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c-erbB-2 Positivity is a factor for poor prognosis in breast cancer and poor response to hormonal or chemotherapy treatment in advanced disease

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Abstract

The aim of this work was to evaluate the prognostic and predictive values of c-erbB-2 in breast cancer. 650 patients were enrolled. The amplification/overexpression of *c-erbB-2* from fresh frozen or paraffin-embedded breast tumour tissue samples was analysed by polymerase chain reaction (PCR) technique (75%), immunohistochemically (17%) or by Southern blot analysis (8%). 126 patients (19%) were positive for c-erbB-2. 148 patients developed metastatic disease, but only 35 were positive for c-erbB-2. Positivity for c-erbB-2 was significantly associated with node positivity, large tumour size, high grade of malignancy, low receptor status, postmenopausal status, and with a shorter overall survival. In multivariate regression analysis, only tumour size and nodal involvement were risk factors for poor survival when analysed separately together with c-erbB-2 and receptor status. Metastatic patients with c-erbB-2 positivity had a significantly shorter survival and disease-free survival (DFS) than the c-erbB-2-negative patients. 29 advanced patients with c-erbB-2 positivity showed a poor response rate to hormonal, non-anthracycline-based and anthracycline-based therapies. Positivity for the c-erbB-2 is a poor prognostic factor in breast cancer, but it also emerges as predictive of the response to hormonal or chemotherapy treatment once the disease has recurred.

Keywords: c-erbB-2; Prognosis; Predictive factor; Breast cancer

1. Introduction

The *c-erbB-2* oncogene encodes a 185-kDa epidermal growth factor receptor-like membrane glycoprotein (EGFR) with an external cellular transmembrane domain and an intracellular domain with tyrosine kinase activity [1]. This oncogene is amplified and overexpressed in approximately 15–30% of breast carcinomas [2], as a result of which cell division is increased and the cell growth rate is higher. Amplification may also be associated with transformation to the cancer cell phenotype [3,4]. Amplification and/or overexpression of *c-erbB-2* in breast cancer occurs most often in axillary node-positive patients [2] and tumours are characterised by a high malignancy grade [5], large size [2], absence of oestrogen and progesterone receptors [2], high S-phase fraction, mutated *TP53* and high nuclear grade [6,7]. An association with early disease recurrence and short survival has been also reported, particularly in patients with axillary node involvement [2]. A limited prognostic value has been described for this gene [8,9], as well as its value as an independent predictor of disease recurrence and survival [5,9].

Retrospective studies have shown that breast cancer patients with tumours overexpressing c-erbB-2 had significantly lower rates of response to hormonal therapies for metastatic disease [10–12] and that disease-free survival (DFS) and overall survival (OS) were also shorter in patients with node-negative disease treated with adjuvant tamoxifen, if there was overexpression of c-erbB-2 in their tumours [12].

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Resistance to the CMF (cyclophosphamide-methotrexate-5-fluorouracil) regimen has been reported by Gusterson and colleagues [13], whose patients with c-erbB-2-positive tumours treated with a combination CMF plus prednisolone exhibited significantly lower rates of DFS and OS 6 years after treatment than the c-erbB-2-negative patients. Similar results were presented by Allred and colleagues [14]. Interpretation of these results must nevertheless be tempered by a reservation on account of the small number of patients involved. The predictive value of c-erbB-2 for the response to anthracyclines also remains controversial, as some studies have been unable to demonstrate any predictive value of c-erbB-2 with regard to standard doses of anthracyclines used for metastatic disease or as neoadjuvant therapy [15,16], whereas others have shown greater benefit for patients overexpressing c-erbB-2 and treated with anthracycline-based regimens in an adjuvant setting [8,17,18]. A relative predictive value for c-erbB-2 overexpression emerges from these studies, which is more related to the use of intensive anthracycline-based regimens (data not shown), but none of them demonstrated any clear benefit of this finding in terms of survival. In fact, they suggest that c-erbB-2 overexpression probably does not correlate with anthracycline resistance.

We set out here to examine c-erbB-2-positive breast cancer patients retrospectively in order to find out whether c-erbB-2 in the tumour cells was associated with poor clinical outcome and to study the association of c-erbB-2-positivity with hormonal or anthracycline-based chemotherapy resistance in a subset of patients with metastatic disease.

2. Patients and methods

2.1. Clinical characteristics of the patients

650 patients operated on for primary breast cancer at Oulu University Hospital or Tampere University Hospital, Finland, during the years 1982–1998 were taken for analysis. The median follow-up time was 36 months (range 1–328 months). The patients had undergone either mastectomy or segmental breast resection and axillary lymph node evacuation. Radiation therapy was given to all those with positive lymph nodes and/or after segmental breast resection. Primary tumour size and axillary lymph node involvement were determined according to the TNM classification from the International Union Against Cancer (UICC). 420 patients were node-negative and 211 node-positive. The nodal status of 19 patients was not known. Histopathology and malignancy grade were recorded according to the World Health Organization (WHO) criteria and using a modification of the Blooming–Richardson system, described by Elson and Ellis [19]. Oestrogen and progesterone receptors were analysed either by radioimmunoassays or by immunohistological methods.

164 c-erbB-2-negative patients and 53 c-erbB-2-positive ones had received adjuvant treatment. 28 of those who were positive for c-erbB-2 were treated with adjuvant chemotherapy and 25 with hormonal therapy, the corresponding numbers in the c-erbB-2 negative group being 67 and 97. The adjuvant-treated patients in the recurrence group are described in Table 1.

148 patients out of the original 650 developed metastatic disease during the follow-up, 81 developed soft tissue metastases and 67 visceral and/or bone metastases (49%). In this group, 17 patients who were c-erb-2-positive developed soft tissue metastases (49%) and visceral and/or bone metastases were found in 18 patients (51%), the figures for c-erB-2-negative patients being 64 (57%) and 49 (43%), respectively.

2.2. c-erbB-2 analyses

c-erbB-2 gene amplification was defined either by the polymerase chain reaction (PCR) or by Southern blot analysis, and c-erbB-2 overexpression by immunohistochemistry. 486 fresh-frozen samples of breast tumour tissue were analysed by PCR. The PCR method used here for the amplification of *c-erbB-2* follows the principle described by Frye and coworkers [20]. This procedure employs co-amplification of the target gene and a single copy reference gene by PCR. Amplification is then evaluated by comparing the PCR product band intensity of the target gene with that of the reference gene. The reference gene used here was the *interferon-gamma* (IFN-γ)) gene (PCR), and the primers used in the assay were *c-erbB-2* 5′primer 5′CCT CTG ACG TCC ATC ATC TC 3′; *c-erbB-2* 3′primer 5′CCT CTG ACG 5′TCT TTT CTT TCC CGA TAG GT 3′, and *IFN-γ* 3′primer 5′CTG GGA TGC TCT TCG ACC TC 3′. PCR amplification of DNA extracted from the breast carcinoma tissue was carried out for 30 cycles of 95°C 1 min, 55°C 1 min and 72°C 1 min in the presence of

Table 1
Numbers of c-erbB-2-positive and negative patients in relation to adjuvant treatment in the recurrence group

Treatment	c-erbB-2-negative patients $n=113$ n (%)	c-erbB-2-positive patients $n=35$ n (%)
CMF	13 (12)	5 (14)
FEC	4 (4)	3 (9)
Intensive FEC[a] + tamoxifen	6 (5)	2 (6)
Tamoxifen	27 (24)	6 (17)
No adjuvant treatment	63 (56)	19 (54)

CMF, cyclophosphamide, methotrexate and 5-fluorouracil; FEC, 5-fluorouracil, epirubicin and cyclophosphamide.

[a] Dose escalation of epirubicin and cyclophosphamide.

Ultrapure deoxynucleotide triphosphate (dNTP) Set (Pharmacia) and Taq DNA polymerase (Promega). The products were then electrophoresed in an ethidium bromide-stained agarose gel and the amplification band intensities evaluated. A molecular weight control, positive and negative amplification controls and a control with water replacing the DNA were run in each assay.

55 paraffin-embedded breast tumour samples were taken for the *c-erbB-2* Southern blot analysis. 10 μg DNA from each tumour specimen was digested with *Hind*|*III* and resolved on a 0.8% agarose gel electrophoresis for 17 h at 40V. After blotting onto a nylon membrane, the DNA specimens were hybridised with rat 1.5 kb *BamHI c-erbB-2* cDNA inserts [21] in a hybridisation mixture containing 50% (v/v) formamide. After washes with 1×SSC, 0.1% (w/v) sodium dodecyl sulphate (SDS) at room temperature and at 60°C, the membranes were subjected to autoradiography for 1–14 days. Each membrane also contained 10 μg of control DNA extracted from blood, which functioned as a reference for normal gene dosage. To double-check the hybridisation results, the membranes were also subjected to rehybridisation with a control probe, representing complete removal of the old signal.

c-erbB-2 protein overexpression was studied immunohistochemically in 109 paraffin-embedded breast carcinomas. A mouse monoclonal c-erbB-2 antibody (NLC-CB11) was obtained from Novacastra Laboratories (Newcastle upon Tyne, UK). After a 30-minute incubation with the primary antibody at a dilution of 1:40, a biotinylated secondary anti-mouse antibody (Dakopatts, Copenhagen, Denmark) was used at a dilution of 1:200–300, followed by an avidin–biotin-peroxidase complex (Dakopatts). The colour was developed with diaminobenzidine, whereafter the sections were lightly counterstained with haematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were carried out by substituting non-immune mouse serum for the primary antibody. A breast carcinoma case previously known to be c-erbB-2-positive was used as a positive control for the immunostainings.

2.3. Statistical analyses

Significance was tested using standard statistical tests: Student *t*-test, Mann–Whitney, U-test, Pearson and Chi-Square. Survival curves were tested for homogeneity by the Kaplan–Meier methodology and compared using the log rank, Breslow or Tarone–Ware test. $P < 0.05$ was considered statistically significant. Cox regression analysis and stepwise regression analysis were used to find significant predictors of survival. Logistic regression analysis was used to analyse the response to hormonal therapy using the receptors and c-erbB-2 status as covariates.

3. Results

3.1. Positive c-erbB-2 in breast cancer patients

126 patients (19%) out of the 650 cases analysed were positive for c-erbB-2, the remaining 524 (81%) being c-erbB-2 negative. Positivity was found in 35 of the recurrence patients (24%). The metastatic sites in the c-erbB-2-positive group were in soft tissue (49%), bone (31%) and visceral tissue (20%), and those in the c-erbB-2-negative group in the same tissues in the proportions 57%, 22% and 22%, respectively. There was no statistically significant correlation between the metastatic site and *c-erbB-2* amplification. The first metastatic site in the whole group appears to be the bone.

3.2. Positive c-erbB-2 in relation to other prognostic factors

Positive c-erbB-2 was significantly associated with axillary node-positive disease ($P = 0.003$), large tumour size ($P = 0.06$), tumours with a high malignancy grade ($P = 0.0003$) and a low oestrogen ($P = 0.0001$) and progesterone ($P = 0.0001$) receptor content (Table 2). *c-erbB-2* amplification also correlated well with menopausal status in postmenopausal women ($P = 0.002$). No correlation with histopathological status was seen.

In multivariate Cox stepwise regression analysis, tumour size and nodal involvement emerged as independent prognostic factors when analysed separately in combination with c-erbB-2, indicating a 2.9 (90% CI 1.9–4.4) risk of death in node-positive patients. For patients with tumour sizes T3 or T4 the risk of death was 2.7 (90% CI 1.4–5.1) and 4.8 (90% CI 2.5–9.5), respectively. c-erbB-2 status did not reach significance in this model, nor when analysed in combination with tumour size, nodal involvement and receptors. There was only one death in the malignancy grade I group, indicating that grade cannot be included in the model, as it does not fulfil the proportional hazards condition.

3.3. Positive c-erbB-2 status and its relationship to survival

Positive c-erbB-2 status was closely related to a shorter DFS ($P = 0.003$) (for all patients with metastatic disease) (Fig. 1) and overall survival (for patients with a positive c-erB-2 status) ($P = 0.02$) (Fig. 2). The median survival time for the c-erbB-2-positive patients was 112 months, versus 226 months for the negative ones. Among the patients with a recurrence of the disease, the median DFS in the c-erbB-2-positive group was 13 months, compared with 23 months among the negative ones and the same relationship was found in the node-positive patients c-erbB-2 positivity being significantly related to a shorter survival time ($P = 0.05$), as was also

the case in the node-negative patients who were positive for the *c-erbB-2* gene ($P = 0.07$).

Survival as a whole was shorter in the premenopausal group ($P = 0.04$), but the shortest time of all was recorded in the postmenopausal patients who were positive for the *c-erbB-2* gene. Patients with advanced disease and an amplifying *c-erbB-2* oncogene show statistically significantly shorter survival than c-erbB-2-negative metastatic patients ($P = 0.01$) (Fig. 3).

Table 2
Positive c-erbB-2 in relation to other breast tumour characteristics

Factor	Patients *n* (%)	c-erbB-2 status	*P* value
Menopausal status			0.002[a]
pre	151 (23)	negative	
	54 (8)	positive	
post	370 (57)	negative	
	72 (11)	positive	
Unknown	3 (0.5)		
Node status			
N+	156 (24)	negative	0.003[a]
	55 (8)	positive	
N−	352 (54)	negative	
	68 (10)	positive	
Unknown	19 (3)		
Tumour size			
T1	282 (43)	negative	0.06[a]
	52 (8)	positive	
T2	197 (30)	negative	
	59 (9)	postitive	
T3	23 (4)	negative	
	9 (1)	postiive	
T4	15 (2)	negative	
	5 (1)	positive	
Unknown	8 (1)		
Histology			
ductal	373 (57)	negative	0.66[a]
	97 (15)	positive	
lobular	89 (14)	negative	
	19 (3)	positive	
other histology	42 (6)	negative	
	7 (1)	positive	
DCIS	15 (2)	negative	
	3 (0.5)	positive	
Unknown	5 (1)		
Grades			
I	62 (10)	negative	0.0003[a]
	7 (1)	positive	
II	146 (22)	negative	
	25 (4)	positive	
III	108 (17)	negative	
	45 (7)	positive	
Unknown	257 (40)		
Receptor status			
ER+	486 (75)	negative	0.0001[b]
ER−	122 (19)	positive	
Unknown	42 (6)		
PR+	486 (75)	negative	0.0001[b]
PR−	120 (18)	positive	
Unknown	44 (7)		

DCIS, ductal carcinoma *in situ*; ER+, oestrogen receptor positive; ER−, oestrogen receptor negative; PR+, progesterone receptor positive; PR−, progesterone receptor negative.

[a] Pearson Chi Square

[b] Mann–Whitney U.

3.4. *c-erbB-2 as a predictor of the response to treatment for metastatic disease*

29 metastatic patients with c-erbB-2-positive and 112 metastatic c-erbB-2-negative patients were treated with either hormonal therapy, or anthracycline-based or non-anthracycline-based chemotherapy. All the treatment modalities for metastatic disease used with every individual patient were included in the analyses. The responses to all these forms of treatment are shown in Table 3. Patients positive with c-erbB-2 had a significantly poorer rate of response to hormonal therapy or anthracycline or non-anthracycline-based chemotherapy (Table 3).

In logistic regression analysis, patients with ER-positivity responded better to the hormonal therapy ($P = 0.08$) than those with negative receptors. The risk of no response to hormonal therapy for patients with a positive c-erbB-2 was 5.2 (90% CI 1.9–15.2) when analysed together with receptor status.

4. Discussion

Since the discovery of the *c-erbB-2* gene, there has been a great deal of interest in finding out its relationship with the clinical course of breast cancer, specifically to already known prognostic parameters. The findings of this study dealing with the correlation of positive erbB-2 with standard prognostic factors, e.g. large tumour size, positive axillary nodal involvement, high malignancy grade and low hormone receptor content in the tumour, are in general consistent with previous studies [2,22,23]. A close correlation with menopausal status was also observed here, in that a high proportion of c-erbB-2 positivity was shown in the tumours of the postmenopausal women and this group was characterised by very short survival by comparison with the other cancer groups.

The finding that c-erbB-2 was found in 19% of the 650 cases analysed here is in-line with the proportions described in previous studies [2,24]. The proportion of c-erbB-2 positivity in patients in whom the disease recurred was 24%. No correlation between metastatic site and c-erbB-2 status was observed in our patients. However, previous studies also showed a certain correlation of this gene with the sites of metastases, in that cases developing visceral metastases expressed this gene three times more frequently than tumours which developed metastases at other sites [9].

Although c-erbB-2 positivity was shown here to be clearly correlated with shorter survival, the strongest

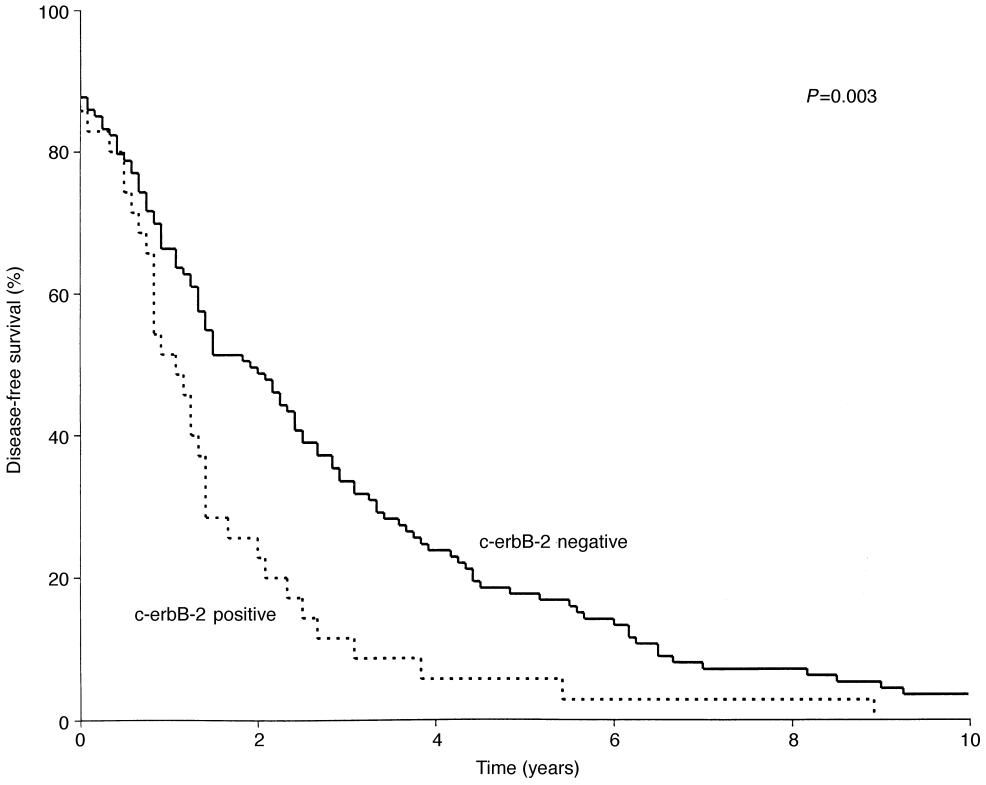

Fig. 1. Disease-free survival (DFS) of patients with metastatic disease and positive for c-erbB-2 (- - - - -) ($n=35$) or negative (—) ($n=113$).

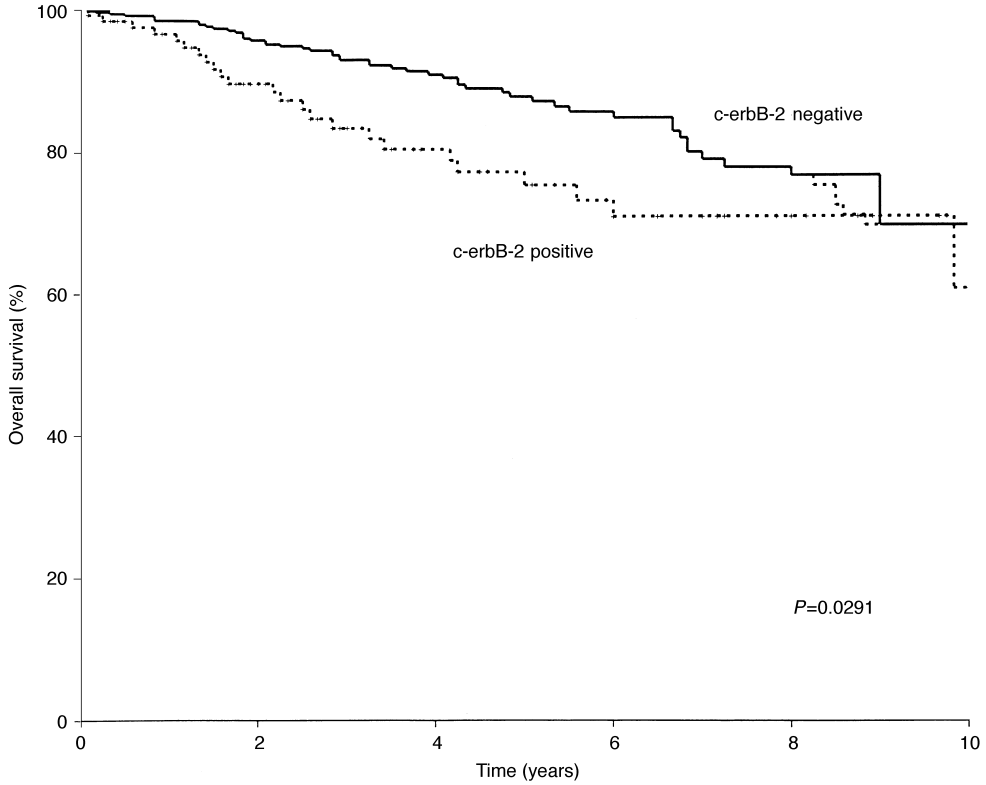

Fig. 2. Overall survival (OS) in the whole group in relation to c-erbB-2; - - - = positive ($n=126$); — = negative ($n=524$).

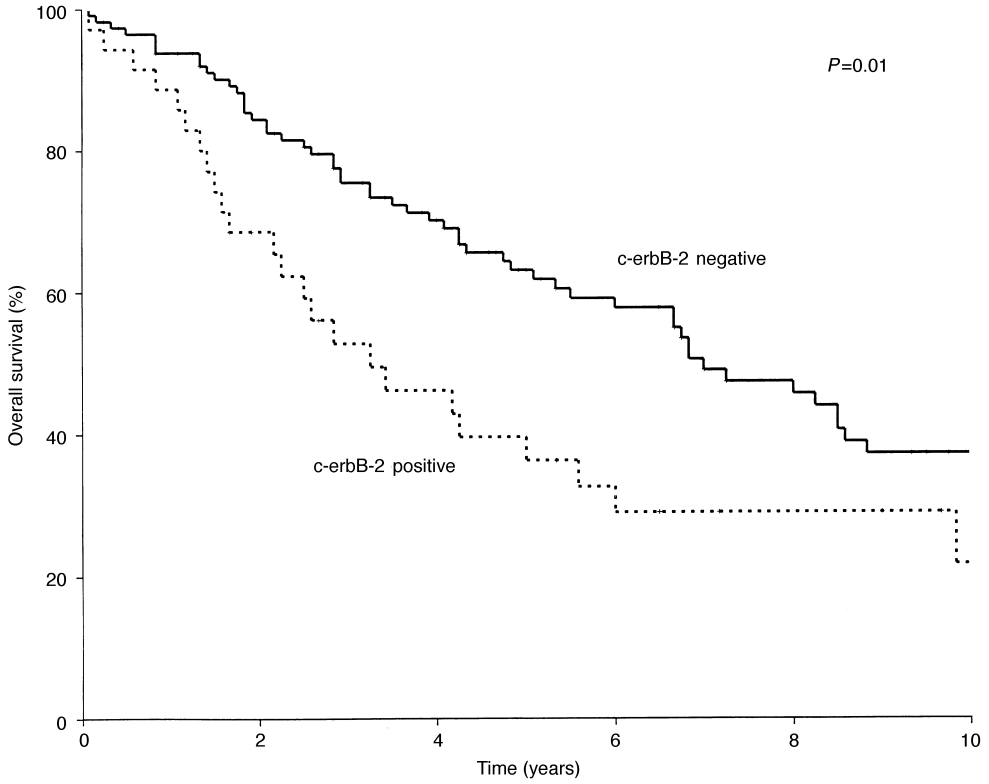

Fig. 3. Overall survival of patients with metastatic disease in relation to c-erbB-2; - - - - = metastatic disease, positive ($n=35$); — = metastatic disease, negative ($n=113$).

factors predicting poor survival in the multivariate Cox stepwise regression analysis were tumour size and positive nodal status. C-erbB-2 does not reach statistical significance as a predictor of poor survival when analysed in combination with tumour size, nodal involvement and receptors. Patients with metastatic disease who were positive for c-erbB-2 in their tumours had a statistically significantly shorter DFS and lower OS than those with metastatic disease who were negative for c-erbB-2. Previous studies have reported these associations with survival particularly in the case of patients with node-positive disease [2,24], but here in this study, node-positive as well as node-negative ones had a shorter survival time, if they were positive for c-erbB-2.

Many reports have suggested a poor response to treatment among patients exhibiting amplification/overexpression of the *c-erbB-2* gene. This was also observed here, with the patients having advanced breast cancer, exhibiting c-erbB-2 positivity and when exposed to hormonal therapy showed very poor response rates. This resistance seems to be almost predictable where hormonal treatment is concerned and particularly applies to the use of tamoxifen. Decreased tamoxifen efficacy both in adjuvant therapy and in the treatment of metastatic disease has been frequently reported [2,10,12,25,26], and it has been hypothesised that c-erbB-2 overexpression could indirectly lessen tumour sensitivity to tamoxifen by inactivating or suppressing

Table 3
Response to chemotherapy and/or hormonal therapy in c-erbB-2-positive or negative patients with metastatic breast cancer[a]

Treatment	c-erbB-2-negative patients			c-erbB-2-positive patients		
	Response to treatment (%)					
	CR + PR	SD	PD	CR + PR	SD	PD
Anthracycline-based therapy	17/49 (35)	7/49 (14)	25/49 (51)	1/23 (4)	3/23 (13)	19/23 (83)
Other chemotherapy	10/37 (27)	4/37 (11)	23/37 (62)	1/15 (7)	0/15	14/15 (93)
Hormonal therapy	40/83 (48)	19/83 (23)	24/83 (29)	3/23 (13)	4/23 (17)	16/23 (70)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

[a] Treatment rates in c-erbB-2-negative patients: anthracycline-based therapy ($n=49$), other chemotherapy ($n=37$) and hormonal therapy ($n=83$) and in c-erbB-2-positive patients: anthracycline ($n=23$), other chemotherapy ($n=15$) and hormonal therapy ($n=23$).

ERs [27]. In a series of studies involving patients with advanced breast cancer treated with megoestrol acetate or fadrozole as second-line hormonal therapy, the response rates were very low in patients with c-erbB-2 positivity [10].

29 of the present c-erbB-2-positive patients with metastatic disease were treated with different chemotherapy modalities, and a poor response was almost the rule among them, the response rates remaining low regardless of the chemotherapy regimen used, e.g. non-anthracycline or anthracycline-based. Substantial differences in response rate were observed relative to a similar set of patients who were negative for c-erbB-2. Preliminary studies have suggested that c-erbB-2 overexpression may be related to partial resistance to adjuvant CMF and adjuvant cyclophosphamide, doxorubicin, 5-fluorouracil (CAF), but that this resistance to chemotherapeutic agents may be overruled by using increasing doses of CAF [8,14,17,18]. Some benefit from adding CAF to tamoxifen in the adjuvant setting in the case of receptor-positive, node-positive and HER2-positive patients has also been described (data not shown). A close correlation has been suggested between c-erbB-2 overexpression and topoisomerase-IIα expression [28]. Topoisomerase-II is a target for doxorubicin action, and thus increased levels of this enzyme are associated with increased sensitivity to doxorubicin *in vitro* [29]. Some other reports, however, did not find any clear correlation between the *c-erbB-2* gene and the response to standard anthracycline-based therapies for metastatic breast cancer, making this issue more controversial [15]. Klijn and colleagues (1999) have also reported that CMF was significantly more effective as first-line therapy for advanced breast cancer with erbB-2-amplified tumours (data not shown). Otherwise the first Milan CMF trial showed that both HER-2-positive and HER-2-negative patients benefited from adjuvant CMF (data not shown).

Many methods have been used over the years for the determination of erbB-2, as is apparent from the present data. The methods used to determine amplification of the *c-erbB-2* gene are reliable, but depend on obtaining representative tumour tissue samples for evaluation purposes and are time-consuming and expensive. On the other hand, immunohistochemical methods are simple to use, inexpensive and applicable to paraffin blocks, but the interpretation of the results is highly subjective because of the enormous heterogenity found in the tumour staining and the lack of standardisation.

Our results indicate a poor prognosis and shorter DFS and OS in patients with breast cancer who are positive for c-erbB-2. Those with advanced disease have a poor response to any treatment whatsoever, hormonal or chemotherapy, and c-erbB-2 thus emerges as a negative predictive factor for breast cancer. Our results support the view that use of the antibody trastuzumab in combination with chemotherapy may provide a step forward in terms of treatment results in cases of advanced disease and in the adjuvant setting for breast cancer patients exhibiting the *c-erbB-2* gene phenotype in their tumours.

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