c-erbB-2/c-erbA co-amplification indicative of lymph node metastasis, and c-myc amplification of high tumour grade, in human breast carcinoma

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Summary A panel of 73 samples, including 52 primary breast carcinomas, 10 normal breast tissues and 11 axillary lymph nodes, has been analysed for the presence of amplifications and gross structural alterations, in the oncogenes c-erbB-2, c-erbA, c-myc, N-myc, c-mos and c-Ha-ras. The tumours were also classified, graded and staged histopathologically and their DNA ploidy (42 samples) was determined by flow cytometry. Three breast cancer cell lines (MCF7, ZR-75-1 and T47D) were also included in the study. Amplification of c-erbB-2 was detected in 28% of the tumours, of which 91% had an increased steady-state level of c-erbB-2 mRNA. Amplification of c-erbA was found in 23% of tumours and was always associated with the amplification of c-erbB-2. Ten out of 12 (83%) tumours which had c-erbB-2 and c-erbA co-amplification had metastasised to axillary lymph nodes ($P<0.006$). However, the human thymidine kinase gene, which is present at the same chromosomal location as these two oncogenes (17q21–22), was amplified in only two tumours. Amplification of c-myc was detected in 21% of the tumours studied, of which 82% ($P<0.005$) were of histopathological grade 3 and none were of grade 1. Flow cytometry showed that 90% ($P<0.01$) of the analysed tumours with c-erbB-2 and c-erbA co-amplification, and 70% ($P<0.1$) of those with c-myc amplification were DNA aneuploid. This study demonstrates the potential value of c-myc amplification in the assessment of the tumour grade, rather than metastatic potential; and of the co-amplification of c-erbB-2 and c-erbA as a strong indicator of metastatic potential, rather than tumour grade.

Abnormalities in either the structure or activity of proto-oncogenes can contribute to the development or progression of the malignant phenotype (Slamon et al., 1984; Nishimura & Sekiya, 1987). Several studies have shown that cellular oncogenes are activated in breast tumours. For example c-myc has been found both amplified and over-expressed in human breast carcinoma (Escot et al., 1986; Varley et al., 1987; Mariani-Costantini et al., 1988), and the loss of c-Ha-ras-1 alleles has been demonstrated in 27% of breast tumours from patients constitutionally heterozygous for this allele (Theillet et al., 1986; Ali et al., 1987). A number of studies have also reported the presence of c-erbB-2 amplification and over-expression in primary breast tumours (Slamon et al., 1987; Venter et al., 1987; Varley et al., 1987; Zhou et al., 1987; Berger et al., 1988; Gusterson et al., 1988a, b; Barnes et al., 1988; van de Vijver et al., 1987, 1988), and breast tumour cell lines (Kraus et al., 1987; van der Vijver et al., 1987). These studies have demonstrated correlations between c-erbB-2 amplification and regional lymph node metastasis and poor prognosis (Slamon et al., 1987; Zhou et al., 1987; Varley et al., 1987) and/or tumour grade (Berger et al., 1988; Barnes et al., 1988).

The c-erbB-2 and c-erbB-1 (epidermal growth factor receptor) are distinct but related genes and are both homologous to the v-erb-B oncogene of avian erythroblastosis virus (AEV) (Semba et al., 1985; Schechter et al., 1985; Yamamoto et al., 1986). The other oncogene of AEV, v-erb-A, is the homologue of c-erbA, one of the steroid receptor family of genes, encoding a nuclear receptor for thyroid hormone (Weinberger et al., 1986). In the human genome both c-erbA and c-erbB-2 are located on chromosome 17q21–22 (Fukushige et al., 1986; Yamamoto et al., 1986; Weinberger et al., 1986). The close proximity of these two genes, the co-existence of their viral homologues in AEV, and the clear evidence of co-operation between v-erb-A and v-erb-B oncogenes (Frykberg et al., 1983; Sealy et al., 1983) all point to possible co-operation between the oncogenically activated c-erbB-2 and c-erbA in the process of malignant transformation.

The purpose of this study was to identify possible alterations in either the structure or expression of a number of oncogenes (myc, mos, ras, erbB-2 and erb-A), in human breast carcinomas and breast tumour cell lines. For controls we have also analysed the thymidine kinase (TK) gene which is on the same chromosomal location as c-erbB-2 and c-erbA, and the dihydrofolate reductase (DHFR) gene which is located on chromosome 5q11–13. Flow cytometric analysis of tumour and lymph node tissue was also performed in order to compare the relationship between gene amplifications and gross DNA abnormalities.

Materials and methods

Fresh excision biopsy or mastectomy specimens containing primary carcinomas from 52 patients were dissected to provide representative samples which were either rapidly frozen and stored in liquid nitrogen, for subsequent DNA and RNA extraction, or fixed and processed for morphological and flow cytometric studies. When available, material from axillary lymph node metastases was also examined and in 10 cases normal breast tissue from the specimen was used to provide a normal control. The tumours were classified according to standard criteria (Page et al., 1987). There were 42 infiltrating ductal, five infiltrating lobular, one tubular, two mucinous and two cribriform ductal carcinomas. Tumour grading was performed by the method of Bloom and Richardson, as modified by Elston (Elston, 1987). The three mammary tumour cell lines used were, MCF7 ZR-75-1 and T47D (Kraus et al., 1987; van de Vijver et al., 1987).

DNA flow cytometry

Nuclear DNA measurements were performed on sections cut from paraffin embedded material from tumour and axillary nodes using a modification of the method of Hedley et al., (1983), as described in detail previously (Quirke et al., 1986).

DNA extraction and hybridisation

High molecular weight DNA was isolated according to standard procedures. DNA samples (7 μg) were then digested with the restriction endonucleases EcoR1, HindIII or BamHI,

separated by gel electrophoresis using 0.7% agarose gels, transferred on to Hybond nylon filters and hybridised to ^{32}P oligo-labelled probes (Feinberg & Vogelstein, 1983). Hybridisation, washing and reprobing of the filters were conducted as recommended by the manufacturers. The level of amplification in each sample was quantified by densitometric scanning of the autoradiographs using a Joyce Loebel microdensitometer. As a control for the equal loading, transfer and hyridisation of the DNA samples, the blots were stripped and re-hybridised with probes for TK and DHFR. The intensities of the TK and DHFR hybridising fragments were used as references for normalisation of the results.

RNA isolation and slot blot analysis

The level of c-erbB-2 RNA was determined in the three cell lines' 14 tumours (in which sufficient tissue was available), one positive lymph node and one peripheral blood lymphocyte sample. Caesium chloride gradient centrifugation was used to isolate the RNA (Tavassoli & Shall, 1988). In the tumour samples from which both RNA and DNA were isolated a single extraction procedure was used (Chirgwin et al., 1979). Ten μg samples of total RNA were used for slot blot analysis (Darling et al., 1989). The filters were then sequentially hybridised to the ^{32}P-labelled c-erbB-2 and c-erbA probes. The level of β-actin RNA was used to standardise the relative amount of RNA loaded in each sample. The level of each RNA species was quantitated by scanning densitometry of the autoradiographs.

Probes

The following probes were used. The c-erbB-2 probe was a 4.6 kb SalI-Hind III cDNA fragment isolated from the plasmid pSV2neuT (Bargmann et al., 1986). The c-erbA probe was the 2.4 kb EcoRI-HindIII fragment of pHE-AI plasmid (Jansson et al., 1983). For the detection of c-myc we used the 1.3 kb ClaI-EcoRI fragment of pmyc plasmid, containing c-myc exon 3 (Dalla-Favera et al., 1982). The N-myc probe was the EcoRI-BamHI insert of the plasmid pNB-I (Schwab et al., 1983). The c-Ha-ras probe was the 2.9 kb SstI fragment from the plasmid pHA-1 (Chang et al., 1982). The TK probe was a 1.3 kb SmaI-BamHI fragment isolated from pTK11 (Bradshaw & Deininger, 1984). The DHFR probe was the 1.8 kb EcoRI fragment from pBH31R-1.8 (Yang et al., 1984). The mos probe was the 1.0 kb HindIII-XbaI fragment of molony murine sarcoma virus, isolated from the plasmid pmos31 (Verma et al., 1980). The beta-actin probe was a 1.1 kb PstI fragment from pAL41, provided by Dr P. Barton (Pasteur Institute, Paris).

Results

Amplification of c-erbB-2, c-erbA and c-myc

Hybridisation with the c-erbB-2 cDNA probe showed amplification in 15 of the 52 tumour samples (Figure 1 and Tables I and II), but there was no evidence of gross genomic rearrangements. The degree of amplification ranged from 4-fold in tumour T50 to 40-fold in tumour T35 (Figure 1a). Rehybridisation of these blots with the c-erbA probe detected between 3 and 40-fold amplification in 12 of the 52 tumours (Figure 1b). Neither the c-erbB-2 nor c-erbA probes detected any amplification in the non-malignant breast tissues from the same patients (10 non-malignant tissues examined). Only two of the eight positive lymph nodes had c-erbB-2 and c-erbA amplification (L25 and L45, see Table I). However, the level of amplification was much lower in the lymph nodes than in the corresponding tumours. None of the three negative lymph nodes had amplification of either c-erbB-2 or c-erbA (data not shown). It is interesting to note that c-erbA amplification was present only in the tumour samples in which the c-erbB-2 gene was also amplified. However, in tumour T26 only the 2.1 kb BamH1 fragment of c-erbB-2

was amplified (approximately 4-fold, compared with the 2.2 kb N-myc fragment in the BamH1 digest–data not shown). By contrast c-erbB-2 amplification was detected in three tumours (Figure 1, T36 and T49; T12 not shown) which did not contain c-erbA amplification.

The analysis of the TK gene, which is on the same chromosomal location as c-erbB-2 and c-erbA (17q21–22), was used to determine the extent of the amplified domain on chromosome 17. TK amplification was detected in two samples only (Figure 1d, T35 and T45). Interestingly, these were tumours in which the highest levels of c-erbB-2 and c-erbA amplification were detected.

The re-hybridisation of the same filters with the c-myc probe demonstrated the amplification of c-myc gene (located on chromosome 8q24) in 11 of the 52 tumours (Figure 1c). The level of c-myc amplification in these samples varied between 3 (T47) and 15-fold (T26). Three of the tumours (T26, T45 and T50) in which c-myc was amplified also had both c-erbB-2 and c-erbA amplification; in tumour T49 c-myc and c-erbB-2 were amplified but not c-erbA (Figure 1). Three of these (T26, T45 and T49) were grade 3 tumours, with positive lymph nodes. Rehybridisation of these samples with the appropriate probes for c-Ha-ras (chromosome 11p15), N-myc (chromosome 2q23–24) and c-mos (chromosome 8q22) did not detect the amplification of these genes in any of the 73 samples analysed (data not shown). The absence of c-mos amplification demonstrates that the c-myc amplified domain (8q24) does not extend to the c-mos locus (8q22) in any of the analysed samples. These blots were finally probed for DHFR (chromosome 5q11–13), which detected no amplification in any of the samples and confirmed the presence of equal amounts of DNA in each track (data not shown). This is also indicated by the comparison of the intensity of the c-myc and TK hybridising bands in Figure 1c and d.

Although there was no evidence of c-Ha-ras amplification in any of the tissues, in two of the seven samples in which BamH1 restriction fragment length polymorphism suggested c-Ha-ras heterozygosity in the non-malignant tissue, there was clear evidence of homozygosity for this locus in the tumour (Figure 2). Approximately 50% of all tumours analysed were homozygous for the c-Ha-ras locus, but since non-tumour tissue was available only in 10 cases, it was not possible to determine what proportion of the homozygosity in the other samples was a result of allelic deletions from a heterozygous background.

c-erbB-2 and c-erbA RNA expression

The steady-state level of c-erbB-2 transcripts was elevated 3 to 15-fold in 11 tumours in which the gene was also found to have been amplified (Figure 3a). The level of c-erbB-2 RNA appears to correlate with the level of gene amplification in these tumours: no increase in the RNA level was detected in any of the tumours without gene amplification (Figure 3, T2 and T40). However, two cell lines, ZR-75-1 and T47D, which did not appear to have c-erbB-2 amplification, both showed about 10-fold higher levels of c-erbB-2 transcripts than was detected in MCF7.

The c-erbA probe did not detect over-expression in any of the tumours with the possible exception of a 2-fold increase in one tumour (Figure 3b and Table I, T24). To eliminate the possibility of loading errors in these studies the slot blots were finally stripped and rehybridised to the beta-actin probe. A similar level of beta-actin RNA was detected in all samples (Figure 3c), thus demonstrating that the detected variations in the steady-state level of c-erbB-2 and c-erbA were not due to differences in the amount of RNA applied to the slot-blots.

Clinical correlation

Comparison of the available clinical data, such as tumour grade, lymph node involvement, tumour size and ploidy, with the analysis of alterations in either the structure or expression
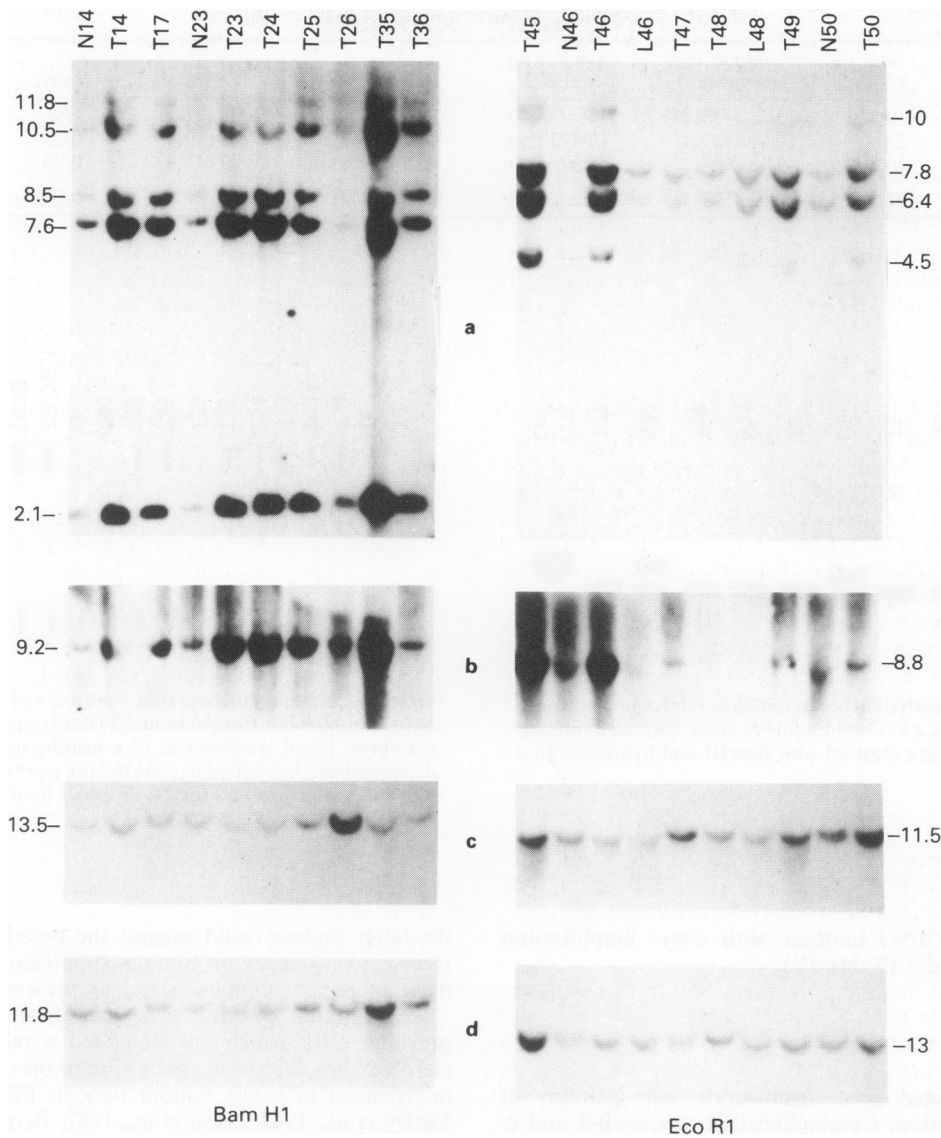
Figure 1 Amplification of c-erbB-2, c-erbA and c-myc genes in human breast carcinomas. DNA samples from breast tumour (T), normal breast (N), lymph nodes (L) were digested with restriction enzyme EcoRI or BAM HI and hybridised to probes for c-erbB-2 (a), c-erbA (b), c-myc (c) and TK (d).

Table I c-erbB-2 and c-erbA gene amplification and RNA levels

Sample no.	Gene copy number		Increase in RNA level	
	c-erbB-2	c-erbA	c-erbB-2	c-erbA
T5	10	5	3	1
T12	5	1	n.d.	n.d.
T14	20	10	10	1
T17	15	10	5	1
T23	25	25	15	1
T24	25	25	4	2
T25	15	15	4	1
L25	4	4	n.d.	n.d.
T26	4	5	n.d.	n.d.
T33	15	15	1	1
T35	40	40	10	1
T36	5	1	10	1
T45	30	30	10	1
L45	5	5	n.d.	n.d.
T46	25	25	10	1
T49	5	1	n.d.	n.d.
T50	4	3	4	1

Numbers indicate the level of gene amplification and the increase in the level of RNA above normal (1 indicates the normal level). n.d., not done.

of the oncogenes examined, suggests a statistically significant correlation between c-myc amplification and tumour grade ($P < 0.005$) (Tables II and IV). Amplification of c-myc was detected in 45% of all grade 3 tumours, but in only 8% of the grade 2 tumours. No c-myc amplification was detected in any of nine grade 1 tumours. There is a similar correlation between c-myc amplification and tumour grade in tumours with positive axillary lymph nodes (Table III).

In the tumours with positive axillary lymph nodes, only one of the 18 grade 1 and 2 tumours (5%) had c-myc amplification, but in five of the seven (71%) grade 3 tumours c-myc was amplified between 3 and 15-fold. Nine of the 11 (82%) tumours with c-myc amplification were of histopathological grade 3 (Table IV).

By contrast to c-myc, the amplification of c-erbB-2 appears to be correlated best with tumour metastasis rather than grade ($P < 0.025$). Eleven of 15 (73%) tumours with c-erbB-2 amplification (between 4 and 40-fold) were node positive (Table IV). This correlation is particularly impressive in tumours with both c-erbB-2 and c-erbA amplification ($P < 0.006$); 10 of 12 (83%) such tumours were node positive. Only six of 11 (54%) node positive tumours showed c-myc amplification (Table IV). Flow cytometry showed that nine of 10 (90%) tumours with c-erbB-2/c-erbA co-amplification

Table II Oncogene amplification in breast tumours: all cases

Tumour grade	No.	DNA aneuploid	c-erbB-2	c-erbA	c-myc	N-myc	c-mos
1	9	3/7 (43%)	4/9 (44%)	4/9 (44%)	0/9 (0%)	0/9	0/9
2	23	10/20 (50%)	4/23 (17%)	2/23 (8%)	2/23 (8%)	0/23	0/23
3	20	9/15 (60%)	7/20 (35%)	6/20 (30%)	9/20 (45%)	0/20	0/20
Total	52	22/42 (52%)	15/52 (29%)	12/52 (23%)	11/52 (21%)	0/52	0/52
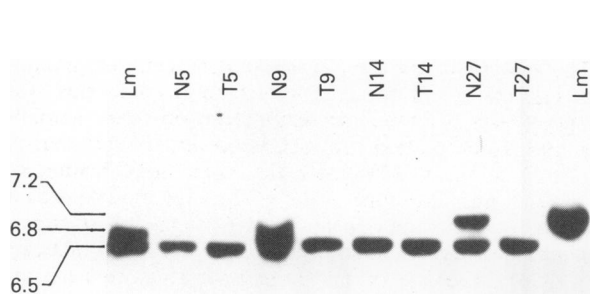
Figure 2 c-Ha-ras analysis in breast tumours. DNA samples from a number of tumours (T), non-tumour tissues (N), and normal lymphocytes (Lm) were digested with BamHI and hybridised to a c-Ha-ras probe.
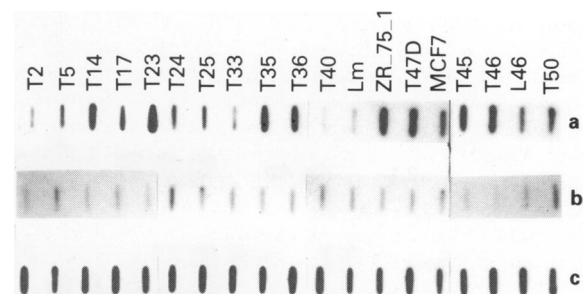
Figure 3 Slot blot analysis of a c-erbB-2 and c-erbA RNA level. 10 µg of total RNA from tumours (T) one lymph node (L) and from peripheral blood lymphocytes of a healthy individual (Lm) were denatured and hybridised to c-erbB-2 (a), c-erbA (b) and β-actin (c). a, b and c were exposed for 24, 72 and 4 hours respectively.

and seven of 10 (70%) tumours with c-myc amplification were DNA aneuploid (Table IV).

Discussion

The results presented here demonstrate two patterns of oncogene amplification. Co-amplification of c-erbB-2 and c-erbA appears to be related to the presence of axillary node metastasis, while c-myc amplification appears to correlate well with high tumour grade. There is also evidence of c-Ha-ras allelic deletion in two of seven tumours in which heterozygosity could be confirmed in normal tissue. Whether

the latter finding could suggest the possible involvement of recessive oncogenes or tumour suppressors in the development of breast tumours is not at present clear.

The detected c-erbB-2 gene amplification correlates with previous work which has suggested a relationship between c-erbB-2 amplification, poor prognosis and lymph node invovlement in breast tumour patients (Slamon et al., 1987; Varley et al., 1987; Zhou et al., 1987; Berger et al., 1988). In the present study we demonstrated that the presence of c-erbA co-amplification in breast tumours, only reported by van de Vijver et al. (1987), is associated with a greater chance of axillary node metastasis. However, it is important to stress that the converse may not be true. That is, the absence of

Table III Oncogene amplification in breast tumours with positive axillary nodes

Tumour grade	No.	DNA aneuploid	c-erbB-2	c-erbA	c-myc	N-myc	c-mos
1	7	3/5 (60%	4/7 (57%)	4/7 (57%)	0/7 (0%)	0/7	0/7
2	11	7/10 (71%)	2/11 (18%)	2/11 (18%)	1/11 (9%)	0/11	0/11
3	7	3/5 (60%)	5/7 (71%)	4/7 (57%)	5/7 (71%)	0/7	0/7
Total	25	13/20 (65%)	11/25 (44%)	10/25 (40)	6/25 (24%)	0/25	0/25

Table IV Analysis of tumours with oncogene amplification

Amplified oncogene	Metastatic/total (ratio)	P	Grade 3/total (ratio)	P	Aneuploid/total (ratio)	P
c-myc	6/11 (54%)	n.s.	9/11 (82%)	<0.005	7/10 (70%)	n.s.
c-erb-B-2	11/15 (73%)	<0.025	7/15 (46%)	n.s.	7/12 (58%)	n.s.
c-erb-A/c-erbB-2	10/12 (83%)	<0.005	6/12 (50%)	<0.025	9/10 (90%)	<0.01

n.s., not significant.

c-erbB-2/c-erbA co-amplification does not necessarily indicate the absence of lymph node metastasis; in 14 of the node positive tumours examined there was no evidence of either c-erbB-2 or c-erbA amplification.

It is interesting to note that c-erbA amplification was present only in tumour samples with c-erbB-2 amplification, suggesting that the latter may. have in fact caused or contributed to the amplification of c-erbA, which happens to be located on the same chromosomal domain. This co-amplification in metastasising breast tumours agrees well with the known properties of the two viral homologues of these genes. The first, v-erb-B, is both necessary and sufficient for the initiation and maintenance of cellular transformation in chick embryo fibroblasts and erythroid cells (Frykberg et al., 1983; Sealy et al., 1983). The second, v-erb-A which by itself is devoid of any transforming activity, co-operates with the v-erb-B, resulting in the formation of transformed cells of increased malignancy and metastatic ability. Whether the c-erbA amplification is only a fortuitous consequence of its close proximity to the amplified c-erbB-2 gene, as suggested by van der Vijver et al. (1987), or whether its amplification has any significance for the development and progression of the breast tumour can not be determined from the present study. However, the fact that the TK gene, which is located on the same chromosomal domain, appears to be amplified in only two tumours, both of which had the highest level of erbB-2/erbA co-amplification, and the co-operativity of these oncogenes in chicken fibroblasts and erythroid cells, suggest that the co-amplification may have contributed to the more aggressive nature of these tumours. The presence of positive axillary lymph nodes in 10 of the 12 tumours (83%) with the c-erbB-2/c-erbA co-amplification strengthens this notion.

Despite the good correlation between the c-erbB-2 and c-erbA amplification, particularly in the metastatic breast tumours, the molecular significance of this alteration for tumour development and progression remains unclear. We have not detected the over-expression of c-erbA in any of the tumour samples (with the possible exception of tumour T24) or the breast tumour cell lines. Whether this is due to absence of increased expression or the failure of detection, possibly due to the short half-life of the message, is not clear. By contrast to the c-erbA, the amplification of c-erbB-2 was associated with a 3 to 15-fold increase in c-erbB-2 RNA levels; no increase in the c-erbB-2 RNA level was detected in any of the breast tumours without the amplification. Increased steady-state c-erbB-2 mRNA and protein level and their association with lymph node involvement, metastasis and/or poor prognosis have also been reported (Venter et al., 1987; van der Vijver et al., 1987; Berger et al., 1988). However, in two other studies a closer correlation was found between increased c-erbB-2 protein level and tumour pathogenesis, in particular with in situ ductal carcinoma (Gusterson et al., 1988a,b) and tumour size (van der Vijver et al., 1988) rather than lymph node involvement.

In agreement with previously published data, in none of the three breast tumour cell lines examined (MCF7, ZR-75-1 and T47D) was there any evidence of either c-erbB-2 or c-erbA amplification. Nevertheless, two of these lines (ZR-75-1 and T47D) did have a much higher steady-state level of c-erbB-2 RNA than MCF7 cells. However, we note that van der Vijver et al. (1987) did not detect c-erbB-2 over expression in the T47D cells. The over-expression of c-erbB-2, in the absence of gene amplification, has also been

detected in three other breast tumour cell lines (BT483, MDA-MB175 and ZR-75-30) (Kraus et al., 1987). Therefore, different mechanisms, one of which appears to be amplification, could lead to the increased levels of c-erbB-2 RNA. The involvement of c-erbB-2 in breast tumours may not be restricted only to those cases in which either amplification or over-expression is detectable. It has been demonstrated that the rat homologue of c-erbB-2, the neu oncogene, can be activated by a point mutation altering a valine residue to a glutamic acid residue in its predicted transmembrane domain (Bargmann et al., 1986). We are currently investigating whether such mutations are present in the c-erbB-2 gene of either the MCF7 cell line or the tumour samples in which neither amplification nor the over-expression of c-erbB-2 was detected.

The analysis of the c-myc domain demonstrated amplification in 21% of all breast tumours analysed in this study. There appears to be no correlation between c-myc amplification and lymph node metastasis. However, the fact that nine of the 11 tumours (82%) with the c-myc amplification were of grade 3, and that none of the grade 1 tumours showed any evidence of c-myc amplification, does suggest a strong correlation between tumour grade and c-myc amplification ($P < 0.005$). Escot et al. (1986) have also detected the amplification of c-myc in human breast tumours but find a better correlation with age rather than tumour grade ($P < 0.02$). However, Varley et al. (1987), who have also detected c-myc amplification in human breast tumours, have not found such a correlation with age (menopausal status), but demonstrate a significant correlation between c-myc alteration (amplification and/or rearrangement) and poor short-term prognosis ($P < 0.02$).

The study of the c-Ha-ras oncogene, which in in vitro studies has been found to co-operate with the myc oncogene in the malignant transformation of primary rodent fibroblasts (Land et al., 1983), demonstrates the allelic deletion of the c-Ha-ras in two of the seven tumours in which there was clear evidence of heterozygosity in this domain. Both of these tumours were of grade 3, and one (T27) also showed 5-fold amplifiction of the c-myc gene. The allelic deletion of c-Ha-ras has also been detected in approximately 25% of all breast tumours; about 74% of the tumours with the deletion were grade 3 (Theillet et al., 1986; Ali et al., 1987). These studies have found a good correlation between c-Ha-ras allelic deletion and tumours of histopathological grade 3. As suggested by these authors, the allelic deletion of c-Ha-ras (chromosome 11p15) may be indicative of the possible involvement of recessive oncogene (tumour suppressor genes), including the Wilm's tumour gene which is located on chromosome 11p13 (Koufos et al., 1984), in the aetiology of breast tumours.

In conclusion, the studies reported here demonstrate a good correlation between c-erbB-2/c-erbA co-amplification and the metastatic ability of the breast tumours, while c-myc amplification correlates well with high tumour grade, but not metastasis.

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