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Relationship Between EGF-R, c-*erb*B-2 Protein Expression and Ki67 Immunostaining in Breast Cancer and Hormone Sensitivity

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The expression of the epidermal growth factor receptor (EGF-R), c-*erb*B-2 protein product and Ki67 have been evaluated in 105 breast cancers of known responsiveness to endocrine therapy using immunohistochemistry. EGF-R staining was observed in 62 of the tumours and was significantly associated with elevated rates of cell proliferation (%Ki67 positive cells) and loss of hormone sensitivity. In contrast, c-*erb*B-2 expression was not correlated with cell proliferation rates and was less strongly related to hormone insensitivity. Subdivision of the EGF-R data according to c-*erb*B-2 measurements revealed an association between c-*erb*B-2 immunostaining and worsened patient outlook and hormone insensitivity in moderately EGF-R-positive tumours. c-*erb*B-2 immunostaining in highly EGF-R-positive tumours did not further contribute to the already poor prognosis of these patients. These data confirm the prognostic importance of EGF-R measurements in breast cancer and may infer a functional interaction between this protein and the c-*erb*B-2 protein product in the aberrant growth of a subset of breast tumours.
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INTRODUCTION

SEVERAL PAPERS have reported structural similarities and interactions in normal and transformed rodent and human cell lines between the epidermal growth factor receptor (EGF-R) and the c-*erb*B-2 protein [1–6]. These include EGF-induced tyrosine phosphorylation of normal *neu* (c-*erb*B-2, equivalent gene product in the rat), oncogenic *neu* and the human c-*erb*B-2 products. Indeed, Kokai *et al.* [7] have demonstrated a synergistic interaction of *neu* and EGF-R in the transformation of rodent fibroblasts. Since each of these proteins possesses tyrosine kinase activity, activation of which can facilitate cell proliferation [8], the co-expression of EGF-R and c-*erb*B-2 in approximately 10% of human breast cancers and their correlation with poorer survival than with the expression of either protein alone [9], may implicate a functional interaction between these two proteins [10] which may potentially be involved in governing the growth of some breast cancers. Moreover, since elevated EGF-R expression [11, 12] and a raised cell proliferation rate in oestrogen receptor-positive tumours [13] correlates with the loss of hormone sensitivity in breast cancer, the concomitant expression of EGF-R and c-*erb*B-2 may similarly influence the responsiveness of the disease to endocrine-based therapies [14, 15].

In this light, the current communication examines in breast cancer specimens (i) the association between EGF-R and c-*erb*B-2 expression, (ii) their relationship to cell proliferation, measured using the Ki67 antibody [16] and (iii) their association with the responsiveness of breast cancer to endocrine therapy. Previous studies have shown that the Ki67 antibody reacts with a nuclear antigen that is present throughout the cell cycle of proliferating cells, but is absent in quiescent cells [16] and its expression in breast cancer specimens correlates with mitotic activity and their rate of recurrence after mastectomy [11].

The principal procedure used in these studies is immunohistochemistry. This has the advantage of directly monitoring proteins in individual tumour cells. Earlier investigations using this technique on breast cancers have established a relationship between the presence of EGF-R [18], c-*erb*B-2 protein product [19–22] and Ki67 immunostaining [17] and short disease-free interval after mastectomy, with c-*erb*B-2 expression correlating with gene amplification [23, 24].

PATIENTS AND METHODS

One hundred and five samples of primary tumour tissues obtained from patients with histologically proven breast cancer presenting to Prof. R.W. Blamey at the City Hospital, Nottingham during the period July 1984 to August 1987 were included in the study. No patient had previously received any form of adjuvant endocrine or cytotoxic therapy.

All patients received systemic endocrine therapy either for locally advanced primary tumours (maximum diameter 5 cm) or for local or distant recurrences. Pre- or perimenopausal patients received the leuteinizing hormone-releasing hormone (LH-RH) agonist goserelin (Zoladex, ICI 118630, 3.6 mg depot/28 days) alone (n = 21) or in combination with the antioestrogen tamoxifen (Nolvadex, ICI 46474, 20 mg twice daily, n = 3), while the majority of postmenopausal women received tamoxifen (20 mg twice daily, n = 83). Four postmenopausal women received the progestin, Megace (160 mg twice daily).

Patients were assessed for complete or partial responses, static disease (no change) or progression at 2–3 monthly intervals by

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UICC criteria [25]. As recommended by the British Breast Group, responsive and static disease were only reported following a minimum duration of remission of 6 months [26].

Preparation of tissue and immunohistochemical procedures

Samples of primary tumour tissue were rapidly frozen upon excision, stored at −70°C and transported on dry ice to the Tenovus Institute, Cardiff, for analysis. A representative portion of the tissue was blocked for cryostat sectioning and immunohistochemistry.

EGF-R immunostaining

Cryostat sections (5 μm) were thaw-mounted onto poly-L-lysine-coated glass slides and air dried for 1 h prior to storage at −70°C for up to 2 weeks before assay. Rehydration in phosphate-buffered saline (PBS) and fixation in chloroform:acetone (1:1 v/v at 4°C for 10 min) was undertaken immediately prior to assay. Slides were then washed twice in PBS. Blocking of non-specific antibody binding was achieved using 10% normal goat serum (10% v/v) in PBS at room temperature for 10 min. Excess serum was removed and mouse monoclonal primary anti-EGF-R1 antibody (Amersham International, U.K.) added (1 μg/μl in PBS containing 10% normal goat serum and 5% normal human serum) for 60 min at room temperature. A control antiserum mouse antisheep erythrocyte antibody (Sigma, U.K.) was added to parallel sections at a similar concentration to the anti-EGF-R1 antibody to enable levels of non-specific staining to be evaluated. Following primary antibody incubations the slides were washed twice for 5 min in PBS and rabbit anti-mouse peroxidase conjugated antibody (Dakopatts, U.K.) added at a dilution of 1/50 in PBS containing 10% normal goat serum and 5% normal human serum for 30 min. Slides were then extensively washed and immunoreactivity revealed with diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide. Slides were washed with distilled water, counterstained with 1% aqueous methyl green, washed again, dehydrated and mounted in a xylene-soluble mountant.

c-erbB-2 product immunostaining

Frozen breast tumour sections (5 μm) were fixed in 3.7% formaldehyde in PBS for 15 min followed by their sequential immersions in PBS for 10 min, in cold methanol (−15 to −25°C) for 5 min and in cold acetone for 2–3 min. Slides were then washed twice in PBS and assayed immediately or stored in a sucrose–glycerol medium at −20°C for up to 2 weeks. Following rehydration in PBS, the sections were incubated at room temperature with 10% normal goat serum in PBS for 15 min to block non-specific antibody attachments. Excess serum was removed and primary rabbit affinity purified polyclonal anti-*erb*B-2 (21N) antibody added [27] (3 μg/ml in PBS containing 5% normal goat serum) for 60 min at room temperature. Parallel sections were incubated with PBS containing serum as controls. Slides were subsequently washed twice in PBS for 5 min and goat anti-rabbit IgG-peroxidase conjugated antibody (Sigma) added at a dilution of 1/50 in PBS for 30 min. Following further PBS washes immunostaining was revealed by incubation of the sections with DAB/hydrogen peroxide. Sections were washed with distilled water, counterstained with 1% methyl green (aqueous), washed, dehydrated and mounted in a xylene-soluble mountant.

Ki67 immunostaining

The details of this assay have been previously described [17]. Briefly, fixation of 5 μm frozen sections was in cold (−10 to −25°C) acetone (10 min) followed by air drying for 30 min. Slides were stored, when necessary, at −70°C for up to 2 weeks. Sections were rehydrated in PBS and then incubated at room temperature with 10% normal goat serum for 15 min. Excess serum was removed and the slides incubated for a further 45 min with a mouse monoclonal Ki67 antibody (1.4 μg/ml, Dakopatts, Denmark). The slides were subsequently rinsed in PBS and primary antibody binding was revealed by an indirect peroxidase anti-peroxidase (PAP) procedure using goat antimouse bridging antibody containing normal human serum followed, after washing in PBS, with a mouse peroxidase antiperoxidase complex.

Specimen evaluation

All specimen evaluation was performed on an Olympus microscope (BH-2) first using an ocular magnification of ×10 in order to enable the localisation and subsequent avoidance of normal and benign areas within the section. This initial examination also allows the heterogeneity of immunostaining within the tumour components to be assessed and thus ensures that adequate sampling of all areas is performed. All subsequent evaluations were carried out using an ocular magnification of ×40. EGF-R, c-*erb*B-2 product and Ki67 immunostaining patterns were evaluated by two personnel using a dual-viewing attachment to the microscope. Control slides were checked for non-specific binding before assessing the percentage of tumour cells stained by the primary antibody (minimum of 2000 tumour cells evaluated).

RESULTS

Immunohistochemical staining

One hundred and five breast cancers have been examined for the expression of EGF-R and c-*erb*B-2 product and the antigen detected by the Ki67 antibody. Tumour cell membrane staining for c-*erb*B-2 protein was clearly recognisable in 26% (27/105) of the tumours with almost all of the cells immunostaining (Fig. 1a). Although weak cytoplasmic staining was observed in approximately 40% of the remaining tumours, it was unaccompanied by membrane staining and was also frequently seen in control sections. The presence of such staining was, therefore, excluded from further analysis.

An evaluation of EGF-R immunostaining showed 41% (43/105) of the tumours to be negative. The remainder contained cell membrane staining for EGF-R that was not present on control slides (Fig. 1b). Of these, 28% (29/105) were highly EGF-R positive with over 60% of the cells showing intense membrane staining patterns (Fig. 1b). Assay cut-off points of 0, 60% and ≥ 60% tumour cells immunostaining have previously been observed by our group to be associated with an increased likelihood of failure to respond to endocrine measures [12].

Tumour cell nuclear staining using the Ki67 antibody was observed in 97% (102/105) of the tumours. The numbers of tumour cells expressing the antigen was, however, highly variable with 33 (31%) and 29 (28%) of the tumours showing 11–29% and ≥ 30% Ki67-positive cells (Fig. 1c). These cut-off points have previously been established by our group to be associated with an increased likelihood of failure to respond to endocrine measures [13].

Relationship between EGF-R, c-erbB-2 protein and Ki67

No significant correlations were observed between either EGF-R status (Fig. 2a) or increasing levels of EGF-R expression (Fig. 2b) and c-*erb*B-2 immunostaining (χ^2 1.97, $P < 0.2$; 3.44, $P < 0.3$, respectively) or between Ki67 and c-*erb*B-2 expression

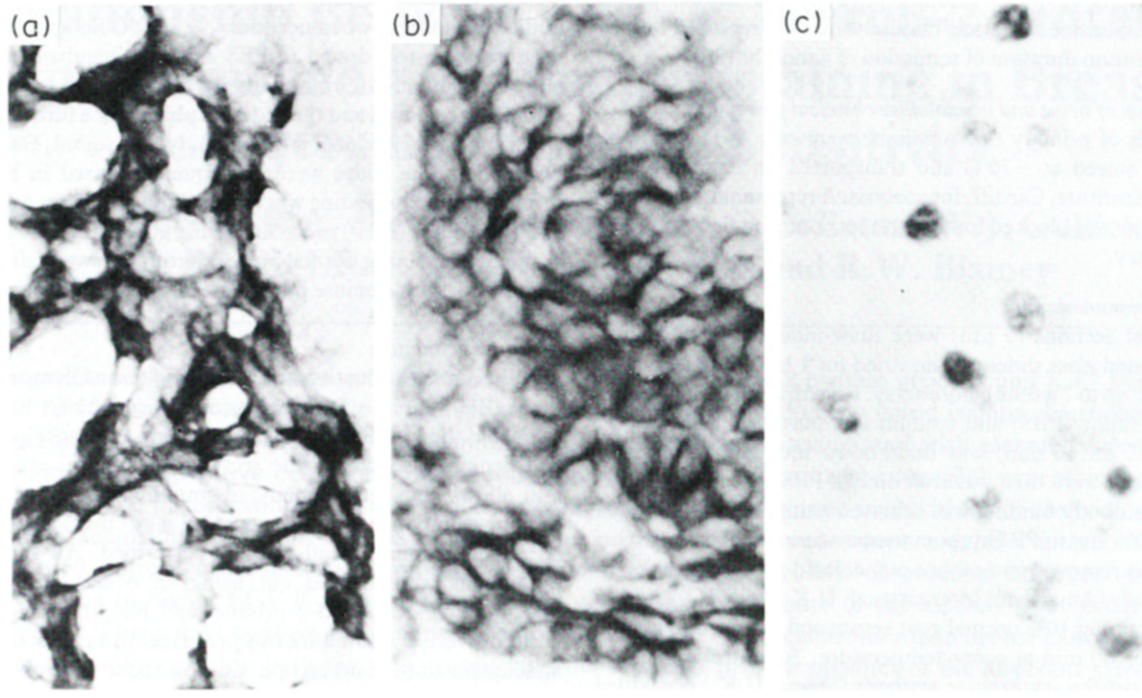

Fig. 1. Immunohistochemical staining of tumours for (a) c-*erb*B-2, (b) EGF-R and (c) Ki67.

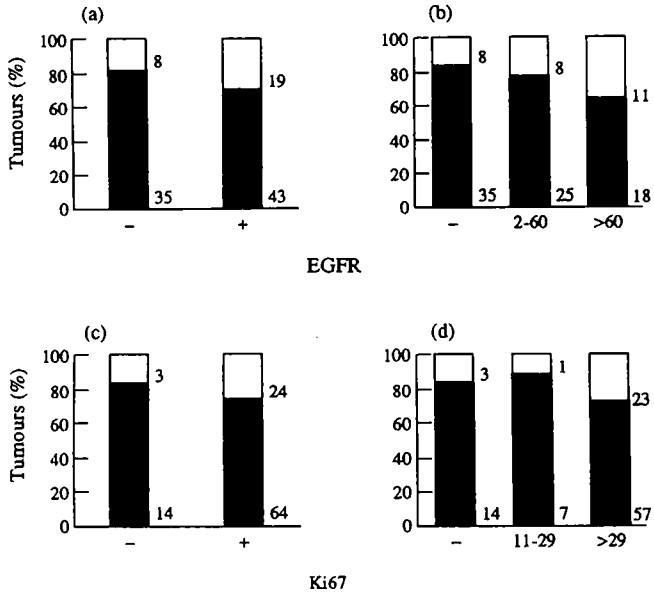

Fig. 2. Relationship between c-*erb*B-2 and EGF-R and Ki67. Correlations were sought between (a) EGF-R status, (b) EGF-R level, (c) Ki67 status, (d) Ki67 level and c-*erb*B-2 status. Tumours were considered to be c-*erb*B-2 and EGF-R positive if they showed any specific immunostaining and Ki67 positive if they showed greater than 10% positive cells. Additional cut-off points for the assays were set at > 60% EGF-R positive cells and ≥ 30% Ki67 positive cells in order to facilitate a quantitative evaluation of the data. The results are displayed as the percentage of tumours that are c-*erb*B-2 positive (□) or negative (■). The results have been analysed using a χ^2 statistic for contingency table analysis.

(Fig. 2c, d; χ^2 0.69, $P < 0.5$; 1.69, $P < 0.5$, respectively). An association was, however, recorded between either EGF-R status (Fig. 3a) or level (Fig. 3b, c) and nuclear Ki67 immunostaining (χ^2 12.67, $P < 0.001$; 13.66, $P < 0.01$; 20.89, $P < 0.07$, respectively). The mean level of Ki67 immunostaining in EGF-R positive and negative disease was 36 and 24%, respectively. The corresponding values for c-*erb*B-2 positive and negative tumours were 30 and 24%, respectively.

Analysis of the EGF-R and c-*erb*B-2 protein phenotypes according to the proportion of tumour cells expressing the Ki67 detected antigen showed that the lowest mean level of Ki67 immunostaining was observed in the EGF-R/c-*erb*B-2 negative group (Fig. 4a). Conversely, the co-expression of EGF-R and the c-*erb*B-2 product was on average associated with the highest Ki67 levels. No quantitative effect of EGF-R expression on Ki67 immunostaining was observed in c-*erb*B-2 positive disease (Fig. 4b).

*Correlation of EGF-R and c-*erbB-2 *protein and the responsiveness of breast cancer to endocrine measures*

Figure 5a shows that while EGF-R status is strongly associated with hormone insensitivity in the breast cancer population (χ^2 39.2, $P < 0.001$), with EGF-R expression being observed most frequently in patients failing to respond to endocrine measures, this was only weakly observed for c-*erb*B-2 protein staining (Fig 6a, χ^2 7.8, $P < 0.05$). Examination of the survival of these groups of patients after the initiation of endocrine therapy reflected their responsiveness to the antihormonal measures with patients with EGF-R negative tumours having the most favourable prognosis (Fig. 5b; mean survival time 4 years: $P < 0.001$ vs. EGF-R positive disease). Although there was no significant difference ($P = 0.1$) between the survival curves for patients with c-*erb*B-2 protein positive and negative tumours (Fig. 6b), the median survival time of women with c-*erb*B-2

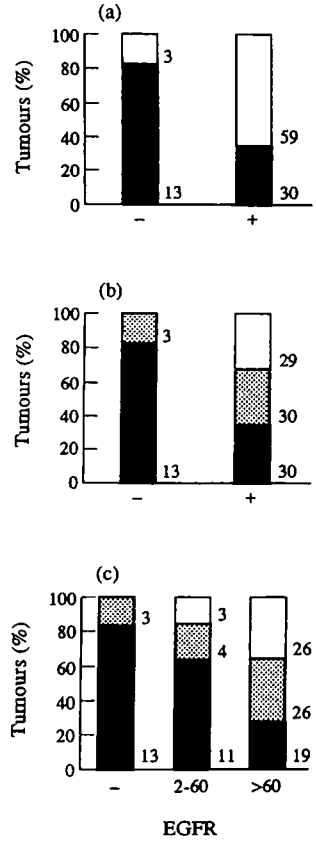

Fig. 3. Relationship between EGF-R and Ki67. Correlations were sought between (a) EGF-R status and Ki67 status, (b) EGF-R level and Ki67 status and (c) EGF-R level and Ki67 level. The tumours were classified and analysed as in Fig. 2. The results are displayed in (a) as the percentage of tumours that are Ki67 positive (> 10% positive cells, □) or negative (■) and in (b) and (c) as the percentage of tumours showing low (□), moderate (▨) and high (■) levels of Ki67 staining.

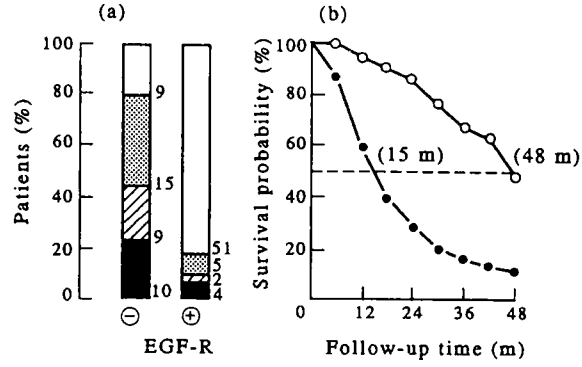

Fig. 5. Relationship between EGF-R status and response to endocrine therapy. The number of responsive (complete, ■, and partial ▨), static (no change, ▨) and progressive (□) tumours in EGF-R positive and negative disease are presented. The results are analysed using a χ^2 statistic for contengency table analysis. After the initiation of endocrine therapy the patients were followed up until death. Survival curves were produced by life table analysis for patients with EGF-R positive (●) and negative (○) disease. The figures in parenthesis show the mean survival time for individual groups of patients.

protein negative disease was 11 months longer than patients with positive tumours (28 and 17 months, respectively).

Subdivision of the EGF-R data according to c-*erb*B-2 expression showed divergent clinical results (Fig. 7a). Thus, while c-*erb*B-2 expression did not influence the proportion of patients responding to endocrine measures in the EGF-R negative group (χ^2 0.66, $P = < 0.5$) and had no detectable influence on their survival ($P = 0.78$), c-*erb*B-2 expression in EGF-R positive tumours appeared to be associated with a further loss of hormone sensitivity (χ^2 3.42, $P < 0.1$), with only 1/19 patients showing any response (partial) to the endocrine measures. No significant influence on their survival was discernible.

Subdivision of the EGF-R positive tumours according to their level of immunostaining shows that the relationships between EGF-R expression and loss of hormone sensitivity (Fig. 8a) is in part quantitative, with patients with highly EGF-R positive disease being predominantly unresponsive to endocrine measures (χ^2 44.1, $P < 0.001$) and having a very unfavourable outlook (Fig. 8b, $P < 0.0001$). Using this classification scheme,

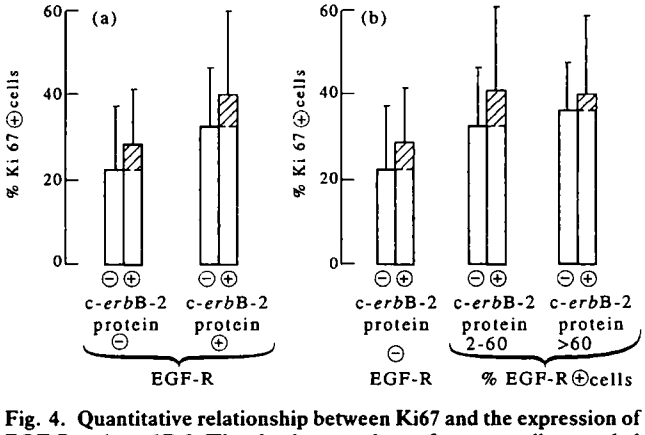

Fig. 4. Quantitative relationship between Ki67 and the expression of EGF-R and c-*erb*B-2. The absolute numbers of tumour cells recorded as Ki67 positive were examined in c-*erb*B-2 positive and negative tumours subclassified according to (a) EGF-R status and (b) EGF-R level. Differences between the groups of data were analysed using a Mann–Whitney non-parametric test and no significant associations were found.

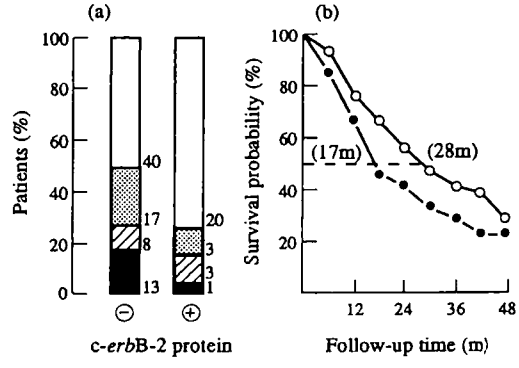

Fig. 6. Relationship between c-*erb*B-2 status and response to endocrine therapy. The number of responsive (complete, ■, and partial, ▨), static (no change, ▨) and progressive (□) tumours in c-*erb*B-2 positive (●) and negative (○) disease, together with patient survival curves following the initiation of therapy are presented. The figures in parenthesis show the mean survival time for individual groups of patients. The results were analysed as in Fig. 5.

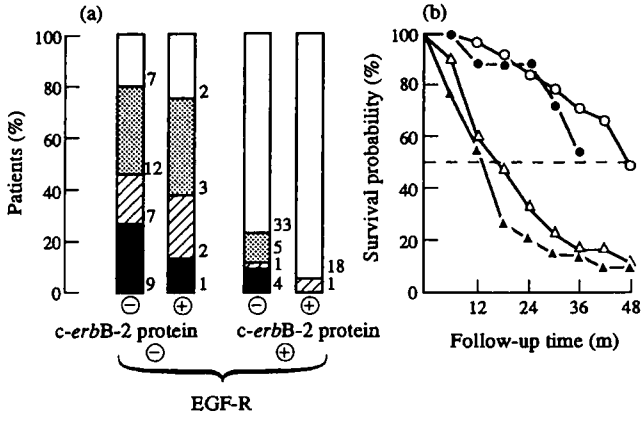

Fig. 7. Relationship between EGF-R and c-*erb*B-2 status and response to endocrine therapy. The number of responsive (complete, ■, and partial, ▨), static (no change, ▩) and progressive (□) tumours in EGF-R negative/c-*erb*B-2 negative (○) and positive (●) and EGF-R positive/c-*erb*B-2 negative (△) and positive (▲) disease, together with patient survival curves following the initiation of therapy are presented. The results were analysed as in Fig. 5.

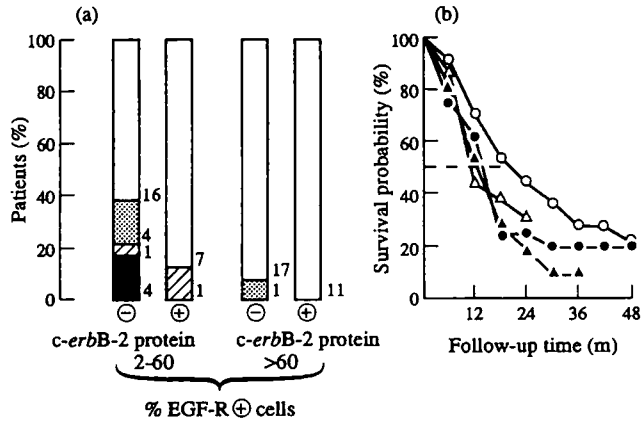

Fig. 9. Relationship between EGF-R level and c-*erb*B-2 status and response to endocrine therapy. The number of responsive (complete, ■, and partial, ▨), static (no change, ▩) and progressive (□) tumours in EGF-R moderate/c-*erb*B-2 negative (○) and positive (●) and EGF-R strong/c-*erb*B-2 negative (△) and positive (▲) disease, together with patient survival curves following the initiation of therapy are presented. The results were analysed as in Fig. 5.

the influence of c-*erb*B-2 protein on hormone sensitivity in EGF-R positive tumours is restricted to women with moderately EGF-R positive disease, with 7/8 patients with double positive samples failing to derive benefit from the endocrine treatments (Fig. 9a). Conversely, of those patients with moderately EGF-R positive disease, the highest response rate was seen in women with c-*erb*B-2 negative tumours (Fig. 9a, χ^2 3.76, $P < 0.1$), leading to an improvement in their survival (Fig. 9b, $P < 0.05$). Patients with highly EGF-R positive tumours have largely hormone independent neoplasms (Fig. 9a, χ^2 0.63, $P < 0.5$) and an unfavourable outlook (Fig. 9b) which is independent of c-*erb*B-2 protein staining. The prognosis of women with moderately EGF-R positive/c-*erbB*-2 positive tumours is similar to that observed for patients with highly EGF-R positive disease.

Subdivision of the data according to the menopausal status of the patients did not reveal any differences in response rates to the endocrine therapies between pre- and postmenopausal women for EGF-R and c-*erbB*-2 status and semiquantitative level (not illustrated). Insufficient patient numbers precluded further substratification of the data.

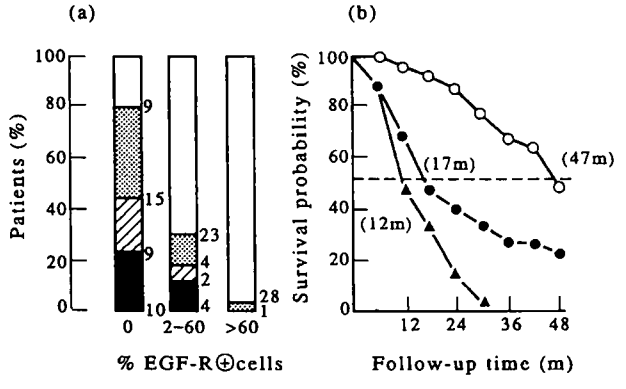

Fig. 8. Relationship between EGF-R level and response to endocrine therapy. The number of responsive (complete, ■, and partial, ▨), static (▩) and progressive (□) tumours in EGF-R negative (○), intermediary positive (●) and highly positive (▲) disease, together with patient survival curves following the initiation of therapy are presented. The figures in parenthesis show the mean survival time for individual groups of patients. The results were analysed as in Fig. 5.

DISCUSSION

The immunohistochemical localisation of the EGF-R [18], c-*erb*B-2 protein product [19–22] and the nuclear antigen detected by the Ki67 antibody [17] have each been shown to provide information on the early recurrence of breast cancer after mastectomy and poor survival. Moreover, significant interactions have recently been reported between two of these moieties with patients with c-*erb*B-2 product and EGF-R positive disease showing an increased number of early recurrences [10]. These data, in conjunction with reports indicating molecular interactions between the proteins [1–6], have been suggested to implicate a direct role for these receptors in the regulation of tumour cell replication and poor prognosis [11]. These concepts are, in part, supported by the current study in which we have observed that the expression of the EGF-R and the c-*erb*B-2 protein product both correlate with loss of hormone sensitivity in breast cancer and that an association exists between c-*erb*B-2 expression and worsened patient outlook and hormone insensitivity in moderately EGF-R positive tumours. Significantly, however, any role for the c-*erb*B-2 protein in directing these aspects of aberrant tumour behaviour appears of secondary importance to the EGF-R status, since its detection in our clinical breast tumour samples is less strongly associated with endocrine insensitivity than EGF-R (Figs 5, 6) and we have not demonstrated any influence of c-*erb*B-2 expression on the hormone sensitivity of breast cancer in either EGF-R negative tumours (Fig. 7) or in those cancers expressing high levels of this protein (> 60% EGF-R positive cells, Fig. 9). Indeed, while EGF-R expression positively correlates with elevated cell proliferation rates, no such significant association is seen with c-*erb*B-2 expression (Figs 2, 4).

Although the current clinical analysis of the c-*erb*B-2 protein product and the EGF-R cannot provide any information on the mechanisms of the proposed interactions between c-*erb*B-2 and the EGF-R, Stern and Kamps [5] and Kokai, *et al.* [4] have demonstrated that the protein encoded by *neu*/*erb*B-2 can act as a substrate for the EGF-R tyrosine kinase. Amplification of the c-*erb*B-2 gene in moderately EGF-R positive tumours could, therefore, be rationalised as providing a means of additionally activating the intracellular signalling pathways involved in

directing hormone insensitive tumour growth. In this respect the potential for the activation of protein kinase C by pathways modulated by both proteins [28, 29] may be significant since threonine/serine kinases appear to link the activities of agents with primary effects at the cell surface with nuclear processes, including the modulation of transcription factor function [30]. How these events are coupled to the control of cell proliferation and the loss of hormone sensitivity in breast cancer can, at the moment, only be a matter for speculation. Recent studies, however, have linked both steroid hormone and EGF-R pathways to modulation of the functional activities of products encoded by the c-*jun* and c-*fos* (proto)-oncogene families [30, 31]. These proteins belong to a group of interactive transcription factors the activation of which is an early event in cellular responses to mitogenic stimuli. Dysfunctional regulation of these genes or inappropriate activation of their products by mutations/aberrations in either steroid hormone or growth factor pathways could lead to elevated rates of cell proliferation and a loss of sensitivity to endocrine therapies.

Clearly our studies should be viewed as a starting point in which we have initially examined "in clinical specimens" proposals that have been largely derived from *in vitro* studies on highly selected cell lines. They have reiterated the importance of EGF-R determinations as a strong prognostic marker for cell proliferation, the loss of hormone sensitivity in breast cancer and poor survival [11] and have shown that in a specific subset of moderately EGF-R positive tumours, c-*erb*B-2 expression may provide additive information. In the future it is obvious that each of these pathways must be studied in greater detail and potential sources of growth factor receptor cross talk examined. Most importantly, there is a need to examine links between receptor content, ligand availability and cellular response pathways. Such studies are currently underway.

1. Stern DF, Heffernan PA, Weinberg RA. p185, a product of the *neu* proto-oncogene, is a receptor-like protein associated with tyrosine kinase activity. *Molec Cell Biol* 1986, **6**, 1729–1740.
2. Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-*erb*B-2 gene: a 185 KD gylcoprotein with tyrosine kinase activity. *Science* 1986, **232**, 1644–1646.
3. Kadowaki T, Kasuga M, Tobe K, *et al.* A M_r = 190 000 glycoprotein phosphorylated on tyrosine residues in epidermal growth factor stimulated cells is the product of the c-*erb*B-2 gene. *Biochem Biophys Res Commun* 1987, **144**, 699–704.
4. King CR, Borrello I, Bellot F, Comoglio P, Schlessinger J. EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3. *EMBO J* 1988, 7, 995–1651.
5. Kokai Y, Dobashi K, Weiner DB, Myers JN, Nowell PC, Greene MI. Phosphorylation process induced by epidermal growth factor alters the oncogenic and cellular *neu* (NGL) gene products. *Proc Natl Acad Sci USA* 1988, **85**, 5389–5393.
6. Stern DF, Kamps MP. *Egf*-stimulated tyrosine phosphorylation of p185*neu*: a potential model for receptor interactions. *EMBO* 1988, 7, 995–1001.
7. Kokai Y, Myers JN, Wada T, *et al.* Synergistic interactions of p-185 c-*neu* and the EGF receptor leads to transformation of rodent fibroblasts. *Cell* 1989, **58**, 287–292.
8. Lehvaslaiho H, Lehtola L, Sisonen L, Alitalo K. A chimeric EGF-R-*neu* oncogene allows EGF to regulate *neu* tyrosine kinase and cell transformation. *EMBO J* 1989, **8**, 159–166.
9. Harris AL, Nicholson S, Sainsbury RC, Fardon J, Wright C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with *neu*. *J Steroid Biochem* 1989, **34**, 123–131.
10. Barker S, Vincent GP. Epidermal growth factor in breast cancer. *Int J Biochem* 1990, **22**, 939–945.
11. Nicholson S, Sainsbury JRC, Halcrow P, *et al.* Expression of EGF receptors associated with lack of response to endocrine therapy in recurrent breast cancer. *Lancet* 1989, **i**, 182–185.
12. McClelland RA, Finlay P, Dixon AR, *et al.* Epidermal growth factor and oestrogen receptor expression in breast cancer: relationship to endocrine sensitivity. Submitted.
13. Nicholson RI, Bouzubar N, Walker KJ, *et al.* Hormone sensitivity in breast cancer: influence of heterogeneity of oestrogen receptor expression and cell proliferation. *Eur J Cancer* 1991, **27**, 908–913.
14. Nicholson S, Wright C, Sainsbury RC, *et al.* Epidermal growth factor receptor as a marker for poor prognosis in node-negative breast cancer patients: *neu* and tamoxifen failure. *J Steroid Biochem Molec Biol* 1990, **37**, 811–814.
15. Wright C, Nicholson S, Angus B, *et al.* Relationship between c-*erb*B-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br J Cancer* 1992, **65**, 118–121.
16. Gerdes J, Lemke H, Baisch H. Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki67. *J Immunol* 1984, **133**, 1710–1715.
17. Bouzubar N, Walker KJ, Griffiths K, Nicholson RI. Ki67 immunostaining in primary breast cancer: pathological and clinical associations. *Br J Cancer* 1989, **59**, 943–947.
18. Lewis S, Locker A, Todd JH, *et al.* Expression of epidermal growth factor receptor in breast carcinoma. *J Clin Pathol* 1990, **43**, 385–389.
19. Barnes DM, Lammie GA, Millis RR, Gullick WL, Allen DS, Altman DG. An immunohistochemical evaluation of c-*erb*B-2 expression in human breast carcinoma. *Br J Cancer* 1988, **58**, 448–452.
20. Walker RA, Gullick WJ, Varley JM. An evaluation of immunoreactivity for c-*erb*B-2 protein as a marker of poor short term prognosis in breast cancer. *Br J Cancer* 1989, **60**, 426–429.
21. De Potter CR, Beghin C, Makar AP, Vanderkerckhove D, Roels HJ. The *neu* oncogene protein as a predictive factor for haematogeneous metastases in breast cancer patients. *Int J Cancer* 1990, **45**, 55–58.
22. Lovekin C, Ellis IO, Locker A, *et al.* c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. *Br J Cancer* 1991, **63**, 439–443.
23. Venter DJ, Tuzi NL, Kumar S, Gullick WL. Overexpression of the c-*erb*B-2 oncoprotein in human breast cancinomas: immunohistochemical assessment correlates with gene amplification. *Lancet* 1987, **ii**, 69–72.
24. Van de Vijver MJ, Mooi WJ, Wisman P, Peterse JL, Nusse R. Immunohistochemical detection of the *neu* protein in tissue sections of human breast tumours with amplified *neu* DNA. *Oncogene* 1988, **2**, 175–178.
25. Hayward JL, Carbonne PP, Heuson JC, Kumaoka S, Rubens R. Assessment of response to therapy in advanced breast cancer. *Cancer* 1977, **39**, 1289–1293.
26. British Breast Group. Assessment of response to treatment in advanced breast cancer. *Lancet* 1974, **ii**, 38–39.
27. Gullick WL, Berger MS, Bennett PLP, Rothbard JB, Waterfield MD. Expression of the c-*erb*B-2 protein in normal and transformed cells. *Int J Cancer* 1987, **40**, 246–254.
28. Pandielli A, Lehvaslaiho H, Magni M, Alitalo K, Meldolesi J. Activation of an EGFR/*neu* chimeric receptor: early intracellular signals and cell proliferation responses. *Oncogene* 1989, **4**, 1299–1305.
29. Koskinen P, Lehvaslaiho H, MacDonald-Bravo H, Alitalo K, Bravo R. Similar early responses to ligand-activated EGF-R and *neu* tyrosine kinases in NIH3T3 cells. *Oncogene* 1990, 5, 615–618.
30. Van de Burg B, de Groot RP, Isbrucker L, Kruiger W, de Laat SW. Stimulation of TPA-response element activity by a co-operative action of insulin and oestrogen in human breast cancer cells. *Molec Endocrinol* 1990, **4**, 1720–1726.
31. Boyle WJ, Smeal T, Defize LHK, *et al.* Activation of protein kinase C decreases phosphorylation of c-*jun* at sites that negatively regulate its DNA-binding activity. *Cell* 1991, **54**, 573–584.

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