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Expression of c-*erbB*-2 Oncoprotein: A Prognostic Indicator in Human Breast Cancer

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ABSTRACT

Sections of formalin-fixed, paraffin-embedded tissue from 185 primary breast carcinomas were stained immunohistochemically using a polyclonal antibody against the c-*erbB*-2 oncoprotein. Positive staining, which is known to correlate with gene amplification, was associated with earlier relapse, shorter postrelapse survival, and shorter overall survival. Lymph node, epidermal growth factor receptor, and estrogen receptor status, tumor size, and histological grade also had prognostic significance but, applying multivariate analysis, only lymph node status was a more important predictor of relapse-free and overall survival than staining for the oncoprotein. Positive staining was correlated with negative estrogen receptor status and high histological grade, but there was no association with either lymph node or epidermal growth factor receptor status or tumor size. Expression of the c-*erbB*-2 oncoprotein appears to be an important independent indicator of prognosis in human breast cancer.

INTRODUCTION

The c-*erbB*-2 or *neu* oncogene, first identified in chemically induced rat neuroblastomas (1), is a member of the tyrosine kinase oncogene family and codes for a M_r 185,000 transmembrane glycoprotein which is structurally similar to, but distinct from, the epidermal growth factor receptor (2–4). Amplification of the gene, which lies on human chromosome 17 (5), has been demonstrated in adenocarcinomas arising at a number of different sites (6–9) and in breast cancer is associated with increased c-*erbB*-2 oncoprotein expression (10, 11). In a study of 86 patients with axillary lymph node-positive breast cancer, amplification was related to earlier recurrence and shorter survival (12), and Berger *et al.* (11) demonstrated a correlation between oncoprotein expression by breast tumors and indicators of poor prognosis (positive lymph node status and high tumor nuclear grade). EGFR[2] status is an independent prognostic indicator in primary breast cancer (13), and there is recent evidence of interaction (transmodulation) between EGFR and c-*erbB*-2 product. In tumor cell lines expressing both receptors, treatment with EGF resulted in phosphorylation of the c-*erbB*-2 protein (14, 15). Therefore, using an immunohistochemical method, we have assessed c-*erbB*-2 oncoprotein expression in a series of breast cancers studied prospectively for EGFR status to investigate further the prognostic significance of the c-*erbB*-2 oncogene and its interaction with EGFR.

PATIENTS AND METHODS

Patients

Tumor tissue from 185 patients with operable breast carcinoma was collected over a 50-mo period. Patients underwent either lumpectomy or simple mastectomy, with removal of axillary nodes if palpable at operation. Radiotherapy to the breast and drainage areas was given to all lumpectomy patients and to node-positive mastectomy patients. Those undergoing lumpectomy also subsequently received an iridium wire implant.

Methods

Within 30 min of surgery, lumpectomy and mastectomy specimens were received in the Pathology Department where tumor dimensions were recorded, routine diagnostic blocks taken, and portions of tumor removed for receptor radioligand binding assays. Levels of epidermal growth factor receptor were measured as described elsewhere (16); ERs were assayed during a dextran-coated charcoal method (17). Cut-off points for EGFR and ER-positive tumors were 10 fmol/mg of membrane protein and 5 fmol/mg of cytosolic protein, respectively.

Expression of c-*erbB*-2 oncoprotein was demonstrated in 3-mm sections of the routine formalin-fixed, paraffin-embedded blocks using a polyclonal antibody raised in rabbits against a synthetic peptide (21N) representing residues 1243 to 1255 of the predicted oncoprotein sequence (18). Sections were incubated at 4°C overnight with primary antibody at a dilution of 1:400 (equivalent to 0.17 μg/ml) and then stained by applying biotinylated swine anti-rabbit immunoglobulin (Dako) at a dilution of 1:1000 followed by streptavidin-biotin-peroxidase complex (Dako) at a dilution of 1:100. The peroxidase reaction was developed using diaminobenzidine as chromagen. Sections were counterstained with hematoxylin and mounted. Tumors were scored by assessing the site of staining (membrane and/or cytoplasm), the proportion of cells staining (0%, 1 to 49%, 50 to 100%), and the intensity of staining [weak (+), strong (++)]. This was carried out independently by two observers, and a consensus on discrepant cases was reached by reassessment on a double-headed microscope. Histological grade was determined using a modification of Bloom and Richardson's method (19). Scoring of 21N staining and histological grade was made without prior knowledge of survival data. Axillary nodes were sampled in 106 patients (57%), allowing histological determination of LN status: the lymph node positive (LN+) group comprised patients with any histological evidence of nodal metastasis.

All 187 patients were included in some analyses, since the frequency of expression of c-*erbB*-2 and its coexpression with EGFR in primary tumors were to be assessed. Since lymph node status is no longer a prognostic factor after relapse (20–22), all patients were included for analysis of postrelapse survival.

Patients were followed up for a maximum of 60 mo; the median follow-up time was 24 mo. Relapse-free and overall survival was measured from the time of surgery. Only deaths due to breast cancer were considered for the purposes of the study. Relapse was defined as any evidence of metastasis or local recurrence.

Survival curves were prepared by the life table method, with comparisons between curves by the log rank test (23) using a program designed for Acorn/BBC microcomputers (B. Angus). Relationships between variables were examined by the χ^2 test. Multivariate analysis was performed using Cox's proportional hazards regression model (24) and the BMDP statistical package (25).

RESULTS

Immunohistochemical Staining. Tumor cell membrane staining of variable intensity and extent was demonstrated in 107 tumors (58%), which could be readily divided into groups showing strong (++; see Fig. 1) or weak (+) staining. In 31 carcinomas (17%), 50% or more of the tumor cells showed ++

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[2] The abbreviations used are: EGFR, epidermal growth factor receptors; ER, estrogen receptor; LN, lymph node; EGF, epidermal growth factor.

membrane staining, both observers identifying the same 31 cases; this group was regarded as "21N positive" (21N+) for the purposes of the study, and all others, including the 76 (41%) showing less intense or more focal membrane staining, as "21N negative" (21N−). In the great majority of tumors, there was also diffuse cytoplasmic staining of tumor cells; only 12 tumors failed to demonstrate convincing cytoplasmic staining of more than half their cells. The intensity of such staining was variable, but only in very occasional cells was it as strong as that seen along the membranes of the 21N+ group. The possible significance of cytoplasmic staining was not addressed further in this study. Staining of tumor cell membranes and cytoplasm was completely abolished by preincubating the antibody with the immunizing peptide (Fig. 1). Weak cytoplasmic staining of normal breast epithelium, myoepithelial cells, smooth muscle, endothelium, sweat gland epithelium, and the more superficial layers of the epidermis was also noted in some sections.

Prognostic Significance of Staining with 21N Antibody. Life table analysis demonstrated an increased risk of earlier recurrence ($P < 0.005$; Fig. 2*a*) and shorter overall survival ($P < 0.001$; Fig. 2*b*) for the 21N+ group which was evident from 12 mo onwards. In addition, postrelapse survival was significantly shorter in this group, with the probability of survival falling to zero within 2 yr ($P < 0.05$; Fig. 2*c*). Within the 21N-group, times to recurrence and death for that group of patients ($n = 76$) with tumors showing weak and/or more focal staining were not significantly different from the group ($n = 78$) showing complete absence of tumor staining [χ^2 (log rank) for relapse-free survival = 0.323, for overall survival = 0.12].

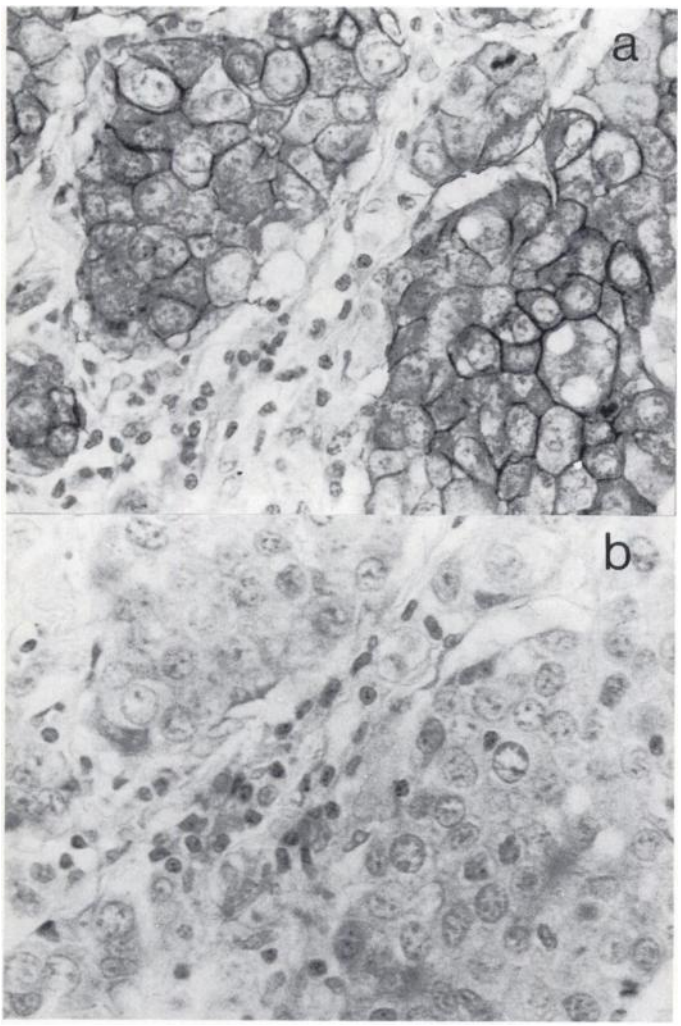

Fig. 1. Invasive ductal carcinoma of the breast: immunohistochemical staining for c-*erbB*-2 oncoprotein without (*a*) and with (*b*) preincubation of antibody with immunizing peptide (21N).

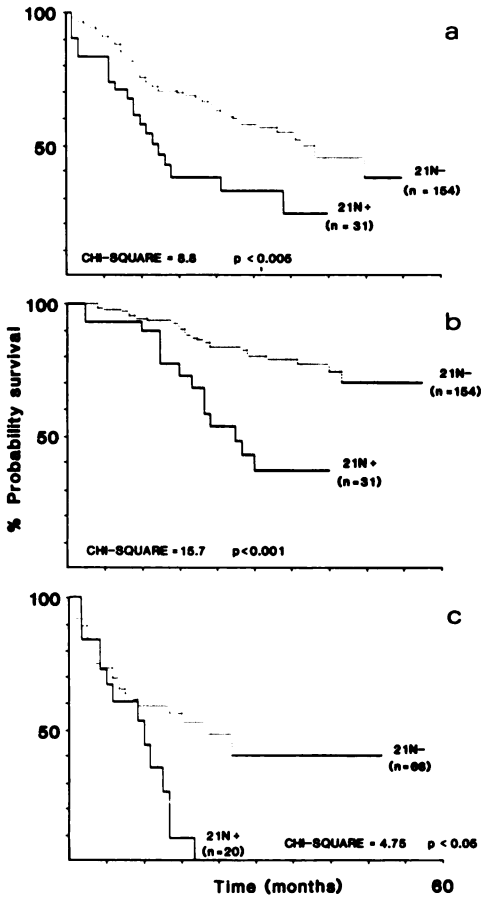

Fig. 2. Curves for relapse-free survival (*a*), overall survival (*b*) and postrelapse survival (*c*), stratified by 21N staining.

Table 1 *Prognostic significance of LN, EGFR, and ER status*

	n	Relapse-free survival (*P*)	Overall survival (*P*)
LN, + *vs.* −	106	<0.005	<0.005
EGFR, + *vs.* −	184	<0.005	<0.005
ER, + *vs.* −	185	<0.05	<0.01

Positive ER status, negative EGFR status, and the absence of lymph node metastasis were individually associated with superior relapse-free and overall survival (Table 1). However, within each of these better prognosis subgroups, patients with 21N+ tumors had a reduced overall survival time (Figs. 3, *a* and *c*, and 4*b*; Table 2). This was true also for those in the worse prognosis ER−, EGFR+, and node-positive subgroups (Figs. 3, *b* and *c*, and 4*b*; Table 2), and 21N staining was associated with shorter relapse-free survival of patients who were ER+, EGFR−, or node positive (Fig. 4*a*; Table 2).

When patients were divided into four groups on the basis of EGFR an 21N status, there was a significant trend for poorer survival with receptor expression, the "double positive" group having the poorest prognosis; this suggests that EGFR and 21N are additive as prognostic indicators (Fig. 3*c*).

Prognostic Significance of Tumor Size and Histological Grade. Tumor size and grade were both significant predictors of overall survival, those patients with large or high grade tumors having a poorer prognosis [χ^2 (trend) for size = 6.152 ($P < 0.025$), for grade = 6.331 ($P < 0.025$)].

Correlation of 21N Staining with Other Prognostic Variables. 21N+ tumors showed a tendency to be more poorly differentiated and to be ER− (Table 3). Although statistically signifi-

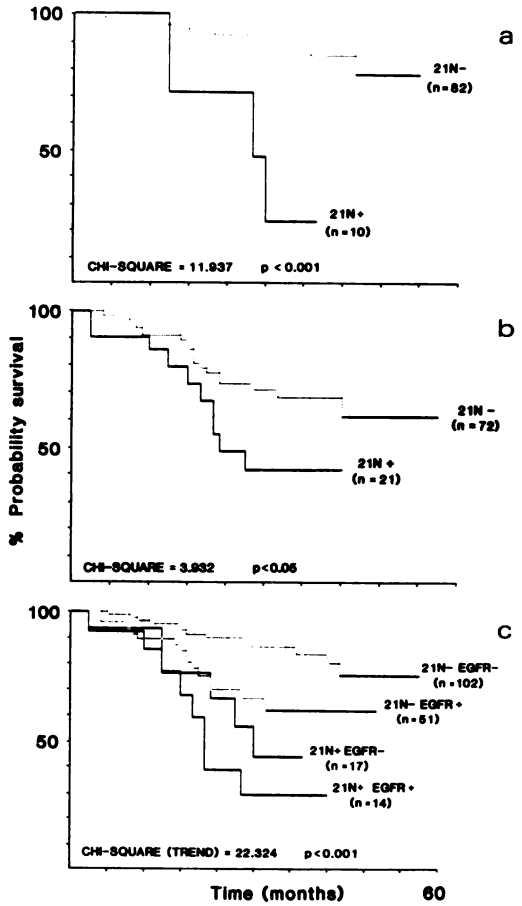

Fig. 3. Curves for overall survival for patients with positive ER status (*a*), negative ER status (*b*), and positive and negative EGFR status (*c*), stratified by 21N staining.

cant, these associations were not close: the great majority of ER− and Grade III tumors were 21N−. There was no correlation between 21N status and either LN involvement, EGFR status, or tumor size.

Multivariate Analysis. To compare the prognostic significance of 21N staining, LN, EGFR, and ER status, tumor size, and grade, multivariate analyses were performed. Full data were available for 102 cases. Significant variables for relapse-free survival were node status ($P = 0.014$), 21N staining ($P = 0.025$), and tumor size ($P = 0.048$), and for overall survival were node status ($P = 0.0025$) and 21N staining ($P = 0.04$). ER status, EGFR status, and tumor grade were not significant in either analysis.

DISCUSSION

Using two synthetic peptides (20N and 21N), Gullick *et al.* have raised antibodies in rabbits against the human c-*erbB*-2 protooncogene product (18). In a recent study using the 20N antibody, Berger *et al.* demonstrated a relationship between immunohistochemical staining of paraffin-embedded sections of breast tumors and both positive node status and high nuclear grade (11). They concluded that determining tumor c-*erbB*-2 oncoprotein levels might be of prognostic importance in breast cancer, and the findings of our study have confirmed this. We assessed oncoprotein expression immunohistochemically using the 21N antibody, which gave excellent results on formalin-fixed, paraffin-embedded material. In a large series of breast cancers which had been studied prospectively in an investigation of EGFR status, intense membrane staining by the 21N antibody of more than 50% of tumor cells was associated with a poor prognosis, being a significant predictor of both early recurrence and short postrelapse survival.

The c-*erbB*-2 protooncogene is amplified in up to a third of primary breast cancers (10–12, 26), and several groups have used the 20N and 21N antibodies to show that there is a correlation between amplification and oncoprotein expression (10, 11, 27). Our results are therefore consistent with those of Slamon and coworkers (12) who found c-*erbB*-2 amplification

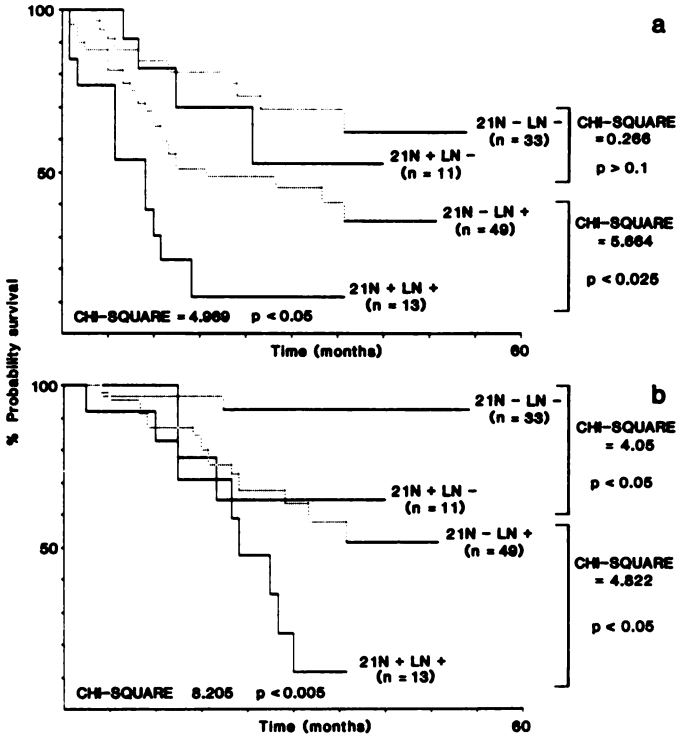

Fig. 4. Curves for relapse-free survival (*a*) and overall survival (*b*) for patients of known lymph node status (lymph nodes involved, LN+; lymph nodes not involved, LN−), stratified by 21N staining.

Table 2 *Prognostic significance of 21N staining within patient subgroups*

	No. of patients			21N+ *vs.* 21N−	
	Total	21N+	21N−	Relapse-free survival (*P*)	Overall survival (*P*)
LN					
+	62	13	49	<0.025	<0.05
−	44	11	33	NS[a]	<0.05
EGFR					
+	65	14	51	NS	<0.05
−	119	17	102	<0.01	<0.005
ER					
+	92	10	82	<0.025	<0.001
−	93	21	72	NS	<0.05

[a] NS, not significant.

Table 3 *Staining with 21N antibody related to histological grade and ER status*

	21N staining	
	−	+
Histological grade		
I + II	77	10
III	68	21
$\chi^2 = 4.44$, $P = 0.035$		
ER status		
−	71	21
+	83	10
$\chi^2 = 4.833$, $P = 0.028$		

to be an indicator of shorter time to relapse and death in a series of lymph node-positive patients. In our study, oncoprotein expression was a predictor of poorer prognosis not only for node-positive patients, but also in the node-negative, ER+ and EGFR− groups. Thus, among patients regarded as having a more favorable prognosis, 21N staining identified those who, it might be hoped, would benefit from more aggressive therapy at an early stage. Multivariate analysis confirmed the prognostic value of 21N staining, which was second in importance only to node status as a predictor of both time to relapse and overall survival. Oncoprotein expression was independent of other prognostic variables, showing a weak correlation only with histological grade and ER status. Machin *et al.* (27), who also used the 21N antibody, found no association between staining and ER, EGFR, or node status. In the same study, staining was not a predictor of time to relapse for a series of 102 tumors (overall survival data were not apparently available). We were also unable to demonstrate a relationship between staining and prognosis in an initial investigation using only 91 of the total series of 185 tumors,[3] but, even in this smaller group, patients with 21N+ tumors showed a tendency to shorter survival.

The c-*erbB*-2 protein is similar in structure to EGFR [the predicted amino acid sequences of the two molecules are approximately 50% homologous (4)] and is presumed to be a membrane receptor, but the nature of its normal function(s) and ligand(s) remains unknown. Given, however, the association of both c-*erbB*-2 and EGFR expression to poorer prognosis and their apparent independence from other variables (13), it is tempting to postulate a role for both proteins as components, rather than simply markers, of mechanisms responsible for breast tumor progression. For example, binding of ligand (growth factor) to, or constitutive functioning of, increased numbers of receptors might lead, via increased protein kinase activity, to promotion of cell replication or metastasis. Kadowaki *et al.* (14) have recently demonstrated EGF-induced tyrosine phosphorylation of the c-*erbB*-2 oncoprotein, suggesting that the latter can act as a substrate for EGFR tyrosine kinase. In this context, it is interesting to note the apparent additive effect in our study of c-*erbB*-2 oncoprotein and EGFR expression on patient prognosis.

To conclude, our results indicate that c-*erbB*-2 expression is an important independent indicator of prognosis in breast cancer. Using the 21N antibody, staining for the oncoprotein can be performed on routine paraffin-embedded material at the time of mastectomy, but it should be possible to use the antibody on diagnostic preoperative fine needle aspirates of breast tumors, and we are currently evaluating this. Finally, as others have noted, the recent demonstration, that a monoclonal antibody to the c-*erbB*-2 oncoprotein could inhibit the growth of c-*erbB*-2-transformed NIH 3T3 cells implanted into nude mice (28), has considerable therapeutic implications. Recently, the prognostic significance of c-*erbB*-2 expression detected by a different antibody was reported (29). There was a significant reduction in overall survival for patients with c-*erbB*-2 overexpression, but significance was not maintained in a multivariate analysis. However, adjuvant chemotherapy was given, in contrast to our study. The possibility that c-*erbB*-2 expression may be related to sensitivity to chemotherapy in poor prognosis patients needs further evaluation in prospective studies.

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[3] Unpublished observations.

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