Journal of Cellular Biochemistry 17G:132–138 (1993)

c-*erb*B-2 Amplification in Mammary Carcinoma

Diana M. Barnes, BSc, PhD

Imperial Cancer Research Fund Clinical Oncology Unit, Guy's Hospital, London, SE1 9RT, United Kingdom

Abstract The c-*erb*B-2 oncogene has been extensively studied in mammary carcinomas since Slamon and colleagues demonstrated the association between amplification and poor prognosis in 1987. Further work found that amplification was accompanied by overexpression of the protein; however, this relationship is not perfect. Recently, Hollywood and Hurst have shown increased transcription in some cell lines containing a single copy of the gene, causing mRNA accumulation in overexpressing cells. Protein expression appears to be a good indicator of various abnormalities in the c-*erb*B-2 gene. Fortunately, c-*erb*B-2 protein, unlike epidermal growth factor (EGF) receptor, survives most fixation procedures used in routine histopathology laboratories. This has enabled immunohistochemical studies to be carried out on archival material.

A higher incidence of c-*erb*B-2 positivity occurs in ductal carcinoma *in situ* (DCIS) than in infiltrating carcinomas. In DCIS there is a very close association between protein expression and high grade (comedo type). This explains the very high incidence of c-*erb*B-2 positivity in Paget's disease of the nipple which is nearly always associated with high grade DCIS. A lower proportion of high grade infiltrating carcinomas express the protein, highlighting the difference in incidence of positivity in the two types of ductal lesion.

As well as having a potential role in the biological classification of mammary carcinomas, c-*erb*B-2 expression has been used to predict response to treatment. There have been reports that tumors expressing c-*erb*B-2 fail to respond to either chemotherapy or endocrine therapy. It is extremely difficult to conduct satisfactory trials to confirm these results since only a quarter of infiltrating mammary carcinomas are c-*erb*B-2-positive, and a very large number of stage-matched patients is necessary in order to achieve comparable treatment and control groups. However, two small studies carried out at Guy's Hospital have found that the presence of c-*erb*B-2 protein does not preclude a successful response to either adjuvant chemotherapy or endocrine therapy for metastatic disease.

Key words: Breast, c-*erb*B-2, DCIS classification, immunohistochemistry, nuclear size, prognosis, treatment response

The c-*erb*B-2 gene codes for a growth factor receptor protein which is very similar in structure to the epidermal growth factor (EGF) receptor. The c-*erb*B-2 oncogene has been extensively studied in mammary carcinoma since Slamon and colleagues [1] demonstrated an association between amplification and poor prognosis in 1987. Following this, Venter *et al.* [2] showed that amplification of the gene was closely associated with overexpression of the protein as detected by immunohistochemistry on frozen tissue sections. Venter also showed that c-*erb*B-2 antibodies were equally effective in formalin-fixed tissue; this was quickly confirmed by van der Vijver *et al.* [3]. This fact has had a significant effect on the introduction of molecular biology into clinical laboratories. Early papers did not always confirm Slamon's original observation that abnormalities in the c-*erb*B-2 gene were associated with poor

Address correspondence to Diana M. Barnes, BSc, PhD, Imperial Cancer Research Fund Clinical Oncology Unit, Guy's Hospital, Saint Thomas Street, London, SE1 9RT, United Kingdom.

prognosis. However, each of these studies examined fewer than 200 patients. When Slamon [4] and Lovekin [5] separately reported two studies including over 500 patients, the association was confirmed irrespective of whether DNA or protein was analyzed.

Although it is generally accepted that amplification of the gene and overexpression of the protein are closely related, careful studies on 274 tumors by Borg *et al.* in 1991 [6] demonstrated overexpression in 16 of 231 (7%) non-amplified tumors. In 1989, Slamon *et al.* [4] had similarly found overexpression in 18 of 136 (13%) non-amplified tumors. Slamon suggested that these cases could represent examples of alteration in the promoter or enhancer element of the c-*erb*B-2 gene. Hollywood and Hurst [7] have recently shown that this is indeed the case. They found that in some cell lines containing a single copy of the gene, increased transcription can cause accumulation of mRNA, resulting in overexpression. They have further identified a DNA binding protein, OB2-1, abundant in cell lines overexpressing c-*erb*B-2 irrespective of gene copy number. It is the expression of a particular protein that is important in tumor behavior; these data clearly vindicate the use of immunohistochemistry for the study of c-*erb*B-2 in mammary carcinoma.

IMMUNOHISTOCHEMICAL STUDIES

We have now stained more than 1,000 samples of mammary carcinoma with antibody 21N [8] using a peroxidase-conjugated streptavidin biotin technique. Only membrane staining has been taken as a positive indication of the presence of c-*erb*B-2 protein; any cytoplasmic staining has always been ignored (Fig. 1). We investigated the relationship between c-*erb*B-2 expression and prognosis in our first study of 195 patients with infiltrating carcinoma [9]. Our overall level of positivity was 29%, but only 9% of the cases exhibited strong staining considered to relate to increased gene copy number. Although there was a very long follow-up (median 10 years), the number of patients in the study was insufficient to show a clear relationship between staining and disease-free or overall survival times, but there was a trend towards a poorer outcome for patients whose tumors showed positive staining.

One patient in this study had Paget's disease of the nipple. Strong positive staining for c-*erb*B-2 was seen in the infiltrating and *in situ* components of the breast carcinoma, as well as in the malignant Paget cells in the nipple. This prompted us to further investigate patients with mammary Paget's disease [10]. In 41 of 45 (91%) cases we found positive c-*erb*B-2 staining of the malignant cells in the nipple (Fig. 2). In 42 of these, the underlying mammary carcinoma was also available. In all but two, the nipple staining was similar to that of the underlying carcinoma (37 positive, 3 negative), irrespective of whether the latter was pure ductal carcinoma *in situ* (DCIS, n = 15) or DCIS accompanied by infiltrating carcinoma (n = 27). The positively stained cases of carcinoma *in situ* were always of the comedo pattern with large pleomorphic nuclei and central necrosis. The two negatively stained tumors had a different histological pattern; in one case there was a solitary focus of cribriform carcinoma with monomorphic cytology, and in the

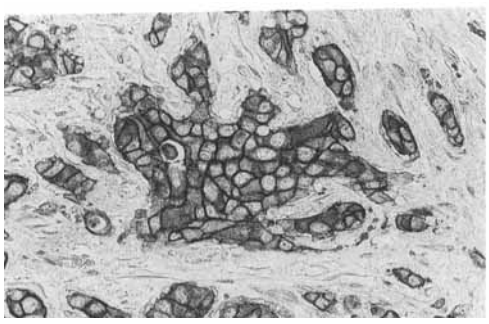

Fig. 1. c-*erb*B-2 expression in an infiltrating ductal mammary carcinoma stained with antibody 21N using a peroxidase-conjugated streptavidin biotin technique.

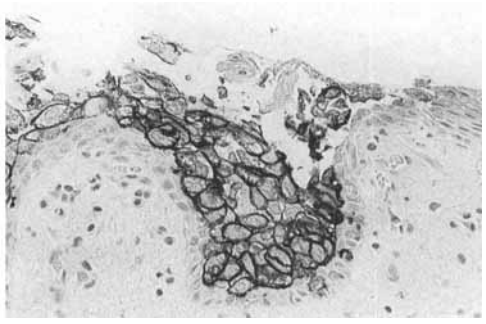

Fig. 2. Nipple epidermis showing c-*erb*B-2 expression in malignant Paget cells.

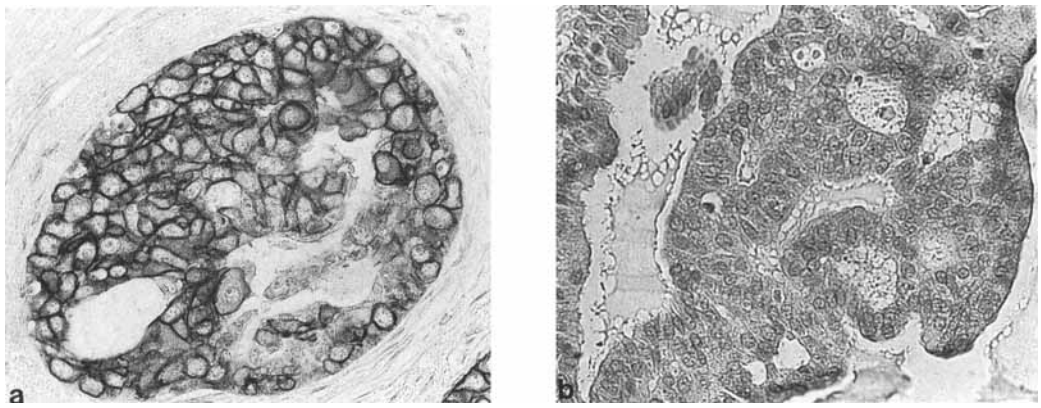

Fig. 3. **(a)** c-*erb*B-2-positive comedo pattern, high grade DCIS with large, pleomorphic nuclei. **(b)** c-*erb*B-2-negative cribriform pattern, low grade DCIS with small, monomorphic nuclei. Both pictures were taken at the same magnification.

other there was a focus of negatively stained adenoid cystic carcinoma adjacent to areas of positively stained comedo type DCIS.

We next investigated the association between overexpression of c-*erb*B-2 and DCIS type [11]. Positive 21N staining was found in 44 of 72 cases of pure DCIS (61%). The very strong association between protein overexpression in large cell comedo pattern DCIS (Fig. 3a) and lack of expression in small cell cribriform/micropapillary pattern DCIS (Fig. 3b) was confirmed ($X^2 = 44.2$, $p < 0.0001$). The average size of the nuclei in the cribriform/micropapillary lesions was 10 μm, whereas that of the comedo pattern DCIS was 20 μm. The correlation between expression of c-*erb*B-2 and large nuclear size was even stronger than the association with histological type ($X^2 = 54.3$, $p < 0.0001$).

It is obvious from these studies that there is a discrepancy between the incidence of c-*erb*B-2-positive staining in *in situ* carcinomas and infiltrating carcinomas. Over 50% of DCIS are positive, whereas only 20–25% of infiltrating carcinomas are positive. To explain this discrepancy, we investigated the relationship between c-*erb*B-2 expression and nuclear size in 148 infiltrating ductal carcinomas with a minimal *in situ* component [12]. In these cases the relationship between c-*erb*B-2 staining and nuclear size was much less clear-cut. Only 30 of the 148 (20%) infiltrating carcinomas had small nuclei, and all but two of these were c-*erb*B-2-negative. The remaining 118 carcinomas all had a varying proportion of large nuclei; only 37 (25%) were c-*erb*B-2-positive, and the other 81 (55%) tumors were c-*erb*B-2-negative. These data suggest the hypothesis that the majority of pure DCIS without any evidence of infiltration have either negatively staining cell membranes and small, uniform nuclei or positively staining cell membranes and large, pleomorphic nuclei. Only a minority of tumors with large nuclei failed to stain. In contrast, two-thirds of the infiltrating carcinomas with large nuclei failed to stain. It is possible that these latter tumors, although arising in the epithelial cells within the ducts, have only a transient *in situ* phase and rapidly become invasive. This would explain their relative absence in any study of pure DCIS, where every attempt is made to exclude tumors with even minimal invasion (Fig. 4). It may be of interest to concentrate future studies on these c-*erb*B-2-negative lesions with large nuclei as they may have an even greater metastatic potential than the c-*erb*B-2-positive tumors with large, pleomorphic nuclei and the c-*erb*B-2-negative tumors with small, monomorphic nuclei.

RECLASSIFICATION OF DCIS TYPE

Work with c-*erb*B-2 in carcinoma *in situ* has contributed to a more rational classification of DCIS. Following an EORTC initiative, some of my colleagues have been involved in formulating such a classification (Roland Holland, personal communication). They found that the most consistent histological feature in cases of DCIS is cytonuclear differentiation. On this basis, DCIS

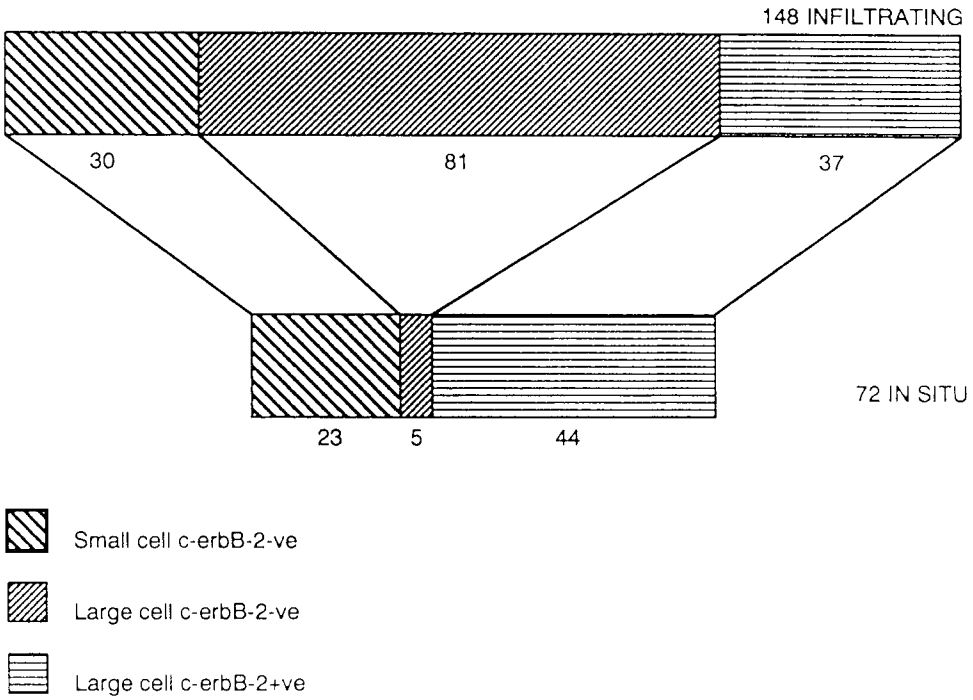

Fig. 4. Comparison of the relative proportions of c-*erb*B-2-positive and -negative infiltrating and pure *in situ* ductal carcinoma of different nuclear size.

can be classified into three groups: well-differentiated (low grade DCIS), poorly differentiated (high grade DCIS), and an intermediate group. Colleagues at Guy's Hospital have studied 102 cases of pure DCIS and found a highly significant correlation between four markers (c-*erb*B-2, p53, progesterone receptor (PR), and proliferation measured by KiS1 immunoreactivity) and the above classification [13]. Poorly differentiated DCIS usually expressed the c-*erb*B-2 oncoprotein and p53 protein, had a high rate of proliferation, and was PR-negative (n = 51). Well-differentiated lesions rarely expressed c-*erb*B-2 or p53, showed low levels of KiS1 staining, and were nearly always PR-positive (n = 23). Intermediate DCIS showed features of both groups (n = 22). This approach towards correlating histological and biological features may help to define a better classification of DCIS, which may in turn be more prognostically and therapeutically relevant.

c-*erb*B-2 AND RESPONSE TO THERAPY

There is some debate about the efficacy of chemotherapy and endocrine therapy in patients with c-*erb*B-2-positive carcinomas. The studies published so far suggest that these tumors are much less likely to be responsive to conventional treatment [14,15]. We have a small amount of data showing that this is not necessarily so. Adjuvant chemotherapy is mainly effective in premenopausal patients; since only a quarter of infiltrating mammary carcinomas are c-*erb*B-2-positive, a very large number of young women must be accrued to show a significant interaction with satisfactory confidence limits between c-*erb*B-2 status and response. We have examined the relationship between c-*erb*B-2 and response to adjuvant chemotherapy [cyclophosphamide, methotrexate, and 5-fluorouracil (CMF)] in 111 premenopausal women from the Guy's Hospital/Manchester adjuvant chemotherapy trial [16]. Twenty-four of the tumors (22%) were c-*erb*B-2-positive. Treatment significantly prolonged relapse-free survival (RFS) for patients with both c-*erb*B-2-negative (X^2 7.40, p = 0.007) and c-*erb*B-2-positive (X^2 4.95, p = 0.03) tumors (Figs. 5a and b). The c-*erb*B-2-positive patients in the non-treatment control arm fared extremely badly, with a median RFS of less than one year. The

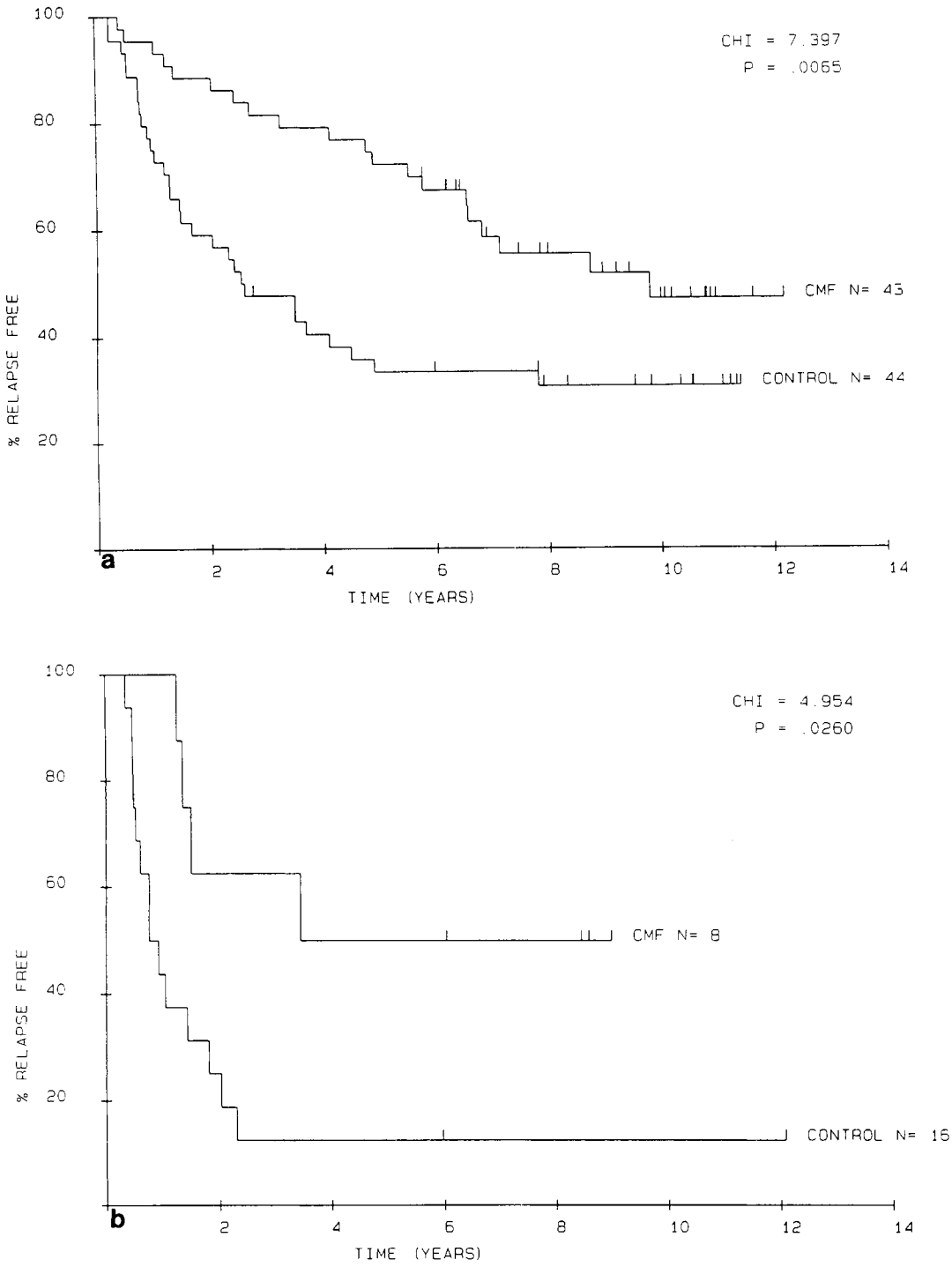

Fig. 5. Log-rank analysis showing the effect of adjuvant CMF on relapse-free survival in **(a)** 87 premenopausal patients with c-*erb*B-2-negative carcinomas and **(b)** 24 premenopausal patients with c-*erb*B-2-positive carcinomas.

TABLE I. Relationship Between c-*erb*B-2 Status and Response to a Variety of Endocrine Therapies for First-Line Treatment of Metastatic Breast Cancer

All Patients			
	CR/PR n (%)	SD/PD n	TOTAL n
–ve c-*erb*B-2	27 (34%)	47	74
+ve	8 (33%)	16	24
TOTAL	35 (36%)	63	93
Tamoxifen-Treated Patients			
	CR/PR n (%)	SD/PD n	TOTAL n
–ve c-*erb*B-2	9 (50%)	9	18
+ve	5 (45%)	6	11
TOTAL	14 (48%)	15	29

CR = complete response
PR = partial response
SD = static disease
PD = progressive disease

–ve = negative
+ve = positive

treated patients with c-*erb*B-2-positive tumors had a median RFS of three years, similar to that in the c-*erb*B-2-negative control arm. From our very small data set it could be argued that all c-*erb*B-2-positive patients should receive adjuvant chemotherapy.

There are also data in the literature showing that c-*erb*B-2-positive patients fail to respond to endocrine therapy [17]. We examined this in 98 women who received a variety of endocrine treatments for metastatic disease. Immunohistochemical staining for c-*erb*B-2 was completed on material from the primary mammary carcinomas, and response to treatment for the subsequent metastatic disease was determined according to UICC criteria [18]. A favorable response (complete or partial) was seen in 35 women, irrespective of c-*erb*B-2 status. The remainder had either static or progressive disease. Twenty-nine of the 98 women (30%) received tamoxifen and 14 responded; again, the effect was seen in both c-*erb*B-2-positive and -negative tumors (Table I). From these data it appears that the presence of the oncoprotein does not preclude a response to either tamoxifen or any other form of endocrine treatment for metastatic disease. There may be other factors which are more important than c-*erb*B-2 in determining the endocrine responsiveness of a particular tumor.

Amplification and overexpression of c-*erb*B-2 is of biological importance in mammary carcinoma. Overexpression without amplification occurs in some 10% of cases, implying that the immunohistochemical detection of c-*erb*B-2 protein is the most clinically relevant technique. In pure DCIS, nearly all of the tumors with large, pleomorphic nuclei express c-*erb*B-2 protein. Although c-*erb*B-2-positive infiltrating ductal carcinomas have large nuclei, there is a large proportion which are c-*erb*B-2-negative. These c-*erb*B-2-negative tumors with large nuclei are rarely seen in pure DCIS, and account for the difference in proportion of tumors with c-*erb*B-2 abnormalities seen in infiltrating and *in situ* ductal carcinomas. Much further work needs to be done assessing the response to both chemotherapy and endocrine therapy by patients with c-*erb*B-2-positive tumors before any rational treatment decisions can be made.

ACKNOWLEDGMENTS

None of this work would have been possible without the collaboration of my clinical, scientific, and statistical colleagues at Guy's Hospital, and at the Imperial Cancer Research Fund laboratories at Lincoln's Inn Fields.

REFERENCES

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987) Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182.
2. Venter DJ, Kumar S, Tuzi NL, Gullick WJ. (1987) Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: Immunohistological assessment correlates with gene amplification. Lancet ii:69–72.
3. van de Vijver MJ, Mooi WJ, Wisman P, Peterse JL, Nusse R. (1988) Immunohistochemical detection of the *neu* protein in tissue sections of human breast

tumours with amplified *neu* DNA. Oncogene 2:175–178.

4. Slamon DJ, Press MF, Godolphin W, Ramos L, Haran P, Shek L, Stuart SG, Ullrich A. (1989) Studies of HER-2/*neu* proto-oncogene in human breast cancer. Cancer Cells 7:371–384.
5. Lovekin C, Ellis IO, Locker A, Robertson JFR, Bell J, Nicholson R, Gullick WJ, Elston CW, Blamey RW. (1991) c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. Br J Cancer 63:439–443.
6. Borg A, Baldetorp B, Ferno M, Killander D, Olsson H, Sigurdsson H. (1991) *erb*B-2 amplification in breast cancer with a high rate of proliferation. Oncogene 60:137–143.
7. *Hollywood DP*, Hurst HC. (1993) A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-*erb*B-2 in mammary tumour lines. EMBO J 12:2369–2375.
8. Gullick WJ, Berger MS, Bennett PLP, Rothbard JB, Waterfield MD. (1987) Expression of the c-*erb*B-2 protein in normal and transformed cells. Int J Cancer 40:246–254.
9. Barnes DM, Lammie GA, Millis RR, Gullick WJ, Allen DS, Altman DG. (1988) An immunohistochemical evaluation of c-*erb*B-2 expression in human breast carcinoma. Br J Cancer 58:448–453.
10. Lammie GA, Barnes DM, Millis RR, Gullick WJ. (1989) An immunohistochemical study of the presence of c-*erb*B-2 protein in Paget's disease of the nipple. Histopathology 15:505–514.
11. Bartkova J, Barnes DM, Millis RR, Gullick WJ. (1990) Immunohistochemical demonstration of c-*erb*B-2 protein in mammary ductal carcinoma *in situ*. Hum Pathol 21:1164–1167.
12. Barnes DM, Bartkova J, Camplejohn RS, Gullick WJ, Smith PJ, Millis RR. (1992) Overexpression of the c-*erb*B-2 oncoprotein: Why does this occur more frequently in ductal carcinoma *in situ* than in invasive mammary carcinoma and is this of prognostic significance? Eur J Cancer 28:644–648.
13. Bobrow LG, Happerfield LC, Springall RJ, Millis RR. (1993) The classification of ductal carcinoma *in situ* and its association with biological markers. J Pathol 169(Suppl):137 (abstract).
14. Allred DC, Clark GM, Tandon AK, Molina R, Tormey DC, Osborne CK, Gilchrist KW, Mansour EG, Abeloff M, Eudey L, McGuire WL. (1992) HER-2/*neu* in node negative breast cancer: Prognostic significance of overexpression influenced by the presence of *in situ* carcinoma. J Clin Oncol 10:599–605.
15. Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderbergh J, Anbazhagan R, Styles J, Rudenstam C-M, Castiglione M, Collins J, Lindtner J, Senn H-J, for the International (Ludwig) Breast Cancer Study Group. (1992) Prognostic importance of c-*erb*B-2 expression in breast cancer. J Clin Oncol 10: 1049–1056.
16. Howell A, George WD, Crowther D, Rubens RD, Bulbrook RD, Bush H, Howat JMT, Sellwood RA, Hayward JL, Fentiman IS, Chaudary MA. (1984) Controlled trial of adjuvant chemotherapy with cyclophosphamide, methotrexate, and fluorouracil for breast cancer. Lancet ii:307–311.
17. Wright C, Angus B, Nicholson S, Sainsbury JRC, Farndon J, Cairns J, Harris AL, Horne CHW. (1992) Expression of c-*erb*B-2 oncoprotein: A prognostic marker in human breast cancer. Cancer Res 49:2087–2091.
18. Hayward JL, Carbone PP, Heuson J-C, Segaloof A, Kumaoka S, Rubens RD. (1977) Assessment of response to therapy in advanced breast cancer. Eur J Cancer 13:89–94.