ELSEVIER

European Journal of Cancer 40 (2004) 1502–1508

European Journal of Cancer

www.ejconline.com

Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy

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Received 15 September 2003; received in revised form 13 February 2004; accepted 12 March 2004
Available online 25 May 2004

Abstract

The cell proliferation rate has been correlated to the response of breast carcinomas to preoperative chemotherapy (CT) and to disease outcome. However, this parameter is not yet used to select which tumours should be treated with preoperative CT. Furthermore, there is no consensus in the method used to evaluate cell proliferation. In poor prognosis breast carcinomas (PPBCs) treated by intensive preoperative CT, we compared the predictive value of S phase fraction (SPF), mitotic index (MI) and Ki67. We also evaluated the prognostic significance of the variation of the MI after CT. A series of 55 T2-T4N0N1M0 breast carcinomas were treated with 4 cycles of cyclophosphamide, 5-fluorouracil (5-FU) and doxorubicin. SPF was determined by flow cytometry on pre-therapeutic needle aspiration products. MI and Ki67 were evaluated on pre-therapeutic biopsy samples and on the tumours after CT. Fifteen patients (27%) had a pathological complete response (pCR), whereas 40 (73%) had residual disease. All three proliferative markers were found to have predictive value, but this value was higher for MI than for SPF ($P = 0.04$) and Ki67 ($P = 0.03$): the rate of pCR was 50% in cases with MI $> 17/3.3$ mm^2, but was only 7% in cases with MI under this threshold ($P = 0.0003$). A significant decrease of MI (mean 10.97) was observed after CT ($P = 0.001$). Furthermore, we observed that even for patients with residual tumour, the variation of MI after CT was a prognostic parameter and overall survival. The sequential analysis of MI in breast cancers treated by preoperative CT thus provides a surrogate for predicting long-term outcome.

Keywords: Preoperative chemotherapy; Mitotic index; Predictive markers; Proliferation markers; Prognostic markers; Pathological response

1. Introduction

Preoperative chemotherapy (CT) is increasingly used in the management of patients with large (>3 cm) breast carcinomas in order to increase both the possibility of breast conservative surgery, through a reduction of the tumour mass, and the patient's survival by the early treatment of occult metastases. Furthermore, pathological analysis after CT provides information on tumour cell sensitivity to the drugs used. The reported rates of complete pathological response (pCR) to CT varied from 7% to 32% according to the literature [1–9]. The pathological response to treatment and axillary

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doi:10.1016/j.ejca.2004.03.014

lymph node status are the most powerful prognostic factors for breast cancers after treatment with preoperative CT [4,5,10,11]. In order to select the patients who could benefit most from preoperative CT, the predictive value of different markers such as p53 [12,13], HER2 [11,14,15], Bcl2 [12] and /or the oestrogen receptor (ER) [12,15,16] have been assessed. These studies have resulted in inconsistent conclusions, but proliferation markers have consistently been found to be associated with tumour sensitivity to CT: a positive correlation was observed between the tumour response and cell proliferation, as assessed by the mitotic index (MI) [17,18], Ki67 expression [12,15,19] or the S phase fraction (SPF) [20–22]. In a pilot study, based on Ki67 immunostaining of tumour cells obtained by fine needle aspiration (FNA) before and after the first course of CT, a decrease of more than 25% in the proliferative fraction of the carcinoma cells significantly correlated with a decreased risk of disease recurrence [23]. This work and a few other studies [20,24] have prompted interest in the dynamic assessment of tumour cell proliferation after CT for the evaluation of the prognosis of breast cancers. However, despite these results, proliferation is not currently used as to help in indicating the need (or not) for preoperative CT. This may be related (in part) to a lack of standardisation in the techniques used to assess tumour cell proliferation.

A high proliferation ratio of tumour cells has previously been shown to result in an improved response to preoperative CT in our institution [21,25]. Therefore, a clinical trial of preoperative neoadjuvant CT including tumours with a high proliferation rate was designed. We report here, the results of the analysis of the sequential assessment of the tumour proliferation rate in the group of patients of that trial who were treated with anthracycline-based preoperative CT. In each case, the proliferation rate was assessed both on a pretherapeutic biopsy specimen and on tumour removed during surgery after CT. The aims of this study were: (1) to determine which of the three commonly used proliferation markers (SPF, MI, Ki67) was the most accurate in predicting the pathological response to CT and (2) to look whether the modification of proliferation markers following CT was a prognostic parameter of disease outcome.

2. Patients and methods

The patients studied ($n = 55$) were part of a former clinical trial (S10) including 80 patients presenting with a poor prognosis breast carcinoma (PPBC) with a high proliferation rate and treated at the Institut Curie between 1995 and 1998. The secondary surgery had not been performed in the Institut Curie for 25 patients and these patients were excluded from this analysis. PPBC corresponded to tumours larger than 3 cm and smaller than 7 cm, occurring in patients under 55 years of age, and with a SPF $> 4\%$. When the SPF was not determined, criteria of inclusion were age under 35 years and/or a high histological grade and/or histological evidence of vascular invasion. Patients presenting with initial metastases or with an inflammatory breast carcinoma were excluded. The median length of follow-up was 52 months (range: 21–70 months).

Treatment included the administration of 4 cycles of CT, every 21 days. Patients received doxorubicin 70 mg/m^2 day 1; cyclophosphamide 700 mg/m^2 days 1, 8; fluoro-uracil 700 mg/m^2, days 1 and 5. Following CT, all 55 patients underwent surgery. Breast-conserving surgery was carried out in 33/55 (60%) patients: a wide surgical resection of the residual mass was done as well as an axillary lymph node clearance. Whole breast irradiation was then delivered, using either Cobalt 60 or 5–6 MV photons to a median dose of 52 Grays (range 50–54 Grays). An additional radiation dose (boost) was given to the tumour bed in 27/3 (22%) patients, to a median total dose of 65 Grays (range: 60–75 Grays). 22/55 (40%) patients underwent a mastectomy after CT, followed by chest wall irradiation (median dose: 48 Grays, range: 45–52 Grays). In addition, radiotherapy to the internal mammary and supraclavicular nodes was administrated in 52 and 46 patients, respectively and axillary irradiation in 25/55 (45%) patients.

2.1. Evaluation of the response to treatment

For all patients, the response to CT was determined clinically and pathologically. Clinical response was considered as complete (cCR) when no residual tumour mass was palpable in the breast and was considered as major when the residual mass was less than 50% of the initial volume.

Pathological response assessment, performed as previously described [4,11], took into account both the proportion of residual epithelial neoplastic cells in the tumour mass, the location of this malignant component (invasive versus intraductal), the MI in malignant cells and the status of the metastatic axillary nodes. The response was considered as pathologically complete (pCR) when there was no residual invasive malignant epithelial cells in both the breast and the axillary lymph nodes. Tumours with an epithelial malignant residual component strictly *in situ* or representing less than 5% of the breast and/or axillary tumour mass and without any mitosis were also classified in the group of pCR. The response was considered as absent when no histological modification of the tumour tissue could be related to therapy and as partial in the remaining cases. Tumour characteristics are summarised in Table 1.

Table 1
Patient and tumour characteristics ($n = 55$)

Characteristics	No.	(%)
Age (years)		
Median	41.1	–
Mean	41	–
(range)	(28–60)	–
TUICC		
T2	32	(58)
T3	18	(33)
T4	5	(9)
Histological grade		
I	3	(5)
II	12	(22)
III	40	(73)
Histological type		
Invasive ductal	48	(87)
Invasive lobular	2	(4)
others	5	(9)
Node status		
N0	6	(11)
N1a	13	(24)
N1b	32	(58)
N2	4	(7)
Hormonal status		
ER+/PR+	22	(41)
ER+/PR−	2	(4)
ER−/PR+	10	(19)
ER−/PR−	20	(37)
ND	1	

ND, not determined; No, number of cases; ER, oestrogen receptor; PR, progesterone receptor; TUICC, Internationale Union Contre le Cancer.

2.2. Evaluation of proliferation

Before surgery, proliferation was evaluated according to three parameters: SPF in 51 of the 55 (93%) cases, MI and Ki67 nuclear immunostaining for all cases. After surgery, proliferation was evaluated on the residual tumour by MI for 38 patients and Ki67 staining for 39 patients. Proliferation was considered to be zero in 15 other cases with pCR.

2.3. SPF measurement by flow cytometry

SPF was assessed by flow cytometry on FNA products. Cells were placed into tubes containing Roswell Park Memorial Institute (RPMI) with 10% foetal calf serum and 10% dimethyl sulphoxide (DMSO) and frozen at −20 °C. After thawing, the cells were washed once in phosphate buffered solution (PBS) and suspended in 300 μl PBS containing 0.1% Tween 80, 30 μl RNase (100 μg/ml final) and 50 μg/ml propidium iodide. The cells were then filtered through nylon mesh and analysed with a Becton–Dickinson FACSCAN (Becton–Dickinson, Mountainview, CA). SPF were computed using Cellfit software (Becton–Dickinson) including the background subtraction.

2.4. Mitotic index

MI was established, using large core needle biopsy samples taken before CT with a needle larger than 14-gauge, as the number of mitoses in 10 successive high power fields (HPFs) using a Leitz DMRB microscope (Leica, Germany) with a 40×/0.70 objective and a 10× ocular. Each field corresponded to a surface of 3.3 mm^2. Mitotic count was performed on histological sections prepared from the biopsy sample of the diagnotic biopsy and stained by Hematein, Eosin and Saffron. The criteria of Van Diest and collegues were used to define the mitotic figures [26]. The amount of sample available allowed a mitotic count on 10 HPF to be achieved in all cases.

2.5. Ki67 immunohistochemistry

Ki67 immunostaining was performed on histological sections prepared from a biopsy sample taken before treatment and from the surgical specimens removed after treatment. Tissue sections were first digested in 0.1% (w/v) trypsin and 0.1% (w/v) calcium chloride in triphosphate buffer saline pH 7.6 for 5 min. Antigen-retrieval was performed by incubating tissue sections for 20 min in citrate buffer 10 mM (pH 6.1) in 850 W microwaves. Tissue sections were then incubated for one hour with an anti-Ki67 monoclonal antibody (Clone MIB1, Dako A/S, Glostrup, Denmark) at a 1/100 dilution. The staining was revealed using the Vectastain Elite ABC peroxidase mouse IgG kit (Vector Burlingame, CA) and diamino-benzidine (DAB) (Dako A/S, Glostrup, Denmark) as a chromogen. Ki67 staining was assessed semi-quantitatively by estimating the percentage of positive neoplastic nuclei within the area of highest positivity chosen after scanning the entire tumour surface at low power (10× objective). All nuclei with homogeneous staining, even with a light staining or only a nucleolar staining, were interpreted as positive.

The respective predictive values of the SPF, MI and Ki67 scoring were evaluated. The variation of MI and of Ki67 scoring before and after treatment was also evaluated. The prognostic value of the pathological response and of the variation of proliferation markers after CT were also determined.

2.6. Statistical analysis

Correlations between the proliferation markers were assessed with a Spearman correlation rank test. Frequency distributions were tested using a χ^2 test and Yates's correction was applied when appropriate. Kaplan–Meier estimates were used to calculate the survival

and metastases rates. Comparisons between the curves were assessed by the log-rank test. Relative risks and their 95% confidence intervals (CIs) were estimated using a Cox proportional hazards model. To assess if there was a link between the decrease of the MI after CT with disease outcome, a percentage of reduction was calculated (defined as the ratio between MI before treatment minus MI after treatment over MI before treatment) and 50% was used as a cut off point.

3. Results

3.1. Clinical and pathological characteristics

The characteristics of patients and tumours are summarised in Table 1. Median age was 41.1 years. Patients were premenopausal in 46 cases (84%) and presented a clinical axillary nodal involvement in 49 cases (89%). Median tumour size was 52.5 mm (range from 20.0 to 120.0 mm). Tumours corresponded to invasive ductal carcinoma in 87% of the cases with a high histological grade in 73%.

3.2. Response to treatment

Clinical assessment was performed in 54 of the 55 patients and showed a complete and major response to CT in 47 (87%) of the cases. Pathological analysis of the breast and of lymph node specimens showed a pCR in 15 of the 55 cases (27%). In two of these cases, the residual tumour was composed of carcinoma *in situ* only.

3.3. Predictive value of proliferation markers for PCR

SPF was evaluated only before treatment, whereas MI and Ki67 could be assessed on sequential specimens taken before and after treatment (Table 2). For SPF, the median value was 8.2% (mean 8.8%, range 2.6–20%). The median value of MI before treatment was 17 (mean 27, range 1–99) and it was 2 (mean 11, range 0–55) after CT. For Ki67 immunostaining, the median score was 42% (mean 41%, range 0–99%) before treatment and 5% (mean 15%, range 0–74%) after CT. The median values were used as cut-offs for the statistical analysis.

3.4. Correlation between the markers

On pretherapeutic specimens, a significant positive correlation was found between the SPF and MI ($r_{spearman} = 0.46$, $P = 0.001$) or SPF and Ki67 scores ($r_{spearman} = 0.32$, $P = 0.023$). This correlation was the strongest between MI and Ki67 score ($r_{spearman} = 0.51$, $P = 2 \times 10^{-4}$). A significant link was observed between high histological grade and high MI ($P \leqslant 0.0001$) or high Ki67 score ($P = 0.02$), but not with SPF. No statistical significant link was observed between any of the three proliferation markers and age, clinical stage or axillary nodal involvement.

Table 2
Proliferation markers before and after CT in breast carcinomas

Markers	Before CT		After CT		*P* value
	N	(%)	*N*	(%)	
SPF					
⩽8%	25	(49)	–	–	–
>8%	26	(51)	–	–	–
ND	4				
MI[a]					
⩽17	29	(53)	44[b]	(83)	
>17	26	(47)	9	(17)	10^{-3}
ND			2		
Ki67					
⩽42%	28	(51)	49[b]	(91)	
>42%	27	(49)	5	(9)	$<10^{-4}$
ND			1		

ND, not determined; CT, chemotherapy; SPF, S phase fraction; HPF, high power field.
[a] Number of mitosis per 10 HPF.
[b] Including the 15 cases with complete pathological response.

3.5. Variations in the MI and Ki67 scores following treatment

A significant decrease in the value of the proliferation markers was observed after CT. The mean value of this decrease was 10.7 points for MI ($P = 0.001$) (Fig. 1) and 21.6% for Ki67 score ($P < 0.0001$). The proliferation rate was considered to be zero in cases with pCR. SPF could not be assessed on the tumour removed during surgery.

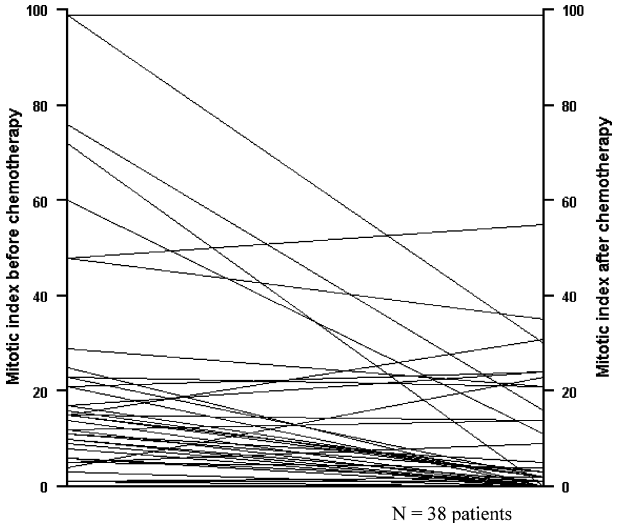

Fig. 1. Diagram showing variation of MI under chemotherapy.

3.6. Predictive value of proliferation markers on the pathological response to treatment

All three proliferative markers were predictive of the pathological response to treatment, but MI had a stronger discriminative value than either Ki67 or SPF (Table 3). The rate of pCR was 50% in the group of tumours with more than 17 mitosis per 10 HPF, whereas this rate was only 7% in tumours with a MI $\leqslant$ 17 ($P = 0.0003$). The rate of pCR was 42% in the group of tumours with SPF $>$ 8% and was 16% in tumours with SPF $\leqslant$ 8% ($P = 0.04$). For Ki67, the rate of pCR was 41% in tumours with a score >42% and 14% in tumours with a score $\leqslant$ 42% ($P = 0.03$).

3.7. Predictive value of other parameters

Age, tumour size and clinical stage were not associated with the pathological response to treatment. Oestrogen and progesterone receptor negativity were both associated with a higher pathological response rate: 36.7% for ER− compared with 12.5% for ER+, ($P = 0.044$) and 40.9% for PR− compared with 15.6% for PR+, ($P = 0.037$).

3.8. Prognostic value of the pathological response to treatment

Among the 55 patients included in this series, 12 died of their disease and 17 developed metastases during the follow-up period.

In the univariate analysis, no significant link was observed between disease outcome and pathological or clinical response, histological grade or clinical stage. After 42 months of follow-up, the metastasis-free interval was shorter for patients under 40 years of age (survival rate of 52%) than for patients aged over 40 years (survival rate 85%) ($P = 0.04$). Patients with tumours that achieved a pCR were found to have better survival rates without metastases but this association did not reach statistical significance ($P = 0.07$). In contrast, the overall survival rate at 52 months was longer (84%) for patients with a residual tumour showing a MI reduction >50% than that observed for patients pre-

Table 3
Pathological response to preoperative chemotherapy according to the proliferation markers

Pathological response	SPF		*P* value	Ki67		*P* value	MI		*P* value
	$\leqslant$ 8%	>8%		$\leqslant$ 42%	>42%		$\leqslant$ 17	>17	
Incomplete	21 (84%)	15 (58%)	4×10^{-2}	24 (86%)	16 (59%)	3×10^{-2}	27 (93%)	13 (50%)	3×10^{-4}
Complete	4 (16%)	11 (42%)		4 (14%)	11 (41%)		2 (7%)	13 (50%)	
	25	26		28	27		29	26	

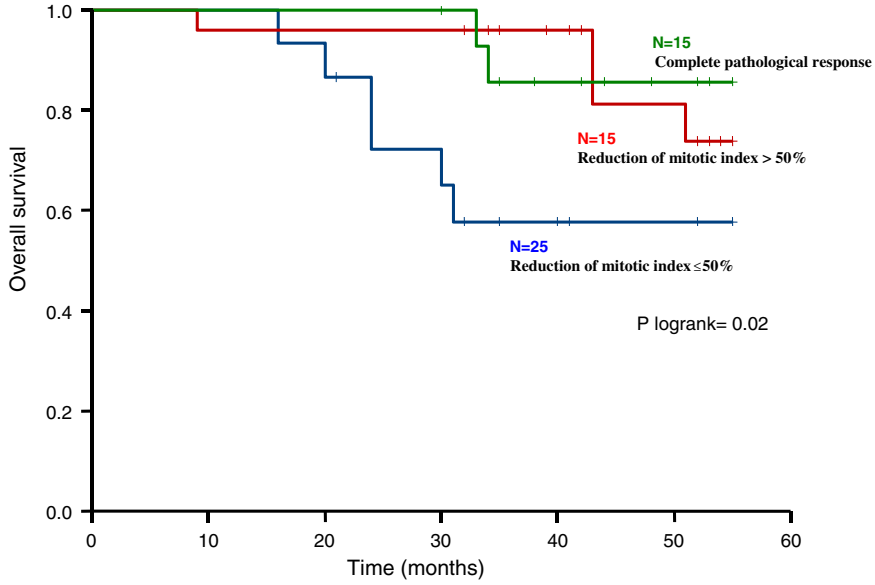

Fig. 2. Overall survival according to the modification of MI under chemotherapy.

senting a residual tumour showing a MI reduction of $\leqslant$50% (58%) ($P = 0.02$) (Fig. 2).

The small number of events observed in our pilot series did not allow us to perform a multivariate analysis.

4. Discussion

Our study shows that the rate of tumour proliferation is a biological parameter which has to be taken into account both to indicate the primary CT and to assess the efficacy of this treatment in breast cancer. In our series of cases, MI proved to be a major predictive criteria of the sensitivity of breast carcinomas to anthracycline-based CT: a pCR was obtained in 50% of the cases with a high MI, whereas only 7% of tumours with a low MI showed a complete response. Similarly, Colleoni observed a pCR of approximately 47% for patients presenting with a tumour that had a Ki67 rate superior to 20% [27]. The negative predictive value of the low proliferative rate was thus even stronger than the positive predictive value of a high rate. The link between tumour cell proliferation and sensitivity to CT has been reported by others [12,16,17, 19,21]. In these studies, the rates of pCR varied from 3% [12] to 28% [17]. The global rate of pCR observed in the present work is in the upper range (27%) of these figures, and in our study the axillary lymph node status was taken into account when assessing the pCR rate. In contrast with previous results [25,28] we did not find that a high proliferation rate was an indicator for a poor prognosis. This may be related to differences in the anthracyclines doses administered to patients, that were higher in our study, and/or to differences in the methods used to evaluate tumour cell proliferation.

In agreement with the observations of others [16], an increased rate of response to anthracycline-based CT was associated with hormonal receptor negativity. This may be linked to the fact that ER/PR− negative tumours preferentially correspond to poorly differentiated and highly proliferative carcinomas.

However, our data indicate that the proliferation rate in breast carcinomas is for the most part, more predictive than any other biological parameter analysed so far such as nuclear grade [17], ER [12,16], HER2 [11,12,16,29] or p53 [13,29]. A reliable assessment of the overall proliferation of tumour cells may be obtained by an analysis of a biopsy specimen [30] and this parameter should thus be taken into account in the indication of preoperative CT in breast carcinomas.

In agreement with other reports [12], we also observed an association between a pCR, following CT and a favourable outcome, but this trend did not reach statistical significance. This might be related to the fact that, in PPBC, subclinical metastases should critically influence the outcome, independent of the local risk of relapse. Alternatively, the response to CT in terms of tumour mass may be delayed in some cases and the existence of residual tumour is not always an indication of cell insensitivity to the drugs used. Therefore, a decrease in the proliferation rate of tumour cells as a direct effect of CT might be a more reliable parameter of drug sensitivity than a reduction in the tumour mass [12,22,24,31]. We observed that, in cases with residual tumour at surgery, a modification of the proliferation rate after treatment was a significant prognostic parameter. The evaluation of tumour response to primary CT should thus take into account not only the importance of the residual tumour mass, but also the effect of therapy on the tumour cell proliferation [12].

In conclusion, the rate of tumour cell proliferation may indicate the possibility of obtaining a pCR following preoperative CT. Few cases of pCR were observed in tumours with a low proliferation rate, whereas up to 50% of the cases with a high MI reached a pCR. It would be interesting to assess the rate of pCR in the same population of tumours treated by taxanes which recently provided a high rate of pCR in unselected breast carcinomas [5]. Our analysis also suggested that the modification of the proliferation rate following treatment could be a prognostic biological parameter, but this prognostic value should be confirmed in a larger number of cases. These data and studies from the literature indicate that the selection of patients likely to benefit from preoperative CT should be performed according to the tumour cell MI, assessed on biopsy specimens, according to well defined histological procedures.

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