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Overexpression of c-erbB-2 is related to a higher expression of vascular endothelial growth factor (VEGF) and constitutes an independent prognostic factor in primary node-positive breast cancer after adjuvant systemic treatment

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Abstract

The aim of this study was to investigate possible associations between the expression of c-erbB-2 and the angiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), p53 status, routine breast cancer prognostic factors and survival. Expression of c-erbB-2, VEGF, bFGF, and p53 protein was determined with an enzyme-linked immunosorbent assay (ELISA) in 656 patients with primary breast cancer (median follow-up time of 83 months). In 60 cases, we also used immunohistochemistry (IHC) for c-erbB-2 evaluation, to be used as a reference for the ELISA. Overexpression of c-erbB-2 was significantly related to a higher expression of VEGF, lower bFGF content, negative steroid receptor status, and a high S-phase fraction. In multivariate analysis, c-erbB-2 was an independent prognostic factor for relapse-free survival (RFS) and overall survival (OS) in all patients, and in node-positive patients, irrespective of the adjuvant systemic therapy. Combined survival analyses regarding c-erbB-2 and VEGF yielded additional prognostic information.

Keywords: c-erbB-2; HER-2; Angiogenesis; VEGF; bFGF; p53; Primary breast cancer; Adjuvant therapy; Relapse; Survival

1. Introduction

c-erbB-2 (also known as HER-2/neu or EGFR2) is one of four members in the epidermal growth factor receptor family (EGFR) with tyrosine kinase activity. So far, at least 12 ligands have been identified which can bind to EGFR1, EGFR 3 and EGFR 4, while c-erbB-2 is an orphan receptor [1]. Amplification or overexpression of c-erbB-2 has been identified in approximately 10–20% of invasive breast cancers and is associated with shorter disease-free (DFS), as well as shorter overall survival (OS) times [2,3]. c-erbB-2 is a predictive factor with regard to monoclonal antibody therapy with trastuzumab, but also a possible predictive factor for anthracycline-based chemotherapy [4]. Whether it is a predictive factor for endocrine therapy is more controversial. Vascular endothelial growth factor (VEGF) is suggested as a major angiogenic factor in human cancer, and like c-erbB-2, higher expression of VEGF has been determined to be a negative prognostic factor for breast cancer patients in several retrospective studies [5,6]. c-erbB-2 is known to upregulate VEGF in

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human tumour cell lines [7,8], as well as in human squamous cell carcinomas of the head and neck [9]. At present, blocking of both c-erbB-2 and angiogenesis are possible targets for therapy [10,11]. So far, few large studies have investigated the possible association between these two tumour growth-stimulating factors in human breast cancer. The primary aim of the present study was to determine possible associations between expressions of c-erbB-2, the angiogenic factors, VEGF and basic fibroblast growth factor (bFGF), and the p53 status. A secondary aim was to determine the prognostic role of c-erbB-2 in the total patient population and in relation to the adjuvant systemic therapy given.

2. Patients and methods

2.1. Patient data

Clinical information and tumour homogenates were collected from 887 unselected women with a diagnosis of primary invasive breast carcinoma between 1990 and 1995 in the northern healthcare region of Sweden (525 node-negative and 362 node-positive patients). The results at 56 months of follow-up according to VEGF and p53 and the clinicopathological features of these patients are described earlier in detail in Refs. [6,12,13]. Remaining cytosols for the determination of c-erbB-2 expression were available from 656 patients (383 node-negative and 273 node-positive) from the original patient population. Node-negative patients were treated with a modified radical mastectomy or conservative surgery, and with radiotherapy following conservative surgery. Most node-negative patients received only local treatment ($n = 368$; 96%). 15 patients received adjuvant chemotherapy with nine cycles of intravenous (i.v.) CMF (cyclophosphamide, methotrexate and fluorouracil). All node-positive patients were treated with surgery and radiotherapy. Adjuvant systemic treatment was administered to all node-positive patients [13]. Most postmenopausal patients received adjuvant endocrine therapy. 90 patients received adjuvant chemotherapy, mainly i.v. CMF. Adjuvant endocrine therapy was given to 183 patients, in most cases this was tamoxifen for 2 or 5 years. Prognostic and biological information were available for all patients regarding tumour size, lymph-node status, Oestrogen Receptor (ER), Progesterone Receptor (PgR), c-erbB-2, VEGF, bFGF and p53. Information concerning histopathological grade was missing in 137 patients (21%). As histopathological grade is an important prognostic indicator for breast cancer patients, this is an obvious weakness of our study. The median age was 58 years (range 28–75 years) and the median follow-up time was 83 months (last follow-up date was 31 January 2001). Patients' characteristics are listed in Table 1.

2.2. Preparation of tumour tissue

After pathological perioperative examination, representative tumour tissue was cut out and frozen in liquid nitrogen for later analysis. Frozen tumour tissue was homogenised in a microdish membrator (Braun, Melsungen, Germany) and suspended in cold standard receptor buffer (10 mmol/l Tris pH 7.4, 1.5 mmol/l ethylene diamine tetra acetic acid (EDTA), 10 mmol/l sodium molybdate, 1.0 mmol/l monothioglycerol). Supernatants were collected after refrigerated centrifugation at 20 000g and stored at −70 °C. The pelleted fractions were analysed for DNA content by the method of Burton, in order to evaluate cell concentrations in the samples.

2.3. c-erbB-2 analysis by enzyme-linked immunosorbent assay (ELISA)

The amount of c-erbB-2 in the cytosols was determined using an enzyme immunoassay kit (Oncogen Science, San Francisco, CA, USA). Briefly, 100 μl of standards and diluted patient samples were added to the wells and incubated for 12–18 h at room temperature.

Table 1
Clinicopathological characteristics of the patients

Feature	Number of patients (%)
Patients enrolled	656
Histology type	
Ductal invasive	574 (88)
Lobular invasive	82 (13)
Tumour size	
T1	368 (56)
T2-3	288 (44)
Lymph-node status	
Node-negative	383 (58)
Node-positive	273 (42)
Histopathological grade	
I + II	265 (40)
III	254 (39)
Not analysed	137 (21)
Oestrogen receptor (ER)	
Positive (⩾0.1 fmol/μg DNA)	472 (72)
Negative (<0.1 fmol/μg DNA)	184 (28)
Progesterone receptor (PgR)	
Positive (⩾0.1 fmol/μg DNA)	415 (63)
Negative (<0.1 fmol/μg DNA)	241 (37)
Adjuvant therapy	
Chemotherapy	105[a] (16)
Endocrine therapy	183 (28)
No adjuvant systemic treatment	368 (56)

[a] CMF = (cyclophosphamide, methotrexate, fluorouracil) intravenously (i.v.) every third week ×9 or FEC = (fluorouracil, epirubicin, cyclophosphamide) i.v. every third week ×9.

The wells were precoated with a mouse monoclonal antibody. After washing, a polyclonal detector antibody was added and incubated for 60 min. Both capture and detector reagent reacts with the extracellular domain of c-erbB-2. The amount of detector antibody bound to the antigen was measured by binding it with a goat anti-rabbit IgG/horseradish peroxidase conjugate, which catalyses the conversion of the chromogenic substrate, tetramethylbenzidine (TMD), into a coloured product. The coloured reaction product was quantitated by spectrophotometry (Multiscan, MCC/340, Labsystems, Helsinki, Finland) at 450 nm and reflects the amount of c-erbB-2 in samples. Concentrations of c-erbB-2 were expressed in pg/μg DNA.

2.4. c-erbB-2 analysis by IHC

In 60 consecutive cases, overexpression of c-erbB-2 was also evaluated in a blinded fashion with the commercial immunohistochemistry test (HercepTest®, DAKO) in paraffin-embedded tumour sections (4 μm), following the manufacturer's recommendations. The antigen retrieval method used in this kit involved immersing slides in a preheated (95 °C) DAKO Epitope Retrieval Solution followed by heating in a water bath at 95 °C for 40 min. This was followed by a 20-min cool-down period at room temperature. Slides were incubated with the primary antibody (prediluted rabbit polyclonal antibody, A0485 (diluted 1:200)) for 30 min at room temperature. The slides were then incubated with the DAKO Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins) for 30 min. Diaminobenzidine was used as the chromogen, and the slides were counterstained with haematoxylin. Slides were scored 0, 1+, 2+ and 3+ and only slides evaluated as 3+ were considered to show a c-erbB-2 overexpression.

2.5. VEGF analysis

A VEGF assay was performed using a commercial quantitative immunoassay kit for human VEGF A (Quantikine, human VEGF, R & D Systems, Minneapolis, MN, USA) as described earlier in Ref. [12]. VEGF concentration in the patient's samples was expressed as pg/μg DNA.

2.6. bFGF analysis

bFGF content was measured using a commercial quantitative immunoassay kit for human bFGF (Quantikine, human bFGF, R&D Systems, Minneapolis, MN, USA). bFGF concentration in the cytosols was expressed as pg/μg DNA.

2.7. p53 analysis

Tumour cytosol p53 protein was quantified with an enzyme immunoassay kit (Oncogen Science, San Francisco, CA, USA) as described earlier in Ref. [6]. p53 protein concentration in the tumour samples was expressed as pg/μg DNA.

2.8. Statistical methods

The Pearson Chi-square test was used to investigate associations between c-erbB-2, VEGF, bFGF, accumulated p53 protein, and established prognostic factors. We also used Spearman's non-parametric test to investigate possible associations between quantitatively determined c-erbB-2, bFGF and VEGF. 8 of the 60 cases (13%) analysed by IHC (HercepTest) had a strong (3+) overexpression. We chased the 15% of patients with the highest c-erbB-2 values in the cytosols to represent c-erbB-2-positive patients. p53 status was determined as negative versus positive. VEGF and bFGF were analysed as dichotomous variables with the median value used as the cut-off point. c-erbB-2 and VEGF were also used as continuous variables in the univariate analyses. To estimate possible additional prognostic information of c-erbB-2 and VEGF, bivariate analyses for relapse-free survival (RFS) and OS were performed. Patients were divided in four groups; a low-risk group with low VEGF expression and c-erbB-2-negative, two intermediate groups with either overexpression of c-erbB-2 *or* high VEGF expression, and a high-risk group consisting of patients with both overexpression of c-erbB-2 and high VEGF expression. Survival rates in the uni- and bivariate analyses were estimated using the Kaplan–Meier method, and comparisons between the study groups were performed with the log-rank test. In bivariate analyses, all four groups were compared. Relative risks for RFS and OS were estimated by Cox's proportional hazards in the univariate analysis. Due to the relatively large patient population and events, most factors analysed in the univariate analyses were statistically significant prognostic factors and they were confirmed or rejected in Cox proportional multivariate analyses. In these, tumour size (T1 versus T2-3), lymph-node status (N0 versus N1), ER status (positive versus negative), VEGF ($<$versus $\geqslant$median), bFGF ($<$versus $\geqslant$median), p53 (negative versus positive) were analysed as dichotomous variables. Grade was analysed in two groups (grades I and II versus III) and in three groups (I versus II versus III). In the latter comparison, grade II was used as reference group due to the fact that only a few patients were classified as grade I ($n=57$), compared with II ($n=208$) and III ($n=254$). The survival time was measured from the date of diagnosis to the first docu-

mented relapse or death. In all tests, the significance level was set at 0.05, and all tests were two-sided.

3. Results

3.1. Patients' outcome

With a median follow-up of 83 months (range 61–132 months), a total of 208 recurrences and 170 deaths were recorded. Clinicopathological characteristics of the patients are listed in Table 1. Recurrences were divided in four groups according to the first predominant metastatic site; soft-tissue including local failures, only bone metastasis, visceral including lung and liver metastasis, and brain metastasis.

3.2. Expression of c-erbB-2 determined by IHC and ELISA

8 out of 60 patients (13%) were found to have a strong (3+) c-erbB-2 overexpression by IHC using the HercepTest (DAKO). A wide range of c-erbB-2 content was found when analysed quantitatively with ELISA (Fig. 1). All patients, but 2, had detectable levels. The median value in the total patient population was 0.6 pg/μg/DNA (range 0.0–29.5). The 85th percentile was chosen as the cut-off value, with 85% of patients with the lowest values classified as c-erbB-2-negative and the 15% of patients with the highest values as c-erbB-2-positive. 7 out of 8 patients classified as 3+ with IHC were also classified as c-erbB-2-positive by ELISA.

3.3. Associations between c-erbB-2 and other prognostic factors

The overexpression of c-erbB-2 was statistically significantly associated with a higher VEGF expression and was found in 29 out of 340 (9%) patients with a lower VEGF expression, compared with 70 out of 316 (22%) with a higher VEGF expression. Similar results were seen with the Spearman's correlation test, although with a large spread ($P<0.001$, $r=0.145$) (Fig. 2). Overexpression of c-erbB-2 was also significantly associated with a lower bFGF expression ($P=0.002$), high S-phase fraction ($P=0.001$), negative ER receptor ($P<0.001$) and PR ($P<0.001$), and ductal type ($P=0.006$). 4 (5%) of 82 patients with a lobular type were found to overexpress c-erbB-2.

c-erbB-2 expression was not related to p53-status ($P=0.131$), histopathologic grade ($P=0.744$), tumour size ($P=0.748$), or axillary lymph-node status ($P=0.250$).

3.4. c-erbB-2 in relation to the first predominant metastatic site

Overexpression of c-erbB-2 was not statistically significantly associated with the first predominant metastatic site. 40% (4/10) of the patients developing brain metastases had overexpression of c-erbB-2, compared with 20% (17/84) of the patients that developed visceral metastasis. Patients with loco-regional relapses or solely bone metastasis had a similar frequence of overexpression of c-erbB-2 as patients without documented relapses, 16% (10/61) and 13% (7/53), respectively.

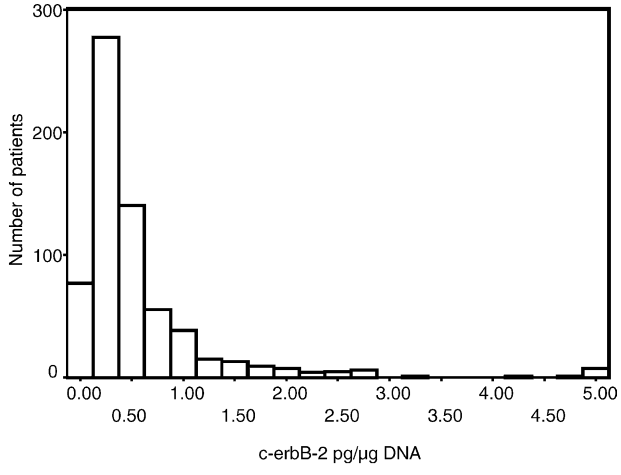

Fig. 1. Distribution of c-erbB-2 content in cytosols from primary breast tumours in the total patient population. Median value 0.6 pg/μg DNA (range 0.0–29.5). 5 patients had values above 5.0 pg/μg DNA (6.3, 7.4, 8.4, 11.9 and 29.5 pg/μg DNA, respectively).

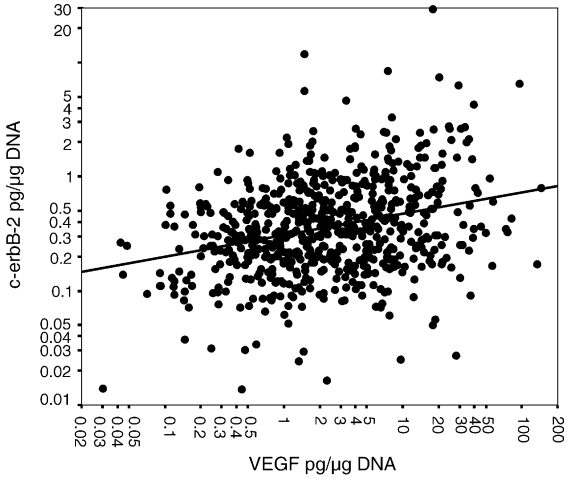

Fig. 2. A statistically significant association was found between the expression of c-erbB-2 and VEGF, but with a large spread ($P=0.001$; $r=0.145$).

3.5. Univariate analysis

Univariate analysis of the total patient population showed a high c-erbB-2 content to be significantly correlated with a shorter RFS ($P=0.043$) (Fig. 3a) and OS ($P=0.014$) (Fig. 3b). Higher expression of c-erbB-2 was also significantly correlated with shorter survival times when tested as a continuous variable, RFS ($P=0.049$), OS ($P=0.054$). c-erbB-2 was a statistically significant prognostic factor for node-positive patients with regard to RFS ($P<0.001$) (Fig. 4a) and OS ($P<0.001$) (Fig. 4b), but not for node-negative patients (data not shown). Other factors with statistically significant prognostic values in the univariate analyses in the total patient population were VEGF, p53 status, S-phase fraction, ER and PR status, histological grade, tumour size and nodal status (data not shown).

3.6. Combined survival analysis according to c-erbB-2 and VEGF expression

Statistically significant differences in RFS ($P=0.009$) and OS ($P<0.001$) were seen in the bivariate analysis according to c-erbB-2 and VEGF expression, with all four groups included in the log-rank test. Patients with negative c-erbB-2 status and a lower VEGF expression ($n=310$) had a 5-year RFS of 72% compared with 57% in the group with expression of both risk factors ($n=69$). Regarding OS, the corresponding figures were 80% in the low-risk group compared with 62% in the high-risk group. These data including the intermediate-risk groups with either c-erbB-2 overexpression or higher VEGF values are presented in Table 2.

3.7. c-erbB-2 in relation to survival after adjuvant systemic therapy

Overexpression of c-erbB-2 was significantly associated with shorter survival times following adjuvant endocrine therapy, RFS ($P=0.016$), OS ($P=0.020$). When ER-negative patients ($n=32$) were excluded from the survival analyses with regard to adjuvant endocrine therapy, c-erbB-2 failed as a prognostic factor, RFS ($P=0.738$), and OS ($P=0.995$). Overexpression of c-erbB-2 was statistically significantly associated with a shorter survival following adjuvant chemotherapy, RFS ($P=0.011$), and OS ($P=0.005$).

Table 2
Survival analyses according to c-erbB-2 and VEGF expression in the total patient population ($n=656$)[a]

	5-year RFS (%)	5-year OS (%)
Low-risk ($n=310$)		
Negative c-erbB-2 and a lower VEGF	72	80
Intermediate-risk		
c-erbB-2-positive and a lower VEGF ($n=30$)	72	76
c-erbB-2-negative and a higher VEGF ($n=247$)	65	70
High-risk ($n=69$)		
c-erbB-2-positive and a higher VEGF	57	62

VEGF, vascular endothelial growth factor; OS, overall survival; RFS, relapse-free survival.

[a] The probability of 5-year RFS ($P=0.009$) and OS ($P<0.001$) in the different groups are presented.

3.8. Multivariate analysis

Cox proportional hazards regression models were used to estimate hazard ratios (HR). The analyses were performed with c-erbB-2 and the other factors included: p53, VEGF content, bFGF content, tumour size, ER content, histological grade and nodal status as single covariates. HRs above 1.0 indicated a greater risk of recurrence or death than for the comparative group set as the reference. The results in the total patient population showed c-erbB-2 to be an independent prognostic factor for RFS (HR = 1.8), and OS (HR = 1.7) (Table 3). Other independent factors for RFS were nodal status, histopathological grade and tumour size. Other independent factors for OS were nodal status, p53 status, tumour size and histopathological grade. Cox multivariate analysis in the node-positive subgroup with the same factors as above included showed c-erbB-2 as an independent prognostic factor with increased HRs for RFS (HR = 2.5), and OS (HR = 2.2) (Table 4). We also performed multivariate analyses with grade in three groups (I versus II versus III), with grade II as the reference group as only a few patients were classified as grade I ($n=57$). These results still showed c-erbB-2 as an independent prognostic factor in the total patient population for RFS (HR 1.8, 95% CI 1.2–2.6) together with nodal status (HR 2.1, 95% CI 1.5–2.9), tumour size (HR 1.5, 95% CI 1.1–2.0) and grade III (HR 1.4, 95% CI 1.0–2.0). For OS, c-erbB-2 (HR 1.8, 95% CI 1.1–2.8) together with nodal status (HR 2.7, 95% CI 1.9–3.9), tumour size (HR 1.5, 95% CI 1.0–2.1) and p53 (HR 1.8, 95% CI 1.3–2.6) were significant, while grade was not. In addition, in node-positive patients c-erbB-2 was an independent prognostic factor; RFS (HR 2.3, 95% CI 1.4-4.0) and OS (HR 2.2, 95% CI 1.2–3.9). By contrast, in node-negative patients, c-erbB-2 failed as an independent factor; RFS (HR 1.2, 95% CI 0.6–2.4) and OS (HR 1.1, 95% CI 0.5–2.6).

4. Discussion

The observed association between the overexpression of c-erbB-2 and higher VEGF expression indicate that c-erbB-2 is involved, at least partly, in the regulation of angiogenesis in human breast cancer. This is in accordance with preclinical data where c-erbB-2 is reported

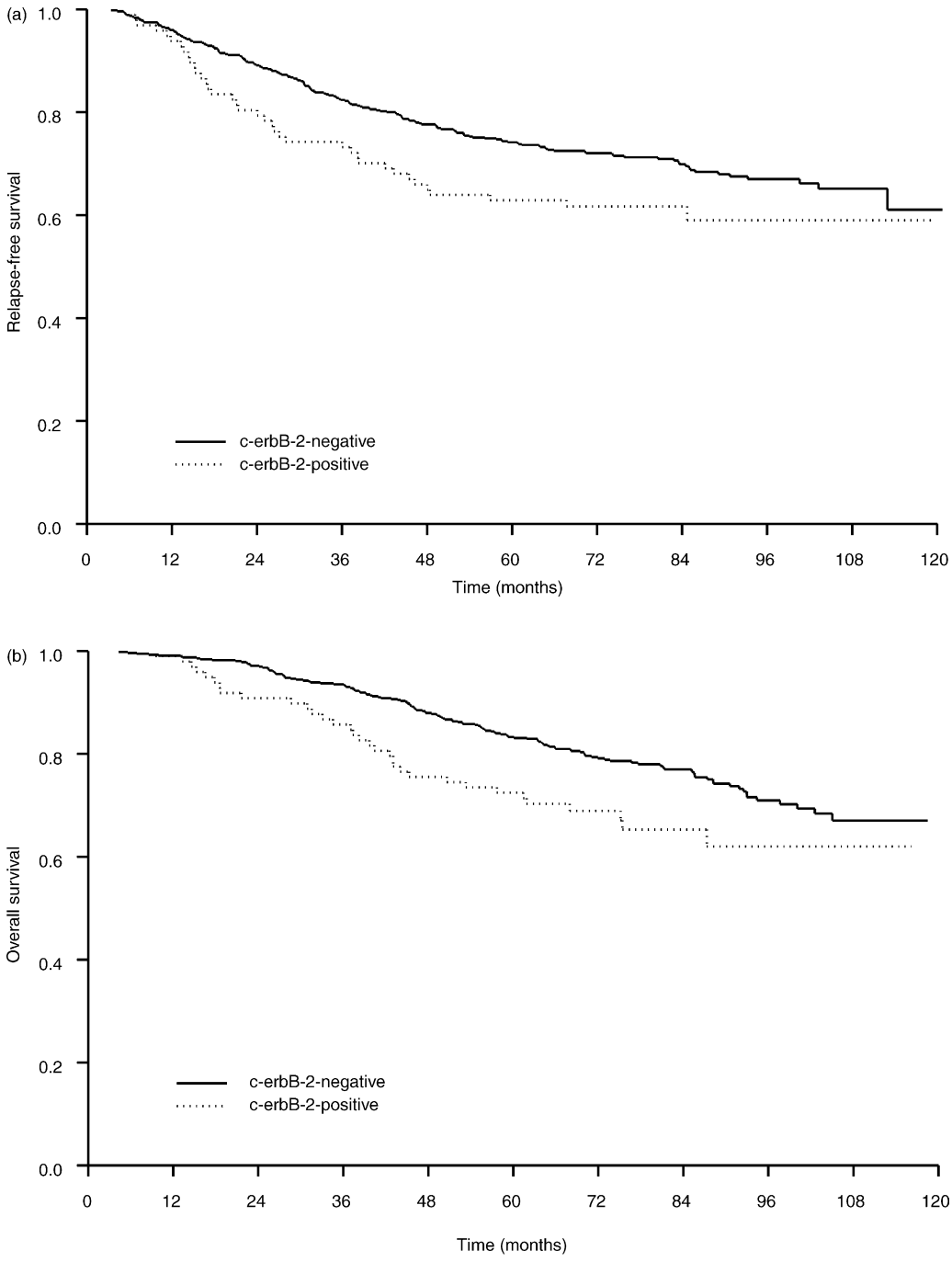

Fig. 3. (a) Relapse-free survival (RFS) and (b) overall survival (OS) in the total patient population according to cytosolic *c*-erbB-2 content in primary breast tumours. c-erbB-2-negative — and c-erbB-2-positive - - -.

to upregulate the expression of VEGF [7,8]. A similar correlation between the expression of VEGF and c-erbB-2 has recently been published in breast cancer cell lines [14]. As c-erbB-2 acts through heterodimerisation with other members of the EGFR family, their known ligands may contribute to the increased VEGF expression. Three c-erbB-receptor ligands, transforming growth factor alpha (TGF-alpha), betacellulin (BTC) and heregulin-beta1 (HRG-beta1), have all been shown to upregulate VEGF-A and VEGF-C in squamous cell carcinomas of the head and neck [15]. In breast cancer, conflicting results are reported. Most studies have used the microvessel density (MVD) count to evaluate angiogenesis. For instance, two studies investigating angiogenesis in relation to other biological markers and survival in node-negative breast cancer, failed to show any correlation between MVD and c-erbB-2 or p53 protein in 211 and 110 patients, respectively [16,17]. The relatively small numbers of patients included may partly explain the differing results, but differences in the

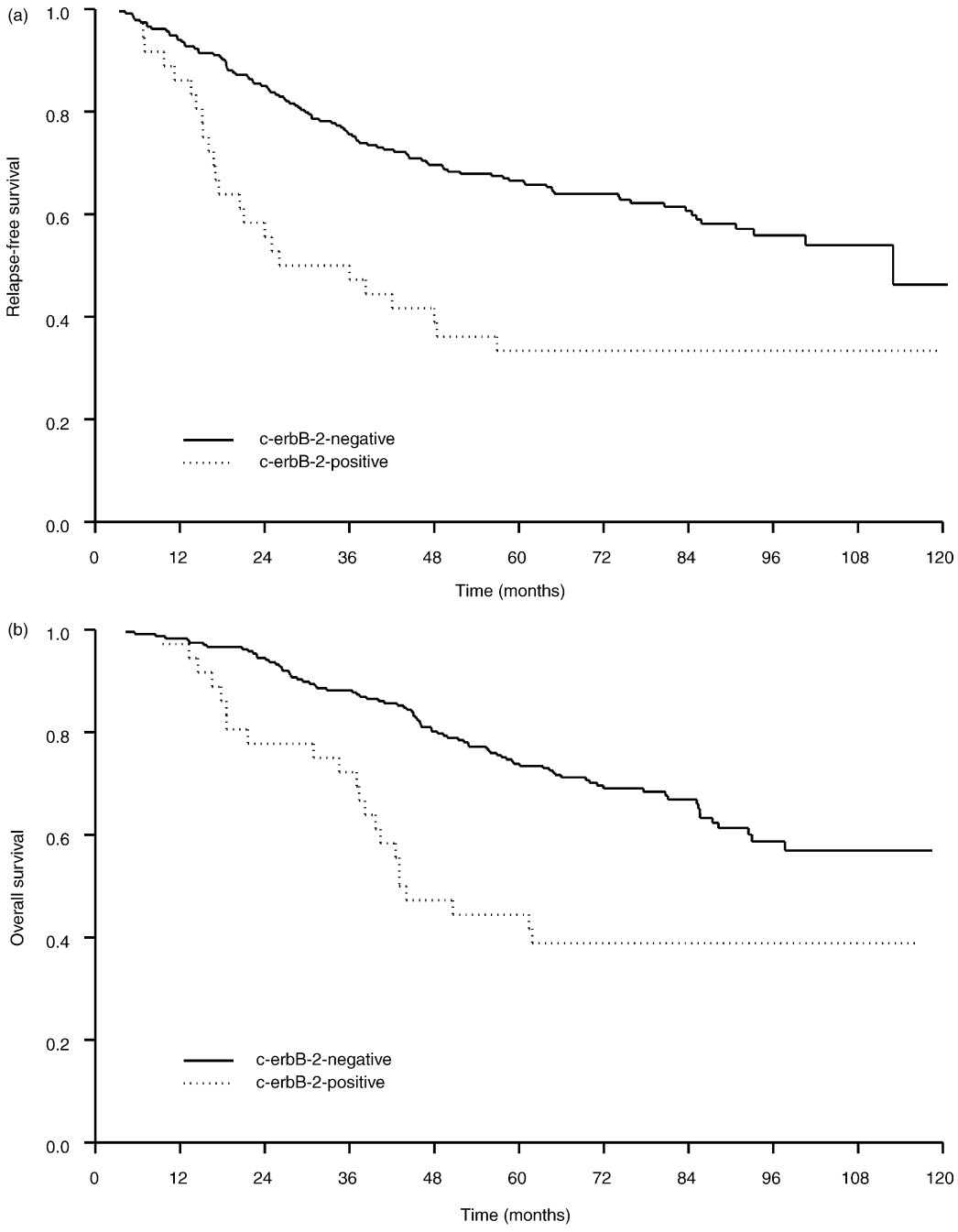

Fig. 4. (a) Relapse-free survival (RFS) and (b) overall survival (OS) in node-positive patients according to cytosolic c-erbB-2 content in primary breast tumours. c-erbB-2-negative — and c-erbB-2-positive - - -.

methods also cannot be excluded. We have recently published data in a smaller patient population ($n = 224$), where VEGF expression was determined by the same method as in the present study. This previously study showed no relationship between VEGF expression and c-erbB-2 determined by IHC using the monoclonal antibody CB11 [18].

Overexpression of c-erbB-2 was significantly related to a lower expression of bFGF in this study. Combined results from previous reports of the prognostic value of bFGF in breast cancer remain inconclusive and the clinical relevance is not clear [19]. We have found bFGF to be associated with other favourable prognostic factors and a trend towards longer survival times in a previous study (Linderholm submitted).

We found a large distribution of c-erbB-2 expression as was observed in the recently published large study by the group of Eppenberger-Castori [20]. Other published studies using ELISA for the determination of c-erbB-2 expression have also showed a significant correlation with survival [20–23]. Three studies were relatively small including a total of 315 patients, while the one by

Table 3
Cox multivariate analysis for (a) RFS and (b) OS in the total population

Variable	Hazard ratio	95% Confidence Interval	*P* value
(a) RFS			
c-erbB-2			
Negative	1.0		
Positive	1.8	1.2–2.7	0.007
bFGF			
<Median	1.0		
⩾Median	0.9	0.7–1.2	0.506
VEGF			
<Median	1.0		
⩾Median	1.1	0.8–1.6	0.454
ER			
Positive	1.0		
Negative	1.1	0.8–1.5	0.681
Tumour size			
<20 mm	1.0		
20–50 mm	1.5	1.1–2.0	0.024
Grade			
I+II	1.0		
III	1.6	1.1–2.1	0.010
Nodal status			
Node-negative	1.0		
Node-positive	2.2	1.6–3.0	<0.001
p53			
Negative	1.0		
Positive	1.4	1.0–1.9	0.069
(b) OS			
c-erbB-2			
Negative	1.0		
Positive	1.7	1.1–2.7	0.016
bFGF			
<Median	1.0		
⩾Median	0.9	0.7–1.4	0.735
VEGF			
<Median	1.0		
⩾median	1.3	0.9–1.9	0.120
ER			
Positive	1.0		
Negative	1.4	1.0–2.0	0.069
Tumour size			
<20 mm	1.0		
20–50 mm	1.5	1.0–2.1	0.033
Grade			
I+II	1.0		
III	1.5	1.0–2.1	0.041
Nodal status			
Node-negative	1.0		
Node-positive	2.8	1.9–4.0	<0.001
p53			
Negative	1.0		
Positive	1.8	1.3–2.6	0.001

Eppenberger-Castori was large, with more than 1000 patients with a known follow-up period. One advantage with immunoassays may be that the potential damage of antigen associated with fixation and embedding tumour samples can be avoided, which may contribute to the results in the above-mentioned studies. However, the ELISA method for c-erbB-2 detection is not standardised or approved by the Food and Drug Administration (FDA), unlike other methods such as fluorescent *in situ* hybridisation (FISH) or IHC (HercepTest® or CB11). Numerous studies have shown overexpression or amplification of c-erbB-2 to be a negative prognostic factor for node-positive patients, while the results in the node-negative group have been less clear. In addition, in our study, overexpression of c-erbB-2 was only a prognostic factor for node-positive patients. One explanation for this might be that the node-negative patients in our material after a relatively short follow-up time have only had a few events so far. Similar results are reported from earlier studies in which c-erbB-2 has failed as a prognostic marker in the node-negative sub-set and two of these reports included a large number of patients [24,25]. The worse prognosis in the node-positive group in the latter study was partly explained by the reduced efficacy of CMF therapy (also seen in our present study) in c-erbB-2-overexpressing node-positive patients [25].

We also found c-erbB-2 to be a negative prognostic factor following adjuvant endocrine therapy. The predictive value for response with regard to adjuvant endocrine therapy is contradictory in the current literature. Preclinical data, where c-erbB-2 has been transfected to achieve gene amplification in ER-positive cell lines, has resulted in an oestrogen-independent growth that did not respond to therapy with tamoxifen. The Naples GUN trial of adjuvant endocrine therapy showed an inferior outcome for c-erbB-2-positive patients receiving adjuvant tamoxifen compared with those receiving no systemic therapy [26]. In contrast, in advanced breast cancer, no lack of efficacy by tamoxifen was seen in 205 c-erbB-2-positive patients [27]. Several retrospective published studies, discussing c-erbB-2 as a negative predictive factor for endocrine therapy, have included patients with ER-negative disease or unknown ER status. It has been postulated that the worse outcome seen for c-erbB-2-positive patients after tamoxifen treatment may reflect, in part, the negative correlation between steroid receptors and c-erbB-2, also seen in our study [27]. It was recently shown that pre-operative endocrine therapy does not affect the proliferation rate in ER-c-erbB-2-positive tumours in contrast to ER-positive/c-erbB-2-negative ones, which over time might lead to an inferior outcome [28]. Thus, an additional treatment predictive value of the c-erbB-2 status to the steroid receptor content in endocrine therapy cannot be excluded.

When c-erbB-2 was added in this material, VEGF was no longer an independent prognostic factor according

to the multivariate analysis. This may partly be explained by the fact that these two variables are, to some extent, closely related to one another, but in our study c-erbB-2 was the dominant prognostic factor. By contrast, other studies, including both angiogenesis and c-erbB-2 in the multivariate analysis, have demonstrated angiogenesis, determined by MVD, to be an independent prognostic factor, while this could not be demonstrated for c-erbB-2 status [16,29].

In summary, our results show an association between the expression of c-erbB-2 and the dominant angiogenic factor, VEGF, in human breast cancer. At present, targeted compounds directed towards VEGF or its receptors have shown activity in metastatic breast cancer, and are presently in clinical phase II and III trials. It is worth investigating if therapy aimed at blocking both pathways results in a clinical benefit.

Table 4
Cox multivariate analysis for (a) RFS and (b) OS in node-positive patients

Variable	Hazard ratio	95% Confidence Interval	*P* value
(a) RFS			
c-erbB-2			
Negative	1.0		
Positive	2.5	1.5–4.2	0.001
bFGF			
$<$Median	1.0		
$\geqslant$Median	0.9	0.6–1.3	0.576
VEGF			
$<$Median	1.0		
$\geqslant$Median	1.2	0.8–1.7	0.496
ER			
Positive	1.0		
Negative	1.6	1.0–2.4	0.050
Tumour size			
$<$20 mm	1.0		
20–50 mm	1.8	1.1–2.8	0.011
Grade			
I+II	1.0		
III	1.2	0.8–1.9	0.373
p53			
Negative	1.0		
Positive	1.7	1.0–2.4	0.035
(b) OS			
c-erbB-2			
Negative	1.0		
Positive	2.2	1.2–4.0	0.007
bFGF			
$<$Median	1.0		
$\geqslant$Median	0.9	0.6–1.3	0.529
VEGF			
$<$Median	1.0		
$\geqslant$Median	1.3	0.8–2.0	0.729
ER			
Positive	1.0		
Negative	2.0	1.4–3.5	0.001
Tumour size			
$<$20 mm	1.0		
20–50 mm	2.0	1.2–3.4	0.006
Grade			
I+II	1.0		
III	1.2	0.8–1.9	0.427
p53			
Negative	1.0		
Positive	1.5	1.0–2.3	0.081

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