C-erbB-3 in human breast carcinoma: expression and relation to prognosis and established prognostic indicators

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Summary A series of 346 patients with primary operable breast cancer and a series of 145 patients with advanced breast cancer were investigated for c-erbB-3 protein expression using the monoclonal antibody RTJ1. Formalin-fixed, paraffin-embedded tumour samples were stained using a standard immunohistochemical method and staining was assessed on a four-point scale. The study aimed to observe the expression of the c-erbB-3 protein and investigate any relationship between expression and established prognostic indicators and prognosis. In both the primary and advanced series breast tumour tissue was found to stain heterogeneously for c-erbB-3. The staining was observed to be predominantly cytoplasmic and the majority of tumours exhibited moderate positivity. However, 15% and 35% of cases in the primary operable and advanced series respectively displayed strong positive staining. No significant difference was found between the staining in the primary and advanced series. In the primary operable breast cancers, no significant associations were demonstrated with overall survival, disease-free interval, regional recurrence, the presence of distant metastases, age, menopausal status, oestrogen receptor status, histological grade, lymph node stage, vascular invasion and c-erbB-2 protein expression. However, a significant association was seen between the degree of c-erbB-3 immunoreactivity and both tumour size ($P<0.01$) and tumour type prognostic group ($P=0.05$). No overall association between local recurrence was seen when the four groups of c-erbB-3 expression were analysed ($P=0.12$), but when those tumours showing no or weak staining were compared with those showing moderate and strong immunoreactivity it was seen that the latter were significantly more likely to develop local recurrence ($P=0.03$). In the series of patients with advanced disease, no significant associations were demonstrated with survival, UICC criteria, age, menopausal status, oestrogen receptor status, histological grade, c-erbB-2 status or the presence of vascular invasion. In conclusion this study found variable expression of c-erbB-3 protein in human breast carcinoma and an association with some recognised prognostic factors in those patients with primary operable breast carcinoma. It seems, however, unlikely that c-erbB-3 protein expression will emerge as a powerful enough prognostic factor to be of value in clinical practice.

Keywords: breast carcinoma; c-erbB-2; c-erbB-3; prognostic factor; immunohistochemistry; growth factor receptor

The c-erbB-3 gene is a recently identified member of the type I family of growth factor receptors. It is located on chromosome 12q13 and codes for a 180 000 molecular weight glycoprotein. The protein product shows considerable sequence homology to other members of the type I family of growth factor receptors, the epidermal growth factor receptor (EGFR) and the c-erbB-2 oncoprotein, especially in the tyrosine kinase domain (Lemoine et al., 1992; Rajkumar et al., 1993).

Expression of both EGFR and the c-erbB-2 oncoproteins have been associated with established prognostic indicators and a poorer prognosis in human breast carcinoma (Sainsbury et al., 1985, 1987; Nicholson et al., 1990; Grimaux et al., 1989; Walker et al., 1989; Wright et al., 1989; Gullick et al., 1991; Slamon et al., 1987). The sequence homology of the c-erbB-3 oncoprotein to the EGFR and c-erbB-2 oncoprotein has lead to interest in c-erbB-3 expression in breast cancer.

Using immunohistochemical techniques, c-erbB-3 expression in normal breast tissue has been shown to be weak to moderate (Prigent et al., 1992). Expression in breast tumour tissue is heterogeneous. However, overexpression, defined as intensity greater than normal tissues, has been demonstrated to occur in approximately 13 to 23% of cases (Lemoine et al., 1992; Prigent et al., 1992) again using immunohistochemical techniques.

Although associations have been found between high expression of c-erbB-3, lymph node metastasis and c-erbB-2 expression, no other associations have been found with established prognostic indicators or prognosis (Lemoine et al., 1992; Gasparini et al., 1994). This study used the RTJ1 antibody for immunocytochemistry to investigate c-erbB-3 expression and the relationship between overexpression and prognostic indicators and prognosis.

Methods

Patients

The patients in this study presented with primary operable or advanced breast cancer to a single surgical team (RWB, JRF) at the City Hospital, Nottingham. A total of 359 patients with primary operable breast cancer and 155 patients with advanced breast cancer were entered into the study. A small number of cases with pure carcinoma in situ and those in which insufficient tumour tissue was available for immunohistochemical assessment were excluded from the study leaving 346 cases in the primary operable series and 145 cases in the advanced series.

Patients with primary operable disease were treated in a standard fashion by simple or subcutaneous mastectomy or tumour excision and radiotherapy. At the time of surgery, nodes were sampled and the tumour staged as described previously (Haybittle et al., 1982). Tumours were measured in the fresh state in three perpendicular planes immediately after excision. Fresh tumour blocks were snap frozen or fixed in neutral buffered formalin and embedded in paraffin wax for...
further assay. Histological grade (Elston and Ellis, 1991), tumour type (Ellis et al., 1992), oestrogen receptor status (ER), vascular invasion (VI) (Pinder et al., 1994) and c-erbB-2 status (Lovekin et al., 1991) were recorded for each tumour sample. ER status was assessed in these patients by a dextran-coated charcoal method and a cut-off of 10 fmol mg⁻¹ protein was used for analysis. For analysis of tumour type four prognostic groups were used as described previously (Pereira et al., 1995).

Patients were followed up after surgery at three monthly intervals for 18 months and then every 6 months for 5 years, then annually. The disease-free interval (DFI) was taken as the time in months from the date of the primary treatment to the first local, regional or distant recurrence. The overall survival (OS) was taken as the time in months from the date of the primary treatment to the time of death.

For the patients with advanced disease UICC criteria were recorded as: 1, complete response; 2, partial response; 3, static; 4, progression of disease. For analysis UICC criteria 1–3 were grouped and compared with those patients who had progressive disease (category 4). In this group of patients a cut-off of 5 fmol mg⁻¹ protein was considered positive for ER analysis.

**Immunohistochemistry**

The tumour tissue was stained with a monoclonal IgM kappa antibody, RT1J, raised to a synthetic peptide from the cytoplasmic domain of the human c-erbB-3 protein (Rajkumar et al., 1993). A standard avidin–biotin immunohistochemical technique was used. Sections (3 μm) were cut from formalin-fixed, paraffin-embedded tumour samples, dewaxed in xylene and taken to alcohol. Endogenous peroxidase activity was blocked with hydrogen peroxide in methanol and non-specific binding sites were blocked with swine serum. Sections were incubated with the RT1J antibody at a 1:10 dilution. This dilution was shown to give optimal staining in pilot experiments. Binding of the primary antibody was demonstrated by a standard avidin–biotin complex technique; biotinylated goat anti-mouse immunoglobulin followed by preformed soluble complexes of avidin and biotinylated horseradish peroxidase (Dako). Diaminobenzidine was used as the chromogen with copper sulphate enhancement and haematoxylin was used as the counterstain. Sections were also processed in the absence of RT1J antibody to act as negative controls and tumours of known c-erbB-3 immunoreactivity were stained as positive controls on each run.

Staining was assessed according to the degree of cytoplasmic staining on a four-point scale: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive. Owing to heterogeneous immunoreactivity within most sections, the whole tumour was systematically assessed by grading fields every 0.2 cm within the section. If the tumour area within the section was small or diffuse throughout the stroma then the whole slide was scanned and graded. The overall intensity for each tumour was taken to be that shown by the majority of fields. Blind reassessment of a random 15% of the sections in each series ensured consistency in assessment.

Adjacent normal breast epithelial tissue was also assessed. Immunoreactivity in normal tissue was found to be heterogeneous, and, if present, of weak or moderate intensity. For the purpose of this study only those tumours exhibiting strong positivity were considered to overexpress c-erbB-3.

**Statistical analysis**

Relationships between variables were sought using chi-squared analyses. Survival data were examined by the life-table method (Mantel–Cox). All statistical analyses were performed using SPSSX software.

**Results**

A variable degree of immunoreactivity was observed in breast tumours. Within the carcinomas staining was found to be heterogeneous and predominantly cytoplasmic with membrane staining seen in less than 1% of cases (Figure 1). The majority of tumours in both series exhibited moderate immunoreactivity but a substantial proportion in both the primary and advanced series exhibited strong positivity (Figure 2). The cytoplasmic appearance of the stain varied from finely granular to diffuse.

**Primary operable breast cancer**

Seventeen of the 346 sections (5%) in the primary series showed no immunoreactivity with the RT1J antibody, 111 (32%) were weakly positive, 167 (48%) showed moderate positivity and 51 (15%) were scored as showing strong positivity.

Associations with other prognostic variables and survival are shown in Table I. No correlation was found between c-erbB-3 overexpression and OS, DFI or regional recurrence, age, menopausal status, ER status, histological grade, lymph node stage, the presence of distant metastases, VI and c-erbB-2 protein expression. An association was seen between the intensity of c-erbB-3 immunostaining with the RT1J antibody and factors indicative of poor prognosis in this series of patients with primary operable breast cancer. A trend was seen between c-erbB-3 immunostaining and tumour type group (P = 0.05); patients in the poor prognostic type group more often showed moderate or strong staining, whereas tumours of...
Table I Associations of c-erbB-3 immunostaining (none, weak, moderate or strong) with other prognostic factors, survival and recurrence in patients with primary operable breast carcinoma

| Factor                | Cut off | Chi-squared | P  |
|-----------------------|---------|-------------|----|
| Age                   | <30, 31–40, 41–50, 51–60, >60 years | 7.0 | 0.86 |
| Menopausal status     |         |             |    |
| ER status             | 10 fmol mg⁻¹ protein | 6.9 | 0.07 |
| Histological grade    | 1, 2, 3 | 11.0 | 0.28 |
| Lymph node stage      | 1, 2, 3 | 12.4 | 0.19 |
| Size                  | <2, 2.1–5.0, >5.0 cm | 18.4 | <0.01 |
| Local recurrence      |         | 5.76 | 0.12 |
| Regional recurrence   |         | 6.5  | 0.37 |
| Distant metastases    |         | 4.3  | 0.22 |
| C-erbB-2 Membrane     |         | 4.0  | 0.26 |
| VI                    | None, probable, definite | 8.4 | 0.21 |
| Tumour type           | 1, 2, 3, 4* | 16.9 | 0.05 |
| OS                    | Overall statistic (3 d.f.) | 0.2 | 0.98 |
| DFI                   | Overall statistic (3 d.f.) | 4.0 | 0.26 |

*See Pereira et al., 1995.

A large and important study on the role of c-erbB-3 expression in breast cancer was conducted by Pereira et al. (1992). The study involved 323 patients with breast cancer and demonstrated that c-erbB-3 expression was associated with worse prognosis. In addition, the study noted a strong correlation between c-erbB-3 expression and other known prognostic factors, such as tumor size, histological grade, and menopausal status.

**Discussion**

There has been considerable interest in the amplification or regulation of members of the type I family of tyrosine kinase growth factor receptors in human breast carcinoma. The first member of the family, epidermal growth factor receptor (EGFR), a 170 kDa transmembrane glycoprotein, has been shown to play a role in normal breast development and differentiation. Overexpression of EGFR has also been correlated with established prognostic indicators, with an important inverse relationship between EGFR and tumor status (Sainsbury et al., 1985, 1987; Nicholson et al., 1990).

The second member of the family, c-erbB-2, is a 185 kDa transmembrane glycoprotein. Comparative molecular and immunohistological studies have demonstrated an association between amplification of the c-erbB-2 gene and strong cell membrane immunoreactivity for the protein in some solid tumors and, in particular, human breast carcinoma. Such amplification of the gene, detected either by molecular investigation or through immunocytochemical demonstration of membrane protein has, in large series of breast cancers, been shown to be associated with a poorer prognosis. In 1987, Slamon et al. carried out an initial study that demonstrated a significant relationship between c-erbB-2 immunoactivity and a shorter DFI and OS (Slamon et al., 1987). Further studies have supported this association (Wright et al., 1989; Gullick et al., 1991; Lovekin et al., 1991). An inverse association has also been demonstrated between c-erbB-2 expression and ER status (Slamon et al., 1987).

The c-erbB-3 gene was first cloned by Kraus et al. in 1989 and subsequently by Plowman et al. in 1990. Its protein product is a 180 kDa transmembrane glycoprotein that shows considerable sequence homology to the EGFR and the c-erbB-2 protein, especially in the tyrosine kinase domain.

A study examining c-erbB-3 protein expression in normal human adult and fetal tissues demonstrated that most developing human tissues, except haemopoietic tissues, express c-erbB-3 and expression is not restricted to proliferating cells. Normal adult breast tissue shows a moderately intense staining of luminal epithelial cells of breast acini and a weaker reactivity of basal myoepithelial cells. This normal distribution is distinctive and different from that observed with EGFR and c-erbB-2. Reactivity is predominantly cytoplasmic and no membrane reactivity of normal tissue has been observed in these early studies (Prigent et al., 1992).

Poller et al. used a polyclonal antibody raised to the c-erbB-3 protein to examine c-erbB-3 expression in a variety of adenocarcinomas. C-erbB-3 protein expression was detected in a series of 13 out of 14 primary breast carcinomas. Expression of the c-erbB-3 protein was found to be a common event in adenocarcinomas but its role in neoplastic progression remained unclear (Poller et al., 1992).

In a more detailed study of breast carcinoma, Lemoine et al. (1992) showed consistently higher levels of the c-erbB-3 oncprotein in cell lines but a wide range of expression in resected primary human breast tumours. In the breast tumours overexpression was seen in 22% of cases, the predominant pattern being cytoplasmic with membrane immunoreactivity being seen in one case only. Investigation of associations with tumour size, histological grade, stage and...
survival showed correlation only with lymph node metastatic disease. No relationship with overall prognosis was demonstrated.

In our series, using the IgM monoclonal antibody RTJ1 (Rajkumar et al., 1993), we have examined the largest series of patients presenting with breast carcinoma to date, including 346 patients with primary operable breast cancer and 145 patients with advanced disease. Cytoplasmic reactivity was the predominant pattern seen and less than 1% of tumours showed positive membrane reactivity. As previously demonstrated by Lemoine et al., the degree of expression varied (Lemoine et al., 1992). The majority of tumours expressed moderate immunoreactivity. Fifteen percent and 35% of cases in the primary and advanced series respectively demonstrated strong positive staining which for the purposes of this study was considered overexpression, as reactivity in normal breast epithelial tissue, although heterogeneous, was confined to negative, weak or moderate levels of intensity. Different levels of frequency of overexpression were identified in the two groups of patients in this study but this failed to reach statistical significance.

No significant associations were demonstrated between c-erbB-3 expression and survival in either patients with primary operable breast cancer or those with advanced disease and no correlation with DFI was found in the former group. In particular there was no relationship with lymph node status in this series which is in contrast to the study carried out by Lemoine et al. (1992). In the patients with operable disease, however, an association was seen between greater intensities of immunoreactivity with RTJ1 antibody and both increased tumour size and a weaker association with poor prognostic type group. In addition those tumours which showed moderate or strong immunoreactivity with c-erbB-3 antibody appeared to be more likely to develop locally recurrent disease. The associations we report here have not been previously documented. Nevertheless, it seems unlikely that immunohistochemical assessment of c-erbB-3 expression will provide sufficiently powerful prognostic information to be clinically useful, the associations with other prognostic factors we describe here are based on differences in intensity of immunoreactivity rather than presence or absence of staining.

A recent study has demonstrated that the growth factor ligand heregulin binds to the c-erbB-3 receptor (Carraway et al., 1994). The same group have also demonstrated little or no tyrosine kinase activity following stimulation and binding of heregulin with the c-erbB-3 receptor. However, in cells expressing both c-erbB-2 and c-erbB-3 a high-affinity binding site is generated and on stimulation produces unique tyrosine residues (Sliwkowski et al., 1994). This is in contrast to the interaction and complex formation between c-erbB-2 and c-erbB-4, where both receptors have active tyrosine kinase components which are capable of autophosphorylation (Plowman et al., 1993). The potential for type I tyrosine kinase receptors to produce different combinations of heregulin-stimulated heterodimeric complexes could explain some of the varied biological activities that have been demonstrated with this group of receptors (Carraway and Cantley, 1994). We have found no association between overexpression of c-erbB-2 and c-erbB-3 assessed immunohistochemically.

In common with other published series we have demonstrated virtually ubiquitous cytoplasmic expression of c-erbB-3 protein at weak to strong levels. The c-erbB-3 gene sequence codes for transmembrane types of protein but membrane localisation of c-erbB-3 protein appears to be a rare phenomenon and is much lower in frequency than EGFR and c-erbB-2 proteins in invasive breast cancer. It is known that EGFR and c-erbB-2 are internalised by endocytosis after ligand binding. The predominant cytoplasmic localisation of c-erbB-3 protein could indicate internalised, non-functional or non-membrane-associated protein. The c-erbB-3 protein may be an orphan receptor (Kraus et al., 1993) but, if the parent of the orphan in terms of signalling were c-erbB-2 or c-erbB-4 as has recently been suggested, then the c-erbB-3 protein may be an important cofactor in the biological effects of type I growth factor receptors.

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