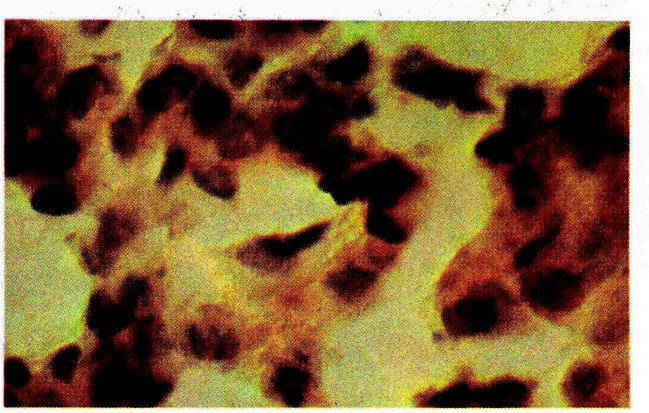

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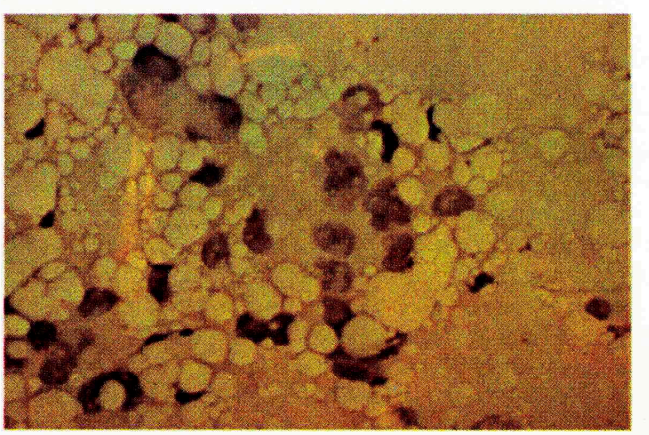

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Figures 1- 8

Table III. *Correlation of p53 protein expression and pathologic type of 40 breast carcinomas.*

Histologic Type	No. of cases	Staining Intensity (-)	(+)	(++)	(+++)
Infiltrating duct Carcinoma NOS*	24	13	7	4	–
Medullary	6	3	–	2	1
Scirrhous	4	2	–	2	–
Papillary	2	–	1	1	–
Mucinous	1	–	1	–	–
Lobular Carcinoma	3	3	–	–	–
Total	40	21	9	9	1

*NOS = not otherwise specified

Table IV. *Correlation between clinical features and p53 protein expression in breast carcinoma cases.*

Clinical Features	No. of cases	Staining Intensity (-)	(+)	(++)	(+++)
Mean Age±SD	40	56±8.4	54±11	51±12	75
Clinico-Pathologic Stage T (Tumor)					
T_1	9	5	3	1	–
T_2	17	10	4	3	–
T_3	14	6	2	5	1
N (Nodal) Status					
N0	19	11	6	2	–
N1	14	6	3	4	1
N2	6	4	–	2	–
N3	1	–	–	1	–

Discussion

In the present study, performed with the monoclonal antibody p53 (pAb421), we have shown that there is immunocytochemical evidence of increased p53 staining in 19 out of 40 (48%) ductal breast carcinomas, whereas only 1 out of 12 fibroadenomas was found to be positive. No positive p53 staining was found in fibrocystic disease or in cells in smears from nipple discharges. These results may indicate that mutations in the p53 gene are late events in the development of breast cancer; however, the sensitivity of this technique may not adequately reflect p53 levels in fibroadenomas, as normal and low levels of p53 are usually undetectable due to the normal protein's very short half life.

Macrophages and neutrophils accompanying malignant cells were occasionally positive for p53. It is possible that the positive staining was due to absorbed or phagocytized antigen from necrotic malignant tumor cells. Mutant p53 proteins are much more metabolically stable than the normal p53 protein and are commonly associated with proteins of the heat shock protein (hsp7) family (25). Although the normal p53 protein is always found in the nucleus, the mutant proteins are found in both cytoplasm and nucleus, perhaps because of their association with hsp 70 (26). The presence of cytoplasmic p53 staining in smears suggests that there are high levels of a form of p53 which is intrinsically abnormal.

Prior studies have demonstrated p53 mutations and elevated p53 expression in a range of human tumors (6, 7, 15, 21, 23, 24, 27, 28). Recently, eleven human breast cancer cell lines have been investigated with a panel of anti p53 monoclonal antibodies (pAb1801, pAb240 and pAb421) and abnormalities in staining were found in all the cell lines (22). It is of particular note that the p53 cDNA which was sequenced from four of the pAb240 cell lines all had missense mutations in the p53 RNA, with no detectable expression of the wild type sequence. These four cell lines, BT 20, BT 549, BT 474 and MDA-MB-231, all showed positive p53 staining for all three antibodies, thereby indicating that elevated p53 expression is synonymous with mutation (22, 29).

Table V. *p53 protein increased expression and clinical characteristics.*

Clinical Characteristic	No. of cases	p53 Protein Expression Positive	Negative No. (%)	
Stages I and II	25	10 (40)	15 (60)	p=0.18
Stages III and IV	15	9 (60)	6 (40)	[NS]
T_1 (<2cm)	9	4 (45)	5 (55)	p=0.56
T_2 and T_3 (>2cm)	31	15 (48)	16 (52)	[NS]
N0	19	8 (42)	11 (58)	p=0.36
N1 and N2, N3	21	11 (52)	10 (48)	[NS]

In a previous study using the p53 pAb421 antibody, 31 out of 200 (16%) breast cancer specimens were found to have positive staining. However, 46% of a subgroup of 88 patients gave positive staining with pAb1801 (23). These authors demonstrated an association between p53 positive staining in breast cancer and positive oestrogen receptor and epidermal growth factor status, as well as high grade tumor. Our results do not demonstrate any correlation between p53 positive staining tumor stage, size and nodal status (Table V). Crawford *et al* (30) demonstrated a correlation between histological grade and p53 levels in breast cancer. They also reported increased expression of p53 in the fibroadenomas, whereas we only found 1 out of 12 with increased p53 expression.

The results presented in this paper indicate that p53 is not overexpressed in fibrocystic disease or in nipple discharges and frequently in fibroadenomas of the breast. However, as the p53 protein is overexpressed in 48% of breast carcinomas, it may be considered that mutations in the p53 gene are a late event in the development of this disease.

Moreover, our results recorded in this study indicate that

the immunocytochemical assay for mutant p53 proteins in FNA cytologic preparations represents a very simple method for the identification of genetic alterations in primary breast cancers, can be useful for preoperative evaluation and may allow the development of new diagnostic and therapeutic agents.

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