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Ras, c-*myc* and c-*erb*B-2 Oncoproteins in Human Breast Cancer

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Abstract. *The expression of* ras, c-myc *and* c-erb*B-2 oncoproteins in 100 human (73 ductal and 27 lobular) breast carcinomas has been examined using an immunohistochemical analysis. The monoclonal antibody Y13 259 has been used for the* ras *p21, the monoclonal antibody Mycl-9E10 for the* c-myc *p62 and the polyclonal antibody pAb1 (from Triton Bioscience Inc.) for the* c-erb*B-2 p185 oncoproteins. The following conclusions can be drawn from the analysis: Of the 100 breast carcinoma cases studied only 14 did not express any of the three oncogenes. The remaining 86 were positive for one or more of the three oncoproteins. Ductal carcinomas expressed oncoproteins in 92% of the cases (67/73), whereas lobular carcinomas expressed them in 70% of the cases (19/27). The most frequently expressed was* c-myc *p62 in 70% of cases followed by* ras *p21, 55% and* c-erb*B-2, 35%. Elevated expression of* ras, myc *or* erb*B-2 oncogenes did not correlate with the presence of metastasis in auxiliary lymph nodes, the numbers of infiltrated lymph nodes the grade of the tumor or hormone status. However, there appears to be a correlation between increased* ras *staining intensity and patient's age, below 50 years.*

The importance of oncogenes in the development of cancer has been suggested by a variety of studies (1). However, the details of their action during the carcinogenesis process remains to be determined (1). Overexpression of proto-oncogenes has been shown to lead to cell transformation (2-7). In particular, studies on the expression of *ras*, c-*myc* and c-*erb*B-2 proto-oncogenes in human breast cancer have suggested that these genes may play an important role in the development of this disease (8-18).

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The human *ras* genes encode for proteins of 21 kd called *ras* p21 which are highly conserved (19). *Ras* p21 proteins are homologous to G proteins, possess GTPase activity, are located in the internal part of the cytoplasmic membrane and are thought to function as signal transducers (19).

Ras genes are expressed at elevated levels in a number of benign and malignant human tumors (for review see Barbacid (19)). Previous studies employing molecular hybridization analysis (8, 9, 10), Western blotting analysis (11), liquid competition radioimmunoassay (13) or monoclonal antibodies to *ras* p21 (14, 15, 16, 17, 18) have demosntrated elevated expression of *ras* p21 in varying numbers of tumours as compared to adjacent normal tissues.

The human c-*myc* gene encodes for a protein of 62 kd called c-*myc* p62 (20) which has been found to be overexpressed in a variety of human tumours (for review see Alitalo *et al* 1987 (21)), including benign and malignant breast tumours (10, 22-26).

The human c-*erb*B-2 gene and the rat equivalent *neu* gene share homology with the closely related epidermal growth factor receptor gene (27). Both types of genes are homologous to the viral *erb*B oncogene. The c-*erb*B-2 gene encodes a 185 Kd receptor-like protein with trypsine protein kinase activity that shares homology with, but is dinstinct from, the EGF receptor (27). Amplification of the c-*erb*B-2 gene has been observed in breast cancer tissues (23, 28-31). Clinical interest in the c-*erb*B-2 gene in breast cancer was initiated by the work of Slamon *et al* (28), which suggested that ampiification of the gene was an indicator of poor prognosis in patients with positive lymph nodes at pathology. However, recent studies by Ali *et al* (32) argue for the lack of evidence for the prognostic significance of c-*erb*B-2 amplification in human breast carcinoma. *In vitro* transfection studies which showed that a 10-fold exression of the transfected c-*erb*B-2 gene in NIH3T3 cells gives rise to transformed cells (6, 7) suggesting that overexpression of this gene in breast tissue may have implications for the progression of the disease. c-*erb*B expression has been evaluated by an immunohistochemical technique in breast cancer (33-36). No correlation was found

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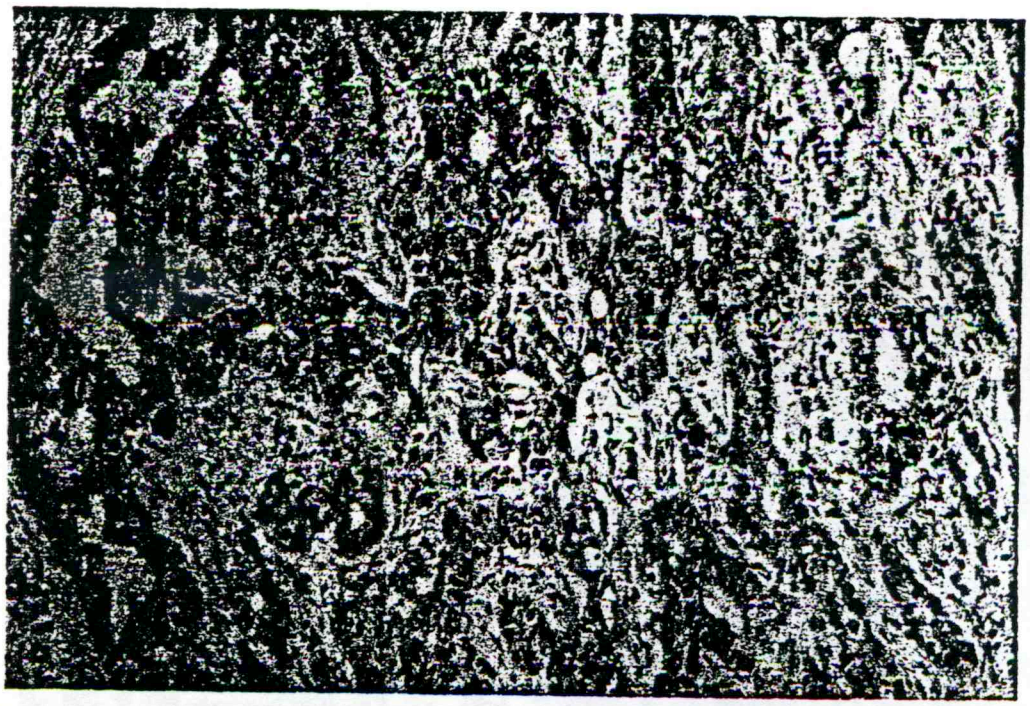

Figure 1. Ras *p21 protein in human ductal carcinoma cells grade II detected by immunohistochemical analysis with the Y13 259 monoclonal antibody (×160).*

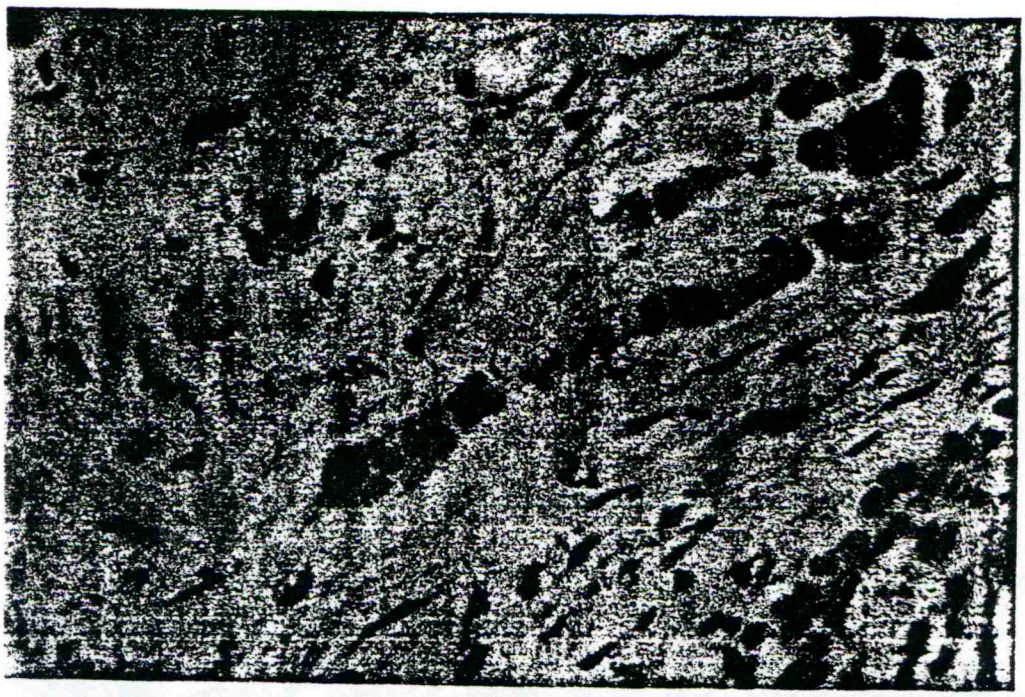

Figure 2. Ras *p21 protein in human lobular carcinoma cells grade II detected by immunohistochemical analysis with the Y13 259 monoclonal antibody (×160).*

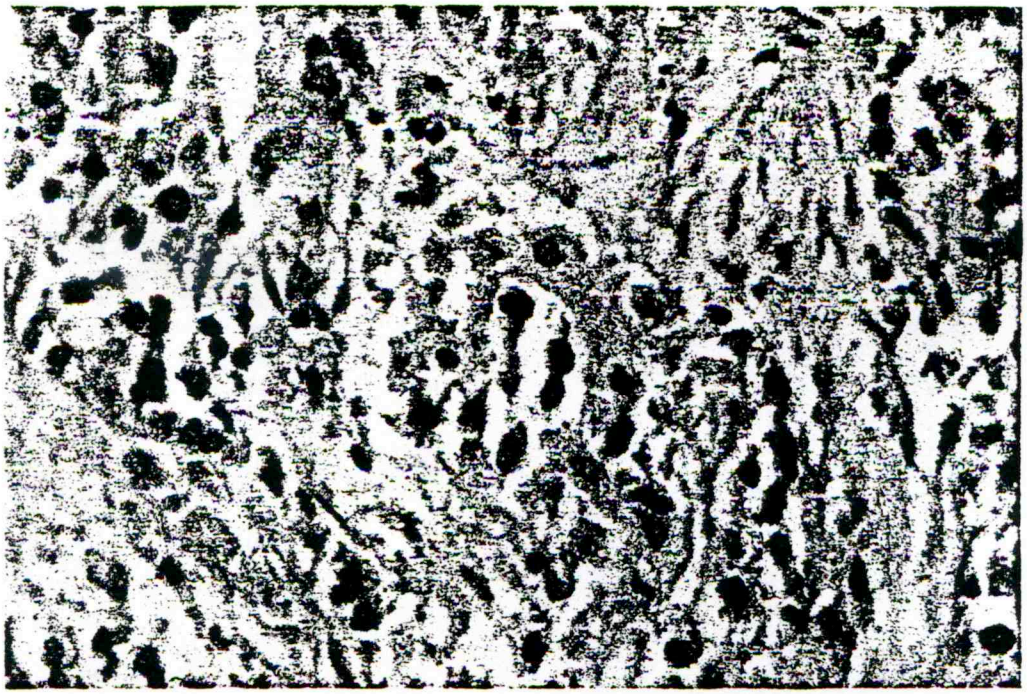

Figure 3. c-myc *p62 protein in human ductal carcinoma cells grade II detected by immunohistochemical analysis with the Myc1-9E10 monoclonal antibody (×40).*

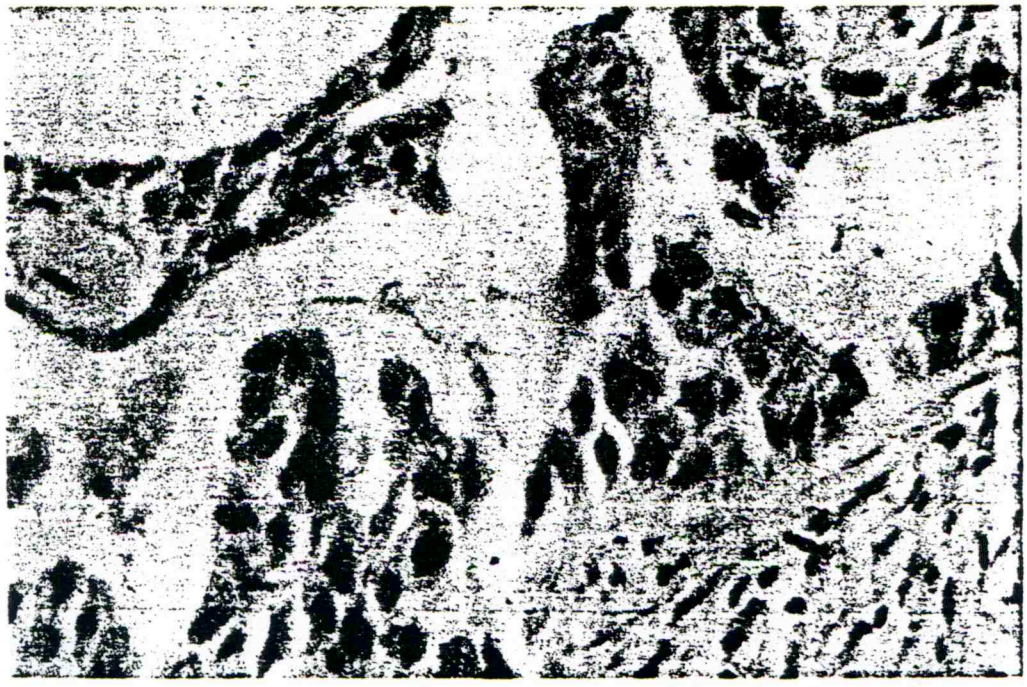

Figure 4. c-myc *p62 protein in human ductal carcinoma cels grade II detected by immunohistochemical analysis with the Myc1-9E10 monoclonal antibody (×160).*

Table I. *Clinicopathological parameters and the expression of* ras, myc, erbB-2 *oncoprotein in patients with breast cancer.*

Patient number	Age	Oestrogen status	Progesterone status	Metastasis of auxillary lymph nodes	Grade	Intensity of staining		
						ras	*myc*	*erbB-2*
1	52	24	10	2	3	1	3	1
2	65	738	1105		1	2	1	1
3	50			0	2	2	1	1
4	76	74	11	0	2	2	2	1
5				1	2	3	3	1
6	56	204	78	2	2	1	3	1
7	57			1	3	3	1	
8	75	56	37		3	2	3	1
9	59	27	40	1	2	3	3	3
10		91	10		2	2	2	1
11	59	74	91	3	2	2	3	1
12	59	11	10	17	3	2	3	1
13	45			2	1	3	3	1
14	43	10	10	3	2	3	3	3
15	73				1	2	1	3
16	34	13	17	3	2	1	1	2
17	55	110	162		2	3	1	3
18	50	18	16	2	3	1	1	3
19					1	1	1	2
20	70				1	1	2	3
21	66			1	1	2	2	2
22	68				2	1	1	2
23					3	1	3	1
24					3	1	2	1
25		10	10	3	3	2	1	3
26					1	1	2	2
27					1	1	1	1
28	50				3	2	2	1
29	43	50	487	7	2	2	2	1
30	53	17	216	12	2	1	2	1
31	59	25	10		2	1	2	2
32	44			3		1	1	1
33	62	10	10		2	1	1	1
34				2	1	1	2	1
35	56			2	1	1	2	1
36	67	300	43		1	1	1	1
37	70					2	3	1
38	49					1	1	1
39					1	1	1	1
40						1	2	1
41	85	87	1299		1	2	2	1
42	67					1	2	1
43					1	3	2	3
44					2	3	3	1
45					1	2	2	1
46	50			0	3	1	2	1
47					1	3	2	3
48	72				3	3	3	2
49	45	22	226		1	2	2	1
50				0	3	2	3	1
51		82	273		2	2	3	2
52					1	2	3	3
53					1	3	2	3
54	65	460	255		1	3	2	2
55					1	2	3	1
56	50	38	103	3	1	2	2	1
57	38	28	53	0	2	3	1	1
58				3	3	3	3	1
59	48	65	45	3	2	3	3	1
60				3	2	3	3	3
61		588	72		2	1	3	3

Continued

Table I *continued*

62					1	1	2	3
63				3	3	1	1	3
64	59	128	32		1	3	3	2
65					1	1	2	1
66		13	4		2	1	2	2
67	44					1	1	3
68	71	135	16		1	2	2	1
69					1	3	3	1
70	67	124	28		2	3	3	1
71	49	10	10		1	2	2	1
72					1	2	3	3
73					3	2	3	2
74	76				1	3	1	1
75	49				1	2	3	1
76					1	1	1	1
77	75				3	1	1	1
78	44	69	21	3	2	3	1	3
79	52			2	3	2	2	1
80	67	160	16	17	3	3	2	2
81	76				2	1	1	1
82	66	171	43		2	1	1	1
83	79				3	1	1	1
84	30	15	10	15	3	1	1	2
85	50				2	1	1	1
86	70				3	1	1	1
87	67				2	1	1	3
88	68			2	2	1	2	1
89	57				1	1	3	1
90	69			3		1	3	1
91	69	23	97	1	2	1	2	1
92	50				2	2	2	1
93	64	26	206		2	1	1	1
94	65	100	10		1	3	3	2
95				3		3	3	1
96	43					3	2	1
97	29	10	59		2	3	3	1
98				3	1	3	3	1
99	70				2	1	3	1
100				0	1	2	2	1

Patients 1-73 lobular breast carcinoma
74-100 ductal breast carcinoma
Oestrogen and Progesterone receptor status determined by the dextran-coated charcoal biochemical method in fmol/mg protein. All values above 10 fmol/mg protein are considered as positive.
Staining intensities of *ras*, *myc* and *erb-B2* oncoproteins
1 = negative or equivocal staining
2 = moderate staining
3 = intense staining
metastasis of auxiliary nodes given as 0 = none recorded at pathology.

between c-*erb*B-2 expression and survival by Gusterson *et al* (33) and Barnes *et al* (35); however, Wright *et al* (36) have reported that overexpression of this oncogene correlated with earlier relapse and shorter overall survival.

Since all three genes, *ras*, c-*myc* c-*erb*B-2, are implicated in human breast cancer we examined the expression of these genes in the same tissue in each patient and assess their clinical significance.

Materials and Methods

Primary breast carcinomas from a total of 100 patients from the Ippokration General Hospital in Athens were analysed. None of these patients had received any radiotherapy or chemotherapy prior to surgery. The tumours were classified following the WHO histological typing of breast tumors (40). They were subsequently followed up at a number of other centers and it has not been possible to collect all the clinical data pertaining to these patients. No follow-up data are included on these patients as the specimens were all taken within the last 18 months.

The following antibodies were used in our studies: for the *ras* p21 the rat monoclonal antibody Y13 259 (37), for the c-*myc* p62 the mouse monoclonal antibody *Myc1*-9E10 (38) and for the c-*erb*B-2 the rabbit anti-peptide polyclonal antibody pAb1 (Purchased from Triton Bioscence Inc). Monoclonal antibodies Y13 259 and *Myc1*9E10 were prepared from the hybridomas as previously described (24, 39).

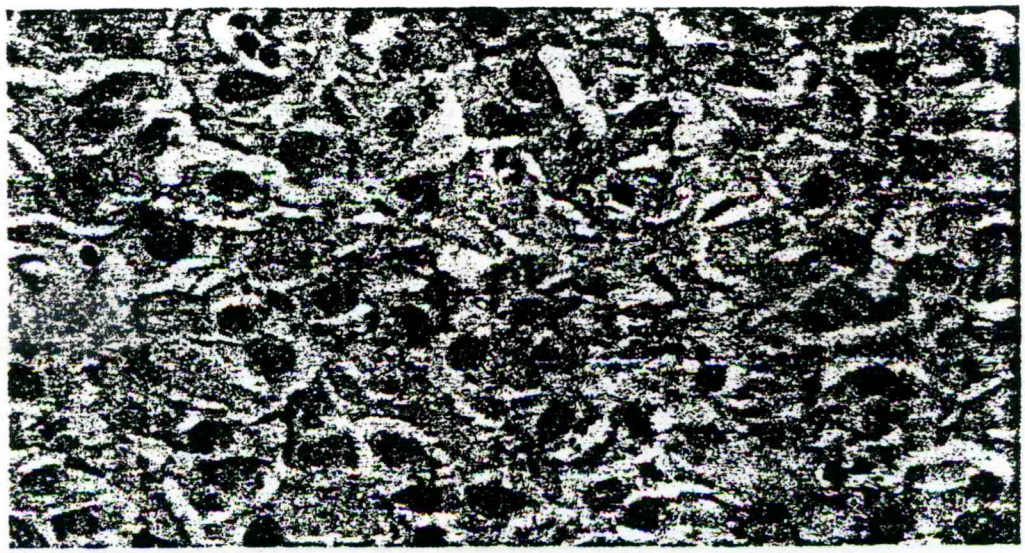

Figure 5. *C-erbB-2 protein in human ductal carcinoma cells grade II detected by immunohistochemical analysis with pAb1 polyclonal antibody (×400).*

Figure 6. *C-erb-2 protein in human lobular carcinoma cells grade II detected by immunohistochemical analysis with the pAb1 polyclonal antibody (×400).*

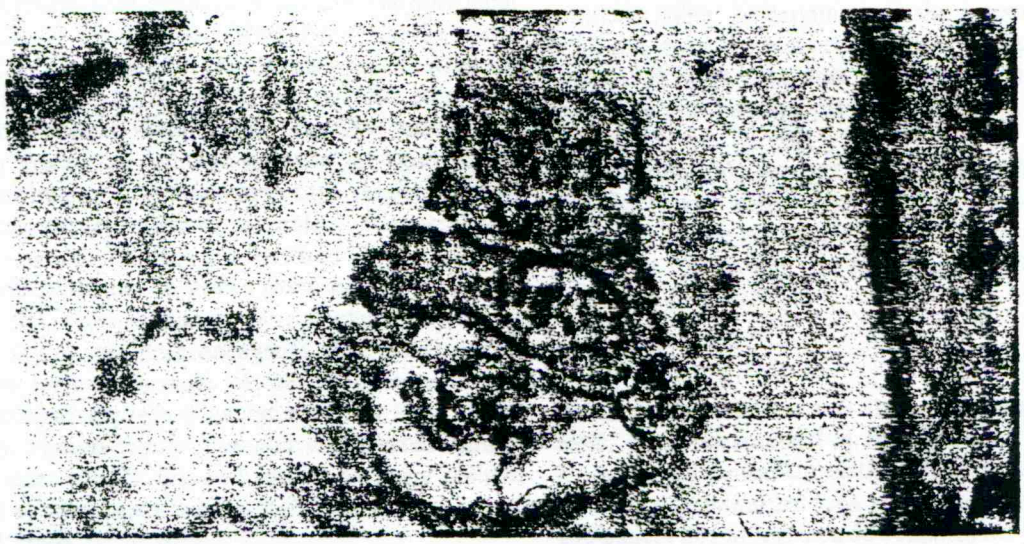

Figure 7. *C-erbB-2 protein in human breast carcinomatous cells detected by immunohistochemical analysis with the pAB1 polyclonal antibody at high magnification (×400).*

Table II. *Selected clinicopathological features and staining intensities seen for* ras, myc *and* erbB-2 *in human breast cancer patients.*

	ras		*myc*		*erbB-2*		*ras/myc*		*ras/erbB-2*		*myc/erbB-2*		*ras/myc /erbB-2*	
	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Number of patients	39	52	27	60	59	35	19	44	26	19	16	22	12	15
Grade I	12	23	8	28	22	16	5	20	8	9	6	12	4	8
Grade II	16	19	12	23	23	12	8	15	10	6	7	7	5	4
Grade III	11	10	7	14	14	7	6	9	8	4	3	3	3	3
Number of patients	27	33	24	36	44	17	15	27	20	9	14	9	10	7
Age ⩽50	3	13	6	13	15	4	3	12	1	2	3	1	8	6
Age >50	24	20	16	26	29	13	12	15	19	7	11	8	2	1
Number of patients	14	25	12	28	26	15	7	21	8	9	6	9	4	6
E^- or P^-	5	6	3	8	7	5	2	7	2	2	1	3	3	5
E^+P^+	9	19	9	20	19	10	5	14	6	7	5	6	1	1

The clinical data were not available for all of the patient specimens used in the immunohistochemical analysis of *ras*, *myc* and *erbB-2*
Staining intensity: N = negative or equivocal staining
P = positive staining
Grade I, II, III, histopathology
E^- or P^- Estrogen or Progesterone Status < 10 fmol/mg protein
E^+P^+ Estrogen and Progesterone Status > 10 fmol/mg protein

For immunostaining, paraffin tissue sections were deparaffinized and mounted on slides. Sections were washed with PBS and treated with the Y13-259 and *Myc1*-9E10 monoclonal antibodies, goat anti-rat (for the *ras* p21) or goat anti-mouse (for the c-*myc* p62), streptavidin-peroxidase and DAB sequentially as previously described (24, 39). The pAb1 antibody to the c-*erb*B-2 p185 was used according to the manufacturer's instuctions.

Results

The ability of monoclonal antibodies Y13 259 and *Myc1*-9E10 to detect enhanced levels of *ras* p21 and c-*myc* p62 respectively has been previously described (8, 9, 24, 25). The polyclonal antibody pAb1 to the c-*erb*B-2 protein has also been described (33, 34). Sections of breast tissues were analyzed for expression of the *ras* p21, c-*myc* p62 and c-*erb*B-2 protein by an immunohistological method (8, 24). The intensity of staining was graded as follows: negative or equivocal 1, moderate 2 or intense 3. A summary of the results obtained with the breast tissues is shown in Table I. Representative immunohistochemical findings are shown in Figures 1-7. The following conclusions can be drawn from the data presented here: of the 100 breast carcinoma cases studied, only 14 did not express any of the three oncogenes. The remaining 86 were positive for one or more of the three oncoproteins. Ductal carcinomas expressed oncoproteins in 92% of the cases (67/73), whereas lobular carcinomas expressed them in 70% of the cases (19/27). Grade I carcinomas were positive for one, two or three oncoproteins in 95% of cases, grade II in 88% and grade III in 85%.

Ras p21 was detected in the cytoplasm (Figures 1 and 2), c-*myc* p62 in the nucleus (Figure 3) and occasionally in the cytoplasm (Figure 4) and c-*erb*B-2 in the cytoplasmic membrane (Figures 5-7), as previously described (8, 15, 24, 25, 33, 34). The most frequently expressed was c-*myc* p62 in 70% of cases followed by *ras* p21, 55% and c-*erb*B-2, 35%.

The staining intensities of the *ras*, c-*myc* and *erb*B-2 oncogenes were correlated with the available clinicopathological data (Tables II, IV). The histopathological grade, patient's age (i.e⩽50 or >50 years) and hormone status were correlated with each oncogene separately and in combination with each other. In certain cases the number of patients is small and therefore reduces the statistical significance of the analysis. However, the only correlation that was found was an association between *ras* expression and the age of the patients (Tables II, III).

The relationship between the incidence of metastasis in the auxiliary lymph nodes was also analysed with the staining intensities of the *ras*, c-*myc* and *erb*B-2 oncogenes, and no significant correlation was found in this group of tumour specimens (Table IV).

Discussion

We have demonstrated that three oncogenes, *ras*, *myc* and *erb*B, are overexpressed in certain breast cancer patients. However, no clinical correlations have as yet emerged apart from an association between increased *ras* staining intensity and patient age of less than 50 years. In the group of patients aged 50 years or under, 3 had negative *ras* staining and 13 had

Table III. *Tumour grade, patient age and hormone status in breast cancer patients, examined for relationship between the staining intensities of: (a)* ras *with* myc; *(b)* ras *with* erbB-2; *(c)* myc *with* erbB-2.

	Grade		Age		Oestrogen/Progesterone Status	
	X^2	P	X^2	P	X^2	P
ras *myc*	3.4	0.75	6.3	9.6×10^{-2}	0.67	0.88
ras *erbB-2*	2.65	0.85	6.7	8.0×10^{-2}	0.80	0.84
myc *erbB-2*	2.13	0.91	0.87	0.8	0.27	0.96

Stastical analysis of the different staining intensities between the *ras*, *myc* and *erbB-2* oncogenes, and certain clinicopathological parameters. Chi squared (X^2) and probability (P) shown.

Table IV. *Relationship between the incidence of metastasis and the staining intensities* ras, myc *and* erbB-2 *oncogenes.*

	ras		*myc*		*erbB-2*	
	N	P	N	P	N	P
Number of patients	45	55	30	70	65	35
Patients with no metastasis recorded	1	4	2	4	5	1
Patients with metastasis in one or more auxiliary nodes recorded	12	18	6	23	18	11
Percentage of patients with data on metastasis	35		35		35	
X^2	$P > 0.05$		$P > 0.05$		$P > 0.05$	

Staining intesity: N = negative or equivocal staining
P = positive staining

positive staining, while in the group of patients aged over 50 years 24 had negative and 20 had a positive *ras* staining pattern. The clinical significance of this finding is unclear, but it may be associated with the clinical outcome of these patients. In contrast to this result, De Biasi et al (13) demonstrated with a direct binding liquid competition radio immunoassay that the tumors with higher levels of *ras* p21 were associated with post menopausal patients. The reason for these different results are unclear. It is also of interest that Escot *et al* (41) reported that amplification of the c-*myc* gene correlated with breast cancer in patients aged over 50 years.

The development of cancer is a multi-stage process. Activation of the dominantly acting oncogenes has been found to occur at various stages in the pathway leading to cancer (1). Such activation could involve the generation of structural mutations which may lead to an altered or activated oncogene protein product (42-44) or altered expression of a proto-oncogene which frequently results in an elevated expression of the proto-oncogene coded protein (2-7). *In vitro* cell transformation studies with *ras* (2, 3) c-*myc* (4, 5) or c-*erb*B-2 (6, 7) proto-oncogenes have demonstrated that overexpression of one or the other of these proto-oncogenes contributes to the generation of the malignant phenotype. Thus both qualitative and/or quantitative changes in the expression of oncogenes play an important role in the development of cancer.

Our results presented here show that multiple oncogenes are activated in human breast carcinomas and suggest multiple pathways in the development of breast cancer. In view of the claims that overexpression of certain oncogenes such as *ras* (12), c-*myc* (23, 45) or c-*erb*B-2 (28, 29, 36) have prognostic significance, it is of great interest to identify the type of tumors which overexpress these genes or to establish a panel of antibodies that would define prognosis. We are currently studying these questions.

Acknowledgements

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