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Expression of *Ras* Rb1 and p53 Proteins in Human Breast Cancer

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Abstract. *The* ras, *Rb and p53 genes have been implicated in the development of human breast cancer. Qualitative or quantitative changes in the expression of the* ras *p21 may lead to cell transformation, and this has been previously demonstrated in breast cancer. Both the retinoblastoma protein (Rb1) and the p53 gene product appear to function as negative regulators of cell division. We have investigated the expression of* ras *p21, Rb1 and p53 proteins in human breast cancer patients immunohistochemically, and correlated the results with a range of clinical and pathological parameters.* Ras *p21 expression was elevated in 65 per cent and p53 in 23 per cent of cases. Rb1 was expressed in 58 per cent of breast cancer tissues and in 75 per cent of normal tissue. Only four patients were found to have loss of Rb1 expression and also overexpression of both p53 and ras gene products. No correlations were found between the expression of these three genes and menopausal status, histological types or tumour grade. However, a correlation was found between Rb1 loss of expression and tumour diameter (> 2cms), and no lymph node metastasis. Also, a significantly higher number of p53 staining specimens were found to be overexpressing the* ras *gene. These results suggest that all three oncogenes are most likely involved in the development of breast cancer but that their role is complex.*

The members of the *ras* family, Harvey (H), Kirsten (K) and N-*ras* genes, code for GTP binding proteins of 21000 daltons (*ras* p21). *Ras* p21 shares sequence homology with G proteins which are known to be signal transducers and they are associated with the inner surface of the plasma membrane and exhibit GTPase activity (1). Qualitative or quantitative changes in the expression of *ras* p21 may lead to cell transformation (2) and have been found to occur in breast cancer (3-5).

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The retinoblastoma gene (Rb1) encodes a family of closely related proteins of molecular weight 110Kd-115Kd (6), which are found predominantly in the nucleus (7). Little is known about its biochemical function, apart from its ability to bind to DNA (8). The Rb gene causes malignant transformation of cells by mutation; however, Rb may also be inactivated by binding to the adenovirus EIA protein or to the SV40 large T antigen, and therefore leads to its classification as a tumour suppressor gene. Structural changes of the human retinoblastoma gene have been demonstrated in retinoblastoma and also in some clinically related tumors, including osteosarcomas (9). Alterations of the retinoblastoma gene have also been found in human breast and lung carcinoma (10-12) and suppression of the malignant phenotype by transferring the RB gene has been achieved (13).

P53 is a nuclear phosphoprotein that was first recognised as a host cell protein bound to the large T tumour antigen of the SV40 tumour virus (14, 15). It is a cellular protein expressed at low levels in non-transformed cells; however, elevated levels are found in certain tumours and in transformed cell lines (16-20). Originally p53 was classified as an oncogene; however, a compelling body of evidence has now been published which demonstrates that p53 is a tumour suppressor gene (21, 22). p53 negatively regulates the cell cycle and loss of function mutations are required for tumour formation (21, 23).

Both the Rb and the p53 gene products appear to negatively regulate cell growth and division. However, mutant p53 gene products appear to gain an ability to stimulate cell division, whereas the Rb gene does not have this extra function. In normal cells the p53 gene product is practically undetectable by immunohistochemical techniques due to the protein's short half life (6-20 minutes), whereas the mutant protein's half life is up to 8 hours (14), and thus it may be inferred that detection of the p53 protein is synonymous with mutant forms of p53.

In the present study we have investigated the expression of *ras* p21, RB1 and p53 proteins in human breast cancer and found evidence for their involvement in this type of malignancy.

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Materials and Methods

Tissue. Tissues were obtained from 100 female breast cancer patients at the "H. Venizelou" Hospital, Athens, Greece. All tissues were fixed in 10% neutral formalin and embedded in paraffin. The diagnosis, including histological classification and stage grouping, was made according to Azzopardi (24). The grading of these tumours was carried out according to Bloom and Richardson (25) (Elston's modification).

Antibodies and immunohistochemistry. The rat monoclonal antibody Y13-259 (26) recognising the *ras* p21 proteins was prepared from the rat hybridoma cell line as previously described (27). The p53 pAB421 monoclonal antibody was purchased from Oncogene Science. A polyclonal antibody to the human Rb gene product (Rb1) was obtained from P. Gallimore. This antibody was produced in rabbit by immunisation with a fusion protein comprising part of Rb1, together with the *E. coli* ß-gal sequence (6).

Immunohistochemical analysis of the *ras* p21 protein was undertaken using the *ras* p21 monoclonal antibody. Tissue sections 5 µm thick were mounted on slides and deparaffinised. Endogenous peroxidase activity was blocked by immersing the sections for 30 min in an aqueous solution of 3% H_2O_2 in the dark. The sections were washed with PBS and treated with the Y13-259 rat monoclonal antibody, goat anti-rat IgG, streptavidin-peroxidase and DAB sequentially as previously described (27).

The immunohistochemical analysis of the Rb1 monoclonal was undertaken using the rabbit polyclonal antibody against the human Rb1 protein (kindly provided by Professor P Galimore). Tissue sections 5 µM thick were mounted on slides and deparaffinised. Endogenous peroxidase activity was blocked by immersing the sections for 30 min in an aqueous solution of 3% H_2O_2 in th dark. The sections were washed with PBS and treated with human serum PBS (1:9) at room temperature for 10 minutes. Then the sections were washed with PBS and treated with the rat antibody against the Rb1 protein (1:10.000), diluted in human serum-PBS (1:9) for 1 hour at 37°C, washed twice with PBS for 5 minutes each, treated with goat anti-rabbit IgG, diluted in human serum-PBS (1:9), at a dilution of 1:100) for 30 minutes at 37°C, washed twice with PBS for 5 minutes each, and then treated with streptavidin-peroxidase, diluted in PBS (at a dilution 1:200) for 15 minutes at 37°C. For localization of the primary antibody 1mg/ml of 3,3′-Diaminobenzidine tetrahydrochloride solution was used. The sections were developed for 10 min at room temperature and then counterstained with Harris Hemotoxylin.

Immunohistochemical analysis of the 53 protein was undertaken using the mouse anti-p53 monoclonal antibody pAb421 (from Oncogene Science). Tissue sections 5 µm thick were mounted on slides and deparaffinised. Endogenous peroxidase activity was blocked by immersing the sections for 30 min in an aqueous solution of 3% H_2O_2 in the dark. The sections were washed with PBS and treated with the mouse anti-p53 monoclonal antibody pAB421 dissolved in 5 volumes of PBS buffer and 5 volumes of bovine serum albumin 1% in ddH_2O for 1 h 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 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 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. The sections were developed for 10 min at room temperature and then counterstained with Harris Hemotoxylin.

Two cell lines were used as control for p53 immunohistochemistry: the spontaneously immortalised 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 (21) and Homer-6 (28). The immunolabelled sections were scored as (−/+) equivocal; (+) moderate; (++) intense staining.

The chi-square and Fishers exact T tests were used to assess relationship between *ras*, Rb1 and p53 expression and the other clinicopathological parameters. Also the weighted logistic regression analysis (29) was used to assess whether there was an interrelationship between the Rb1, p53 and *ras* gene products. In a number of patient cases not all of the clinical data were available.

Results

We have investigated Rb1 p53 and *ras* p21 expression in 100, 85 and 75 breast cancer tumours respectively. Sixty-six per cent of the patients were post-menopausal (cut off > 50 years). Over eighty percent of the tumours were infiltrating ductal breast carcinomas, reflecting that these are the most frequent histological type of breast tumour, with 11% infiltrating lobular, and representative specimens of *in situ* ductal, mucinous, papillary and mixed types. The Bloom's grade of the invasive ductal carcinomas assessed at the time of presentation, were found to be 11/93 (12%) Grade I; 66/93 (71%) Grade II; and 16/93 (17%) Grade III. The clinical data on lymph node status were available for 89 of these patients: 39 per cent had no metastatic nodes, whereas 61 per cent had one or more positive lymph nodes.

The *ras* p21 monoclonal antibody Y13-259 showed good cytoplasmic staining in the positive stained tumour cells (Figures 1-3). The monoclonal against p53 (PAb 421) showed mainly diffuse nuclear staining in a large number of the neoplastic cells in the positive stained tumours (Figures 4, 5). The Rb1 gene product was identified in the cytoplasm of a large number of the normal breast tissues studied, although in 26 of the 92 tumour specimens examined, RB1 staining was not found in the adjacent normal tissue (Figures 6, 7).

In this study the Rb staining data of the tumour specimens were based on the results of both the tumour and its adjacent normal breast tissue, where normal cases were available. The data were analysed along the following lines: 1) In tumour tissue where the Rb gene has been "mutated" or "deleted", resulting in loss of function and where no staining was seen, the tumour data fell into two subgroups, Rb_T-Rb_N- and Rb_T-Rb_N+ (Rb_T = tumour tissue; Rb_N + normal tissue). 2) Where there was no loss of Rb function in either the tumour or in the normal tissue specimen, *i.e.* Rb_T+ Rb_N+, and a normal positive staining pattern was seen. Forty-one per cent of the breast cancer patients analysed had lost the Rb1 gene product as assessed by immunohistochemical staining.

Ras p21 expression was elevated in 63% of the breast carcinomas investigated. No correlations were found between elevated *ras* p21 expression and age, cancer type, tumour diameter, grade, progesterone status or lymph node metastasis (Tables I-VII). However, a correlation was found between *ras* p21 overexpression and positive oestrogen receptor status ($P < 0.05$), Table V. p53 expression was elevated in (20/85) 23 per cent of the breast carcinomas in this study. No correlations were found between p53 staining and the pa-

tient's age cancer type, tumour diameter, grade, hormonal status or lymph node metastasis. The retinoblastoma gene product was lost in 41 per cent of the breast tumours and no correlations were found between the loss of Rb1 function and age, tumour grade or hormonal status. However, on analysing the tumour size in relation to Rb1 expression, we found that tumours of less than 2cm in diameter rarely lost the Rb1 function (5 of 24), whereas the tumours greater than 2 cm in diameter (34 of 75) had lost Rb1 expression ($P < 0.05$) (Table III a, b). On examining Rb expression in relation to lymph node status, a higher incidence of lymph node metastasis was found in the patients whose specimens express the Rb1 gene than in those that have lost the Rb1 gene function ($P < 0.05$) (Table VII).

We also assessed whether there was any relationship between the *ras,* Rb1 and p53 gene products in the study. It is of note that of the 88 breast tumours which were analysed for both Rb1 and p53 expression, 50 per cent of the tumours had either: loss of Rb1 function (23 cases); overexpression of p53 (12 cases); or had two genetic alterations, loss of Rb1 and p53 overexpression (9 cases) (Figure 8a). However, no statistical correlation was found, demonstrating that there was no correlation between these two onco-suppressor genes in this group of breast tumours. Also, no correlation was found between *ras* p21 overexpression and loss of Rb1 expression in these tumours (Figure 8b). In the 75 patients investigated for both Rb1 and *ras* expression, 60 patients had a genetic abnormality in one (12, Rb-; 28, ras+) or both (20, Rb-ras+) of these genes.

However, our findings do indicate an association between positive p53 and *ras* p21 overexpression. Fourteen of the sixteen positive staining p53 breast tumours were also found to be *ras* p21 positive ($P < 0.05$). (Figure 8c), although no correlations were found with any of the clinicopathological parameters when the results were grouped into the categories: ras^+ $p53^+$; ras^+ $p53^-$; ras^- $p53^+$; ras^- $p53^-$ (Table VIII).

Furthermore, only four tumours demonstrated altered gene expression in all three of the genes analysed (*i.e.* Rb-, p53+, ras+) and no association was found using the weighted regression analysis. All four these patients had infiltrating ductal carcinomas and three of them were less than 2cm in diameter and were grade two tumours.

Discussion

Previous studies have indicated that the H-*ras* oncogene may be important in the progression of breast cancer, and that K-*ras* and N-*ras* may also have a role (3). Neither amplification or rearrangements of the H-*ras* gene have been found to be important, but loss of one H-*ras* allele was linked to parameters of tumour aggressiveness and also to oestrogen status (30,32).

Elevated levels of *ras* p21 expression have been found to be associated with invasive breast tumours (33), histological grade (4, 34) menopausal status (35-36), nodal status (34) and with poor prognosis of the disease (37, 38). However, these findings have not been a consistent feature of all the aforementioned studies.

In this study we have found a correlation between *ras* p21 overexpression and positive oestrogen receptor status, but no correlation with menopausal status, cancer type, tumour size, tumour grade, progesterone status or lymph node status.

P53 expression has been previously investigated in breast tumours using a range of p53 antibodies, PAb 421, PAb 1801

Table I. *Distribution of the frequency of Rb1* ras *and p53 expression compared to menopausal status.*

		Age (years) <50	>50	N	P
Rb1	–	14	28	96	NSD
	+	20	38		
ras	–	14	22	74	NSD
	+	22	42		
p53	–	26	50	84	NSD
	+	8	16		

Menopausal status calculated on the basis of < 50 or > 50 years. N = number of patients investigated. Data shown as percentages.

P = Fishers exact T test. NSD No significant difference (P>0.05)

Immunohistochemical staining scored as described in the materials and methods. Rb-, loss of expression;
Rb+ level of staining as seen in normal breast tissue.
p53 (+ and ++) and *ras* (+ and ++) indicates elevated expression as compared to normal breast tissue. p53- and *ras*- indicates no increase in expression.

Table II. *Correlation of Rb1,* ras *and p53 with cancer type.*

		Cancer type IFD		SD	IFL	others	P*
Rb1	–	34	(34)	0	4	3	NSD
	+	47	(47)	1	9	2	
ras	–	22	(29)	1	3	2	NSD
	+	38	(51)	0	6	3	
p53	–	50	(59)	1	9	5	NSD
	+	18	(21)	0	2	0	

Percentages of infiltrating ductal breast cancers are given in parenthesis.
P* = Chi Squared
Cancer types:
IFD = infiltrating ductal
SD = *in situ* ductal
IFL = infiltrating lobular
others = mucinous; papillary; mixted types, *etc.*
Immunohistochemical staining as described in Table I.

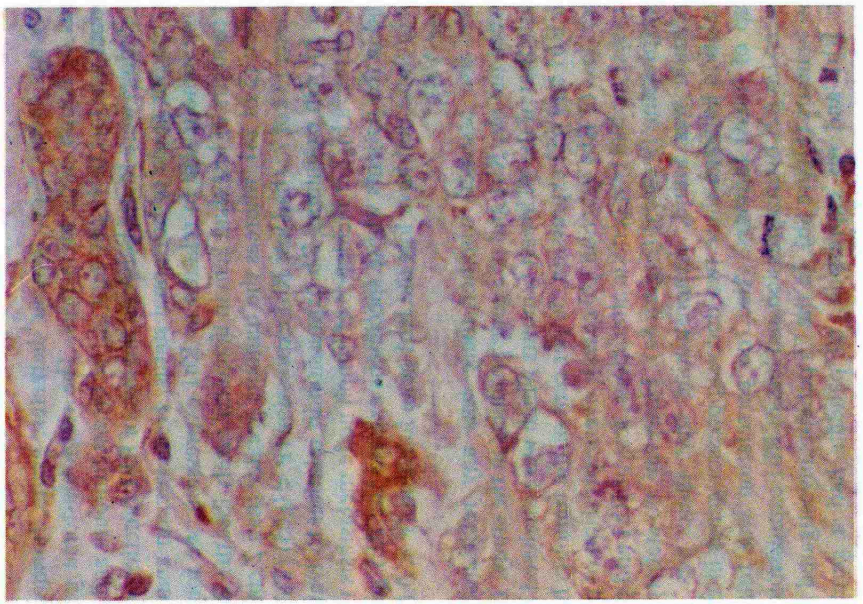

Figure 1. *Ras protein expression (++) in an infiltrating ductal carcinoma (x400).*

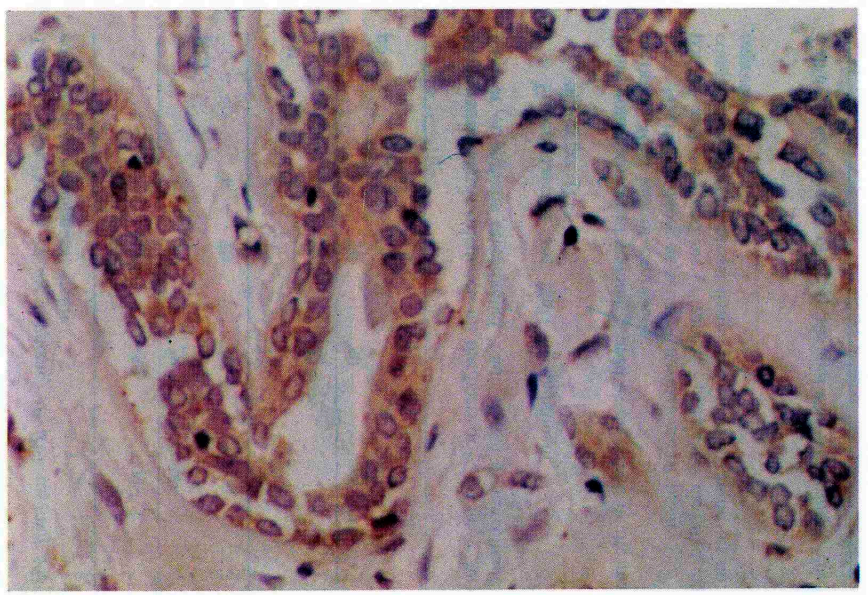

Figure 2. *RAS protein expression (++) in an infiltrating lobular carcinoma (x400).*

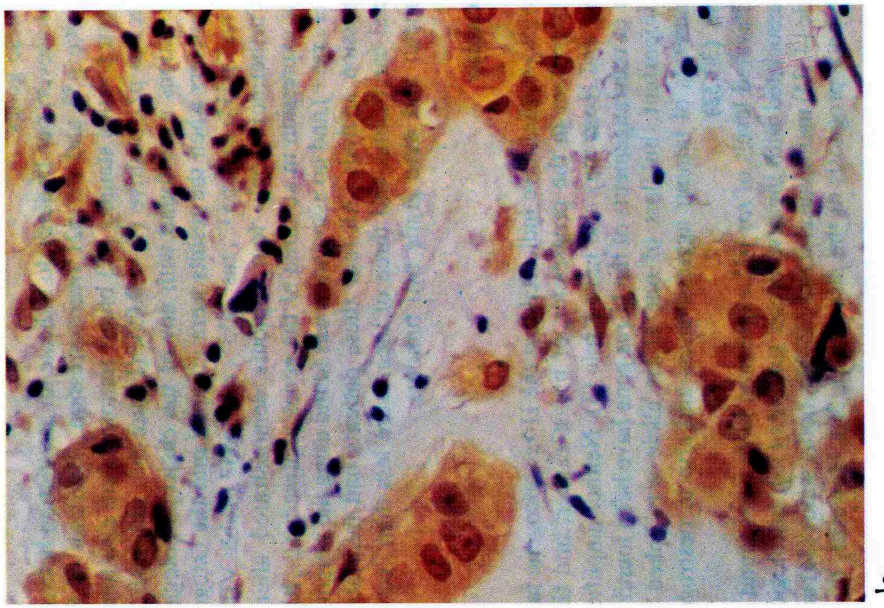

Figure 3. *RAS protein expression (++) in an* in situ *ductal carcinoma (x400).*

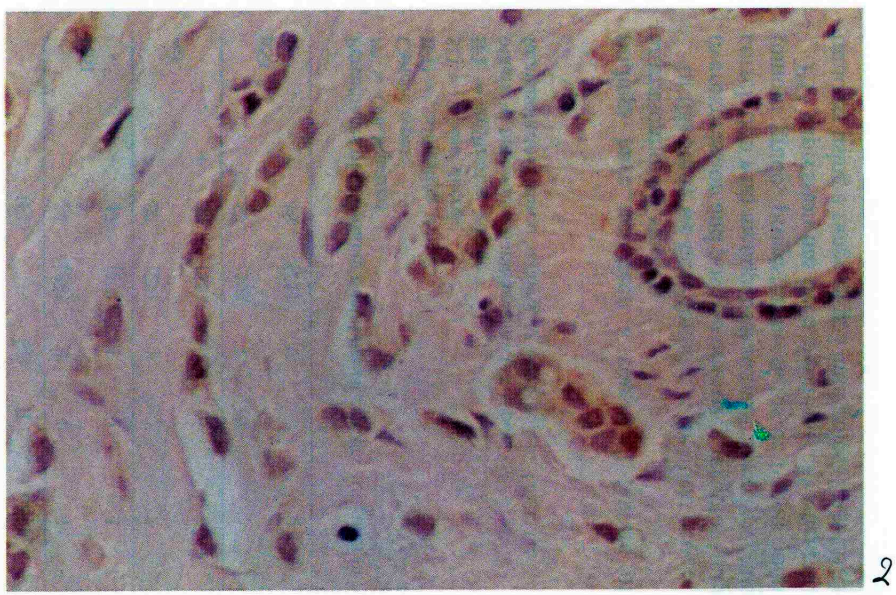

Figure 4. *p53 expression (++) in an infiltrating ductal carcinoma (x400).*

5

3

4

Figure 5. *p53 expression (++) in an* in situ *ductal carcinoma (x400).*

Figure 6. *Rb1 protein expression (++) in a part of normal breast lobule (upper right) adjacent to an infiltrating ductal breast cancer that does not express (−/+) the protein (x400).*

Figure 7. *Rb1 protein expression (++) in a normal mammary lobule (x400).*

Table III. *Correlation of Rb1* ras *and p53 expression with tumor diameter.*
(a) Patient numbers

		Tumour diameter <2	<4	<6	>6cm	P*
Rb1	–	5	21	10	3	S.D. P<0.05
	+	19	34	4	3	
ras	–	4	15	7	1	NSD
	+	11	29	3	2	
p53	–	17	37	8	2	NSD
	+	4	12	2	1	

P* = chi-squared test

(b) Percentage of patients with lesions < or > 2 cms.

		Tumour diameter <2	>2	N	P*
Rb1	–	5	34	99	S.D. P<0.05
	+	19	42		
ras	–	6	32	72	NSD
	+	15	47		
p53	–	20	57	83	NSD
	+	5	18		

P* = Exact T-test.

Immunohistochemical staining as described in Table I.

Table IV. *Distribution of the frequency of Rb1, ras and p53 expression compared to the grade of the tumour.*

		Grade I	II	III	N	P
Rb1	–	5	29	6.5	93	(NSD)
	+	6.5	42	11		
ras	–	6	22	8	72	(NSD)
	+	6	47	11		
p53	–	9	57	11	82	(NSD)
	+	2	15	6		

Immunohistochemical staining as described in Table I.

Table V. *Distribution of the frequency of Rb1, ras and p53 expression compared to oestrogen or progesterone status.*

		ER status –	+	P	PR status –	+	P*
Rb1	–	8	3	NSD	9	30	NSD
	+	12	49		11	50	
ras	–	14	23	S.D. P=0.04	6	31	NSD
	+	10	53		18	45	
p53	–	14	63	NSD	15	62	NSD
	+	7	16		5	18	

P* = Fishers Exact T-test.
ER status: oestrogen status (+=< 10 fmol/mg)
PR status: progesterone status (+=< 10 fmol/mg)
Immunohistochemical staining as described in Table I.

Table VI. *Distribution of the frequency of Rb1, ras and p53 expression compared to hormone status.*

ER/PR	Rb1 –	+	*ras* –	+	p53 –	+
ER^+ PR^-	7	5	3	11	11	1
ER^+ PR^+	23	43	20	39	53	15
ER^- PR^-	3	4	3	7	3	3
ER^- PR^+	7	8	11	6	11	3
N	93		71		81	
P*	NSD		NSD		NSD	

P*=X^2 test.
Immunohistochemical staining as described in Table I.

Table VII. *Distribution of the frequency of Rb1, ras and p53 expression compared to lymph node metastasis.*

		Lymph node metastasis No Nodes	Positive Nodes	N	P*
Rb1	-	22	18	89	P>0.05
	+	17	43		
ras	–	12	26	66	NSD
	+	26	36		
p53	–	27	49	74	NSD
	+	11	13		

P*= Exact T-test.
Immunohistochemical staining as described in Table I.

and PAb 240, using different immunohistochemical techniques (20, 39-44).

P53 expression was elevated in 23 per cent of the tumours investigated in this study using the PAb 421 antibody on paraffin embedded sections. A wide range of experimental data has been published on p53 expression in breast cancers, from 15 to 50 per cent using different antibodies under different immunohistochemical conditions, 15 per cent with PAb 421 on frozen sections (40) to 50 per cent with the same antibody with fine needle aspirations of breast carcinomas (44). Other investigators have found: 36% using the PAb 1801 antibody on frozen sections (42); over 50% with PAb 1801 and PAb 240 on frozen sections and just over 20% with methacarn-fixed sections (20); 15% with PAb 421 and 45% with PAb 1801 on frozen sections (40); 23%, using PAb 1801 with frozen and methacarn sections (45) and 40% with PAb

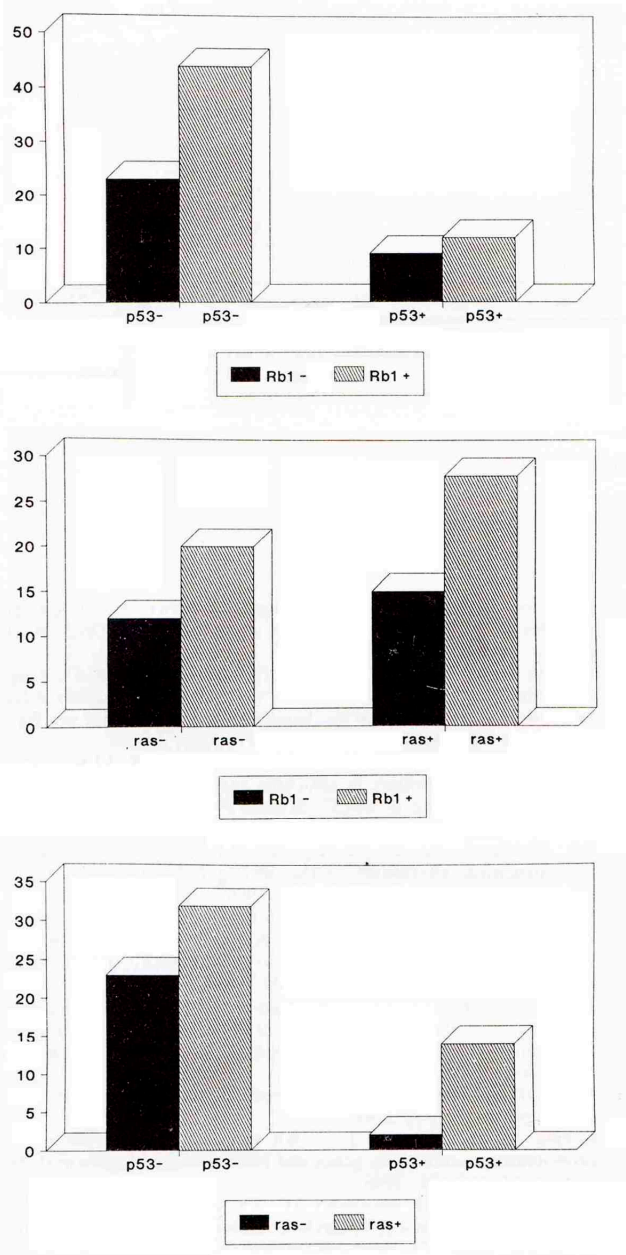

Figure 8 (a, b, c). *Interrelationship between loss of Rb1 expression and (a) p53 expression (P>0.05).*
(b) ras *expression in breast cancer tumours (P>0.05).*
(g) Interrelationship between p53 expression and ras *expression in breast cancer tumours (P<0.05).*

1801 (46); 22%, 11/49 with widespread overexpression and 24% positive nuclear staining in a few cells (46%), in acetone fixed tissue using PAb 1801 (47).

Recently, Varley *et al* (43) have investigated loss of chromosome 17p13 sequences and p53 expression (PAb 1801, PAb 240) in 51 breast carcinomas. Eighty-six percent of the breast carcinomas studied showed some form of p53 gene alteration, by loss of heterozygosity or loss of p53 expression immunohistochemically, but many of the tumours only demonstrated one of these genetic alterations. However, only 14 per cent (7/51) showed no allelic loss of 17p13 and no p53 overexpression. It is of note that Davidoff *et al* (47) found that 61 per cent of the 49 breast tumours investigated had a deletion at or near the p53 locus, but that the allelic loss did not correlate with overexpression of the p53 protein. These findings indicate that the p53 gene is probably the most frequently described genetic change in breast cancer, but that the immunohistochemical studies probably do not provide a complete picture of the p53 gene alterations in this disease.

We found no correlations between p53 expression and menopausal status, tumour size, tumour grade, hormonal status or lymph node status. Previous reports have suggested that p53 positive stained tumours are associated with oestrogen levels (40-43) and high grade tumours (40, 42). Our results indicate no correlation with progesterone status or lymph node status, which is in aggreement with Ostrowski *et al* (42) and Koutselini *et al* (44), nor with tumour type, grade or stage of the tumour, in aggreement with Varley *et al* (43) and Koutselini *et al* (44). The two studies which have assessed p53 overexpression and prognosis have found differing results (42, 43). The retinoblastoma gene has been shown to have frequent gene alterations in a number of human tumours, including sarcomas (11, 48), small cell lung cancer (10), bladder carcinoma cell lines (49), glioblastomas (50), as well as in breast carcinomas (12, 51, 52). Initial studies into Rb1 deletions in breast tumours indicated an incidence of about 7% (51,52). In a larger study, Varley *et al* (53) analysed 72 breast carcinomas and found that 17 per cent had structured abnormalities in the Rb1 gene and no alteration in benign breast lesions. Varley *et al* (12, 53) also analysed Rb1 expression with the Rb1-Ab20 antibody. Sixteen of the 56 breast tumours investigated showed variable proportions of unstained tumour cells. Furthermore, 11 of these tumours were also shown to have an alteration in the Rb1 gene. No correlation was found between either a deletion in Rb1 or loss of expression and poor prognosis. However, these authors (12), reported that there appeared to be a trend between an increase in the frequency of genetic alterations in Rb1 (*i.e.* Rb1 gene alterations, loss of Rb1 expression) and poorer differentiated tumours.

We have found a loss of Rb1 gene activity in 46% of the breast tumours we have analysed, which is a higher percentage than that reported by Varley *et al* (12). Furthermore, our results show a correlation between tumour size and loss of Rb1 expression. However, there appears to be a negative correlation between Rb1 loss of expression and positive lymph node metastasis.

No correlations were found between Rb1 loss of expression and p53 expression or *ras* expression, and only four patients had alterations in all three genes.

It has been previously reported that p53 tumour antigen and the *ras* oncogene co-operate to transform primary rat embryo fibroblasts in culture (23, 54). We have demostrated a correlation between p53 and *ras* overexpression in this

Table VIII. *Combined analysis of ras p21 and p53 expression in breast cancer patients.*

	Tumour Diam.		Grade			ER		PR		LNM	
Staining Pattern	<2 cms	>2	I	II	III	–	+	–	+	–	+
ras$^+$ p53$^+$	2	12	1	12	6	8	12	6	13	10	10
ras$^+$ p53$^-$	12	41	4	36	6	6	39	13	32	16	28
ras$^-$ p53$^+$	0	4	0	1	1	1	1	0	3	0	3
ras$^-$ p53$^-$	5	24	6	22	5	10	23	5	28	12	21
N =	78			67		67		67		61	
P =	NSD			NSD		NSD		NSD		NSD	

Tumour diameter <2, or >2 cm.
ER, oestrogen receptor status; PR, progesterone receptor status. LNM, Lymph Node Metastasis, (–) no nodes; (+) positive nodes. Immunohistochemical staining as described in Table I.

group of tumours, indicating that these two genes may co-operate in the development of a particular sub-set of breast tumours. However, our present data do not help to identify which sub-group may be involved.

We have reported here evidence for loss of Rb1 expression and over-expression of the p53 and *ras* genes in certain subgroups of breast cancer patients. We intend to analyse the expression of these genes in benign breast tumours and determine whether their aberrant expression is a feature of early tumour development. However, a more detailed analysis is required using more sensitive techniques to determine subtle alterations in these genes, such as point mutations and loss of heterozygocity which may indicate the exact incidence of genetic abnormalities in these tumours (43, 49, 55).

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