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## Original Paper

# c-erbB-2 Expression and Benefit From Adjuvant Chemotherapy and Radiotherapy of Breast Cancer

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**Frozen tissue from primary tumours of 152 premenopausal breast cancer patients, who participated in a trial comparing radiotherapy with adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil, CMF), was analysed for c-erbB-2 protein expression, measured by flow cytometry. The relative risk of distant recurrence or death in the chemotherapy group as compared with the radiotherapy group was 3.0 (95% confidence interval (CI) 1.1–7.8) for patients whose tumours showed high c-erbB-2 levels and 0.87 (95% CI 0.43–1.7) for those with tumours with low levels of c-erbB-2 protein. Patients with highly proliferative tumours that did not overexpress c-erbB-2 benefited most, in terms of survival, from CMF. In addition, we found an increased risk of locoregional recurrence for tumours overexpressing c-erbB-2 when radiotherapy was replaced by chemotherapy.**

**Key words: adjuvant treatment, breast cancer, c-erbB-2, chemotherapy, CMF, drug resistance, local recurrence, radiotherapy, S-phase fraction**
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## INTRODUCTION

BOTH RADIATION and chemotherapy are widely used in the treatment of breast cancer. Postoperative radiotherapy is an effective locoregional treatment. Some authors have also claimed that radiation therapy may prevent distant dissemination in subgroups of node-positive patients [1, 2]. It is now well established that adjuvant chemotherapy significantly improves survival of premenopausal patients [3]. However, the response to treatment for individual patients is highly variable and hard to predict with any accuracy, although a large number of factors are able to predict the prognosis of patients with breast cancer.

One factor that has been suggested as a prognostic indicator in breast cancer is c-erbB-2, also known as HER-2/neu. Overexpression of the c-erbB-2 protein is frequently associated with amplification of the corresponding gene in human cancer [4]. Either mutation of *c-erbB-2* or elevated expression of the normal protein can transform cells in culture [5]. The protein is closely related in structure to the epidermal growth factor (EGF) receptor and c-erbB-3 and c-erbB-4 proteins. The members within this family form homodimers as well as heterodimers, and candidate c-erbB-2 ligands, such as the heregulins, have been shown to bind to the c-erbB-4 protein. Overexpression of c-erbB-2 has been associated with oestrogen receptor (ER)-negative tumours [6–9], DNA aneuploidy [8, 9], high cell proliferation [8–10], poor tumour grade [7, 8, 11, 12], and is frequently detected in comedo type ductal cancer *in situ* [10, 13].

Overexpression of c-erbB-2 has indicated a poor prognosis in several studies, but whether this reflects an independent prognostic value among systemically untreated patients or is due to an interaction with response to systemic therapy is currently much debated [6, 7, 12, 14, 15]. In the present study, we analysed c-erbB-2 expression in primary tumours of premenopausal patients who participated in a randomised trial which compared 12 cycles of chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil, CMF) with postoperative radiotherapy. Using flow cytometry, the level of c-erbB-2 expression was assessed together with DNA content.

## PATIENTS AND METHODS

### *Patients*

In 1976, the Stockholm Breast Cancer Group initiated a trial to compare postoperative radiotherapy with adjuvant chemotherapy [1]. The trial included pre- and postmenopausal patients with unilateral, operable breast cancer. Surgery consisted of modified radical mastectomy. The patients were required to

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have either histologically verified lymph node metastases or a tumour diameter, measured on the surgical specimen, exceeding 30 mm. Patient accruel started in November 1976 and ended in April 1990. For 152 of the 545 premenopausal patients included in the trial, frozen tumour samples were available after hormone receptor analysis, and those were used for simultaneous measurement of c-erbB-2 expression and DNA content. The distributions of lymph node status, tumour size and oestrogen receptor status were similar between these available cases and the original series.

Radiotherapy was given with a high-voltage technique [1]. The dose was 46 Gy with 2 Gy per fraction 5 days a week for a total treatment time of approximately 4.5 weeks. The target volume included the chest wall, axilla, supraclavicular fossa and internal mammary nodes. The chemotherapy protocol was the same as in the first Milan trial, that is, 12 courses of CMF (cyclophosphamide 100 mg/m$^2$ orally at days 1–14, methotrexate 40 mg/m$^2$ i.v. on days 1 and 8, and 5-fluorouracil 600 mg/m$^2$ i.v. on days 1 and 8) [1]. The treatment intent was achieved in 98% of the patients randomised to radiotherapy, whereas 9% in the chemotherapy group did not receive the allocated treatment. After the first six courses of CMF, 82% of the patients had received 65% or more of the planned dose. In the original study, there was no significant difference in overall survival in any of the menopausal groups or when all patients were analysed. In premenopausal patients, there was a tendency towards a lower risk of distant failure in the chemotherapy group ($P = 0.08$). 52 of the 152 patients included in the present study had distant recurrence or died during a median follow-up period of 8 years. 18 patients had a locoregional recurrence.

*Preparation for flow cytometry*

The procedure has been described elsewhere [9]. A piece of the tumour specimen was thawed at room temperature and a cell suspension was obtained mechanically by cutting the tissue with a pair of scissors in citrate buffer. The cell suspension was filtered through a 41-μm Nylon mesh before it was fixed in 1% paraformaldehyde (PFA) for 3 min. After addition of phosphate-buffered saline with 0.5% bovine serum albumin (PAB), the sample was centrifuged (890 *g*), resuspended in PAB and separated into two tubes. One tube was incubated with the c-neu antibody (Ab-2, clone 9G6, Oncogene Science Inc., Manhasset, New York, U.S.A.) and the other with $IgG_1$ immunoglobulins (Sigma Chemical Co., St Louis, Missouri, U.S.A.) for 60 min (0.5 μg/ml PAB). Thereafter, both were similarly processed. The secondary fluorescein isothiocyanate-conjugated antibody (FITC, $F(ab)_2$) (Dakopatts Co., Glostrup, Denmark) was added (20 μl/ml PAB) after washing and resuspension in 1 ml PAB. After 30 min, the cells were washed and treated with RNAse (100 μg/ml), aspirated with a syringe (needle diameter 0.6 mm) and filtered as described earlier. DNA was stained with propidium iodide (PI), 15 μg/ml, prior to the flow cytometric analysis.

*Flow cytometry*

Samples were analysed with a FACScan flow cytometer (Becton Dickinson, California, U.S.A.) equipped with a 15-mW argon laser (488 nm) for simultaneous excitation of PI and FITC. Ten thousand events were recorded in list mode. Doublet discrimination was performed by gating on the area and width signals from the red fluorescence. The DNA histogram was evaluated as described elsewhere [9]. Tumours with a single DNA $G_{0/1}$ peak were classified as DNA diploid, and if more than one $G_{0/1}$ peak was present, the tumour was considered DNA aneuploid. A rectangular model was used for estimation of the S-phase fraction (SPF). The SPF was assessed in 137 of the tumours (90%) and ranged between 1.2 and 17%. The mean S-phase value was 6.7% and the median was 5.9%. The median coefficient of variation for $G_{0/1}$ peaks was 4.3% (range 2.9–7.2).

A fluorescence index (FI) was calculated as a quantitative measure of the c-erbB-2 receptor content. For both the c-neu antibody and the isotypic control, a histogram of the FITC fluorescence was generated in log scale. In order to correct for non-specific binding, the mean channel value of the control histogram was subtracted from that of the c-neu histogram. The FI was obtained by transforming the difference in log scale into a ratio in linear scale, so samples negative for c-erbB-2 expression

*Table 1. c-erbB-2 overexpression in relation to other tumour characteristics*

| | Number of patients | Overexpression of c-erbB-2 % FI ≥ 2.0 (*n*) | Test for trend |
|---|---|---|---|
| Total | 152 | 22 (33) | |
| Nodal status, tumour size | | | |
| $N_0$, >30 mm | 12 | 17 (2) | |
| N+, ≤20 mm | 53 | 17 (9) | |
| N+, 21–30 mm | 49 | 24 (12) | $P = 0.24$ |
| N+, >30 mm | 38 | 26 (10) | |
| ER content* | | | |
| <0.1 fmol/μg DNA | 56 | 39 (22) | |
| ≥0.1 fmol/μg DNA | 94 | 11 (10) | $P < 0.0001$ |
| DNA ploidy | | | |
| Diploid | 51 | 10 (5) | |
| Aneuploid | 101 | 28 (28) | $P = 0.011$ |
| S-phase fraction† | | | |
| <5% | 56 | 9 (5) | |
| 5–10% | 48 | 29 (14) | $P = 0.0006$ |
| ≥10% | 33 | 39 (13) | |

*Data were missing in 2 cases. †Data were missing in 15 cases. FI, fluorescence index; ER, oestrogen receptor.

had a FI around 1.0. For DNA aneuploid tumours, the FI was calculated from the aneuploid population. The median FI was 1.5 and the maximum value was 15.1. In a previous study, the FI correlated with the staining intensity of immunohistochemically stained sections when using the same primary antibody [9]. High FI levels corresponded to clear membrane staining, and intermediate levels were often obtained in cases with diffuse cytoplasmic immunohistochemical staining.

*Statistical methods*

The risk of distant recurrence or death and the risk of locoregional recurrence in relation to different variables were estimated using Cox's proportional hazards model [16]. The product-limit method was used for estimation of cumulative probabilities of distant disease-free and local recurrence-free survival [17]. Relationships between c-erbB-2 content and other grouped variables were tested by means of chi-square tests for contingency tables with ordered categories.

## RESULTS

The tumours from 33 patients (22%) had a FI of 2.0 or over, and were classified as overexpressing c-erbB-2 protein. Overexpression was significantly associated with the absence of ERs, DNA aneuploidy and a high S-phase fraction (Table 1), but in some categories the numbers were small, so these data should be interpreted with caution.

Patients whose tumours had low or intermediate c-erbB-2 content tended to benefit from adjuvant chemotherapy compared with radiotherapy, while those with tumours clearly overexpressing c-erbB-2 had a high relapse rate following chemotherapy (Table 2). The relative risk of distant recurrence or death for those who received chemotherapy compared with those who received radiotherapy was 0.87 if the tumours had low c-erbB-2 expression (FI < 2.0, 95% CI 0.43–1.7) and 3.0 (1.1–7.8) if the tumours had overexpression. c-erbB-2 expression (FI < 2.0 versus FI ≥ 2.0) appeared to be related to response to treatment. For patients whose tumours expressed c-erbB-2 at high levels, the distant disease-free survival rate after 7 years was 15% for those who received chemotherapy, and 59% for those given radiotherapy. This was close to the estimated 7-year rate of 67% for radiotherapy-treated patients whose tumours had low c-erbB-2 content (Figure 1).

The survival benefit from chemotherapy was most evident for patients with highly proliferating tumours not overexpressing the c-erbB-2 receptor (Figure 2). Alternatively, patients whose tumours overexpressed c-erbB-2 in combination with a low or moderate S-phase fraction benefitted more from radiotherapy compared with chemotherapy (Figure 2).

For patients whose tumours expressed c-erbB-2 at high levels, there was also a higher rate of locoregional recurrence in the chemotherapy group (6/12) as compared with the radiotherapy group (1/21) (Figure 3). In the c-erbB-2-negative subgroup, 9% (11/119) of the patients relapsed locally with no notable difference between treatment groups. In addition, a high number of positive lymph nodes was associated with an increased risk of local recurrence. Fourteen of the 18 locoregional relapses occurred among the 72 patients having either more than three lymph node metastases or a tumour that expressed c-erbB-2 at high levels.

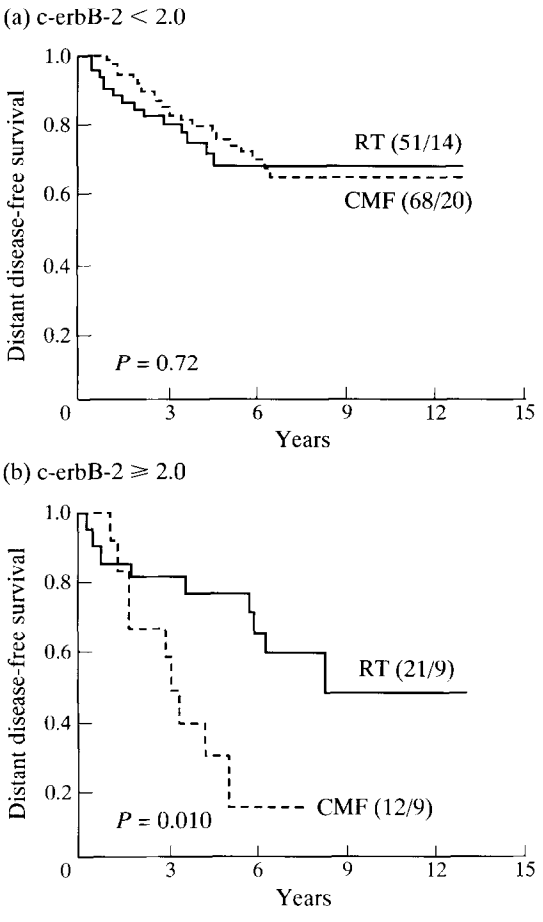


**Figure 1. Distant disease-free survival of patients treated with chemotherapy (CMF) or radiotherapy (RT) in relation to c-erbB-2 content. Numbers of patients and distant recurrences or deaths are presented within parentheses.**

## DISCUSSION

In two previous studies of the effect of adjuvant chemotherapy on survival, there was a trend towards less benefit for patients whose tumours overexpressed c-erbB-2 compared with those having c-erbB-2-negative tumours [7, 14]. Such a relationship is

*Table 2. Relative risk of distant recurrence or death in patients treated with chemotherapy as compared with those who received radiotherapy in relation to c-erbB-2 expression*

| c-erbB-2 (FI) | Number of patients: Chemotherapy | Number of patients: Radiotherapy | Relative risk | 95% confidence interval | Test for trend |
|---|---|---|---|---|---|
| <1.5 | 45 | 31 | 0.89 | 0.36–2.2 | |
| 1.5–2.0 | 23 | 20 | 0.84 | 0.28–2.5 | $\chi^2 = 3.09$ |
| ≥2.0 | 12 | 21 | 3.0 | 1.1–7.8 | $P = 0.079$ |

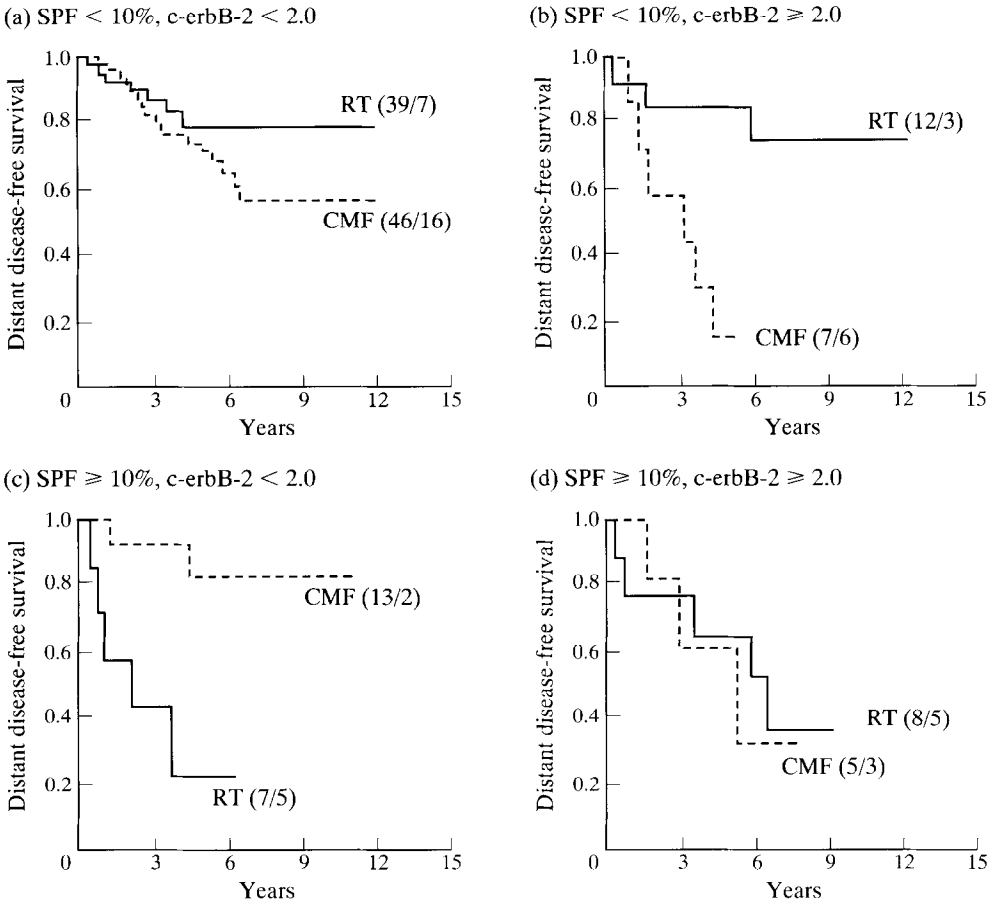


**Figure 2. Distant disease-free survival of patients treated with chemotherapy (CMF) or radiotherapy (RT) in relation to c-erbB-2 content and S-phase fraction (SPF). Numbers of patients and distant recurrences or deaths are presented within parentheses.**

supported by the present results showing a relationship between c-erbB-2 expression and a benefit from chemotherapy compared with radiotherapy. It has also been proposed, however, that c-erbB-2 overexpression may be related to chemosensitivity due to its correlation with S-phase fraction [18]. This idea has some support from one report with few patients [19], but in addition, from a large randomised study where c-erbB-2 positivity indicated a good response to increased dose of chemotherapy [20]. In the former study chemotherapy consisted of CMF, while the regime in the latter study included doxorubicin.

Cell lines *in vitro* overexpressing c-erbB-2 have shown resistance to chemotherapeutic agents [15], and resistance to tumour necrosis factor (TNF) and lymphokine-activated killer (LAK) cells has also been demonstrated [21]. Furthermore, resistance to tamoxifen has been shown in c-erbB-2 transfected cells implanted in mice [22], and has been suggested from clinical studies [6], although c-erbB-2 overexpression does not exclude remission after preoperative tamoxifen treatment of patients [23]. Alternatively, there is support for a relationship between c-erbB-2 overexpression and chemosensitivity to doxorubicin and other drugs acting on topoisomerase II. The gene coding for this enzyme is located close to the *c-erbB-2* gene on chromosome 17q, and co-amplification of the genes has been observed [24]. Amplification probably results in overexpression of topoisomerase II and higher chemosensitivity. Moreover, Arteaga and associates [25] demonstrated that activation of the c-erbB-2 receptor may lead to phosphorylation of topoisomerase II, which increases the activity of the enzyme and may sensitise cells to anthracyclines. Thus, the different results on the relationship between c-erbB-2 expression and the benefit from chemotherapy, obtained in the study of Muss and colleagues [20] and the present study are not conflicting if the type of drug used is also considered.

Our results indicate that there might be an interaction between c-erbB-2 receptor content and response to CMF, in addition to the correlation between treatment response and S-phase fraction that we have recently reported [26]. Although the number of patients in some subgroups was small, we observed a striking difference in distant disease-free survival according to c-erbB-2 expression, S-phase fraction and treatment (Figure 2). Interestingly, patients in the radiotherapy group, whose tumours showed low S-phase levels, had few distant recurrences irrespective of c-erbB-2 status, while c-erbB-2 overexpression indicated a very poor prognosis for patients who received CMF. It has been proposed that radiotherapy not only reduces the risk of local recurrence, but also may improve the distant recurrence-free survival among node-positive patients [1, 2]. Providing that c-erbB-2 overexpression is an indicator of aggressive behaviour, the benefit from locoregional treatment on distant recurrence, which we observed among c-erbB-2-positive patients, could imply that c-erbB-2 expression correlates with locoregional spread. To test this hypothesis, we investigated the risk of local relapse and found a very high risk related to c-erbB-2 overexpression for patients who did not receive radiotherapy.

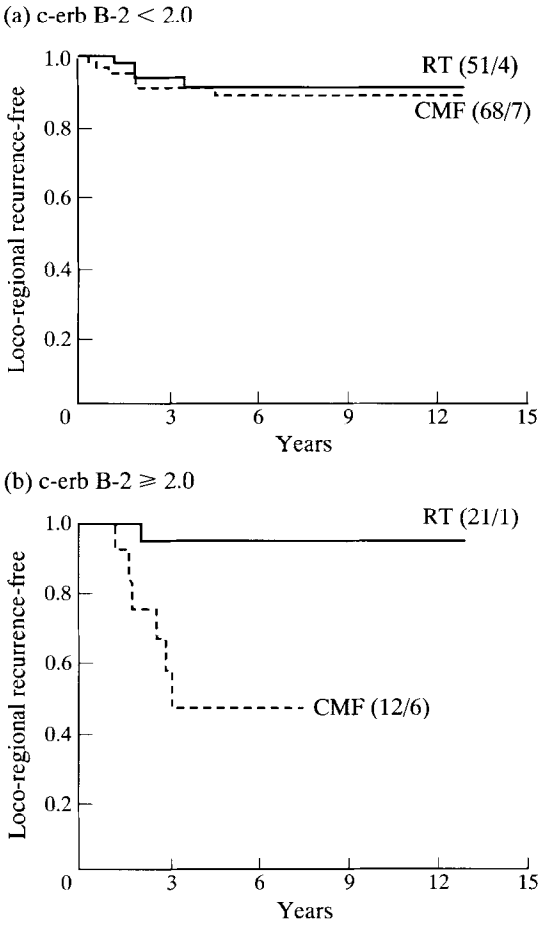


**Figure 3. Local recurrence-free interval of patients treated with chemotherapy (CMF) or radiotherapy (RT) in relation to c-erbB-2 content. Numbers of patients and locoregional recurrences are presented within parentheses.**

To our knowledge, this is a new finding, although it is supported by indirect observations. Overexpression of c-erbB-2 is more common among invasive carcinomas with a large *in situ* component [7, 14], and correlates with young age [6, 8], both being strong indicators of local recurrence [27]. High levels of c-erbB-2 are common in comedo type ductal carcinomas *in situ* and appear to indicate a greater invasive potential at this stage [10, 13]. Furthermore, c-erbB-2 overexpression correlates with the presence of lymph node metastases at primary diagnosis [6, 8, 9].

A significant association of c-erbB-2 positivity with recurrence has frequently been found in studies of node-positive breast cancer, where most patients received adjuvant systemic treatment. The prognostic value of c-erbB-2 found in node-negative breast cancer studies has varied [7, 8, 11, 14, 18, 28–30]. According to the present findings, one may speculate that the prognostic significance of c-erbB-2 expression, in part, reflects the degree of locoregional treatment. Toikkanen and associates [12] observed a worse prognosis related to c-erbB-2 overexpression for patients without systemic therapy after long-term follow-up. However, not all of these patients were given radiotherapy, and axillary dissection was not always performed, which was also the case in another study showing prognostic significance [28]. Long-term survival of node-negative patients treated with surgery alone has shown correlation to c-erbB-2 expression [8, 29]. In contrast, Gasparini and colleagues [30] found no prognostic value for c-erbB-2 in a study of node-negative breast cancer patients who were given extended locoregional treatment.

While a high S-phase fraction seems to identify a subgroup of premenopausal patients who would benefit from adjuvant chemotherapy, high expression of c-erbB-2 by the tumour might indicate poor responsiveness to CMF. We also suggest that there may be a significant interaction between c-erbB-2 expression and response to locoregional treatment. However, these suggestions need to be verified in prospective studies.


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