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Original Paper

Prediction of Response to Primary Chemotherapy for Operable Breast Cancer

M. Colleoni,[1] E. Orvieto,[2] F. Nolé,[1] L. Orlando,[1] I. Minchella,[1] G. Viale,[2] G. Peruzzotti,[1] C. Robertson,[3] C. Noberasco,[1] V. Galimberti,[4] V. Sacchini,[4] P. Veronesi,[4] S. Zurrida,[4] R. Orecchia[1] and A. Goldhirsch[1]

[1]Department of Medicine and Radiotherapy; [2]Division of Pathology and Laboratory Medicine; [3]Division of Epidemiology and Biostatistics; and [4]Division of Senology, Istituto Europeo di Oncologia, Via Ripamonti 435, 2041, Milan, Italy

The use of primary systemic cytotoxics leads to a high remission rate in patients with breast cancer. Response was identified as an important variable associated with survival. Thus, features which predict response, are potentially relevant for planning treatments and improving survival. Retrospectively, we investigated several histopathological features (expression of oestrogen and progesterone receptors, Mib1, bcl-2, c-erbB-2, and p53) prior to two programmes of either sequential preoperative chemotherapy (doxorubicin plus cyclophosphamide) and radiotherapy (Group A), or preoperative chemotherapy (5-fluorouracil, folinic acid and vinorelbine) alone (Group B) in patients with operable breast cancer. After three courses, patients with a partial or complete response were given a further three courses, which was followed for patients in Group A by radiotherapy 50 Gy plus a boost of 10 Gy. All patients were submitted to surgery after completion of preoperative treatment and pathology material from 73 patients (median age, 49 years, range, 30–70; performance status, 0–1; 68 T_2, 5 T_3) was obtained. The overall response rate according to radiological and clinical evaluation was 59% (68% for Group A and 49% for Group B). 12 of 14 patients with p53-positive tumours and 31 of 59 with p53-negative tumours responded ($P = 0.04$). 6 of 7 patients with elevated c-erbB-2 had a response compared with 37 of 66 patients in the group with c-erbB-2 negative tumours ($P = 0.03$). Mib1 expression decreased substantially (≥50%) in 25 patients during treatment, of whom 20 responded compared with 21 of 48 patients with a lower decrease ($P = 0.04$). Response was observed in 28 of 37 patients with high baseline Mib1 (> 20%) and in 15 of 36 patients in the low Mib1 group ($P = 0.05$). Finally, 32 of 44 tumours with low expression of progesterone receptors responded compared with 11 of 29 tumours with high receptors expression ($P = 0.05$). These markers might be useful for tailoring primary and postsurgical systemic treatments. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: doxorubicin, cyclophosphamide, preoperative chemotherapy, predictive features
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INTRODUCTION

IN RECENT years, the concept of treating patients with primary chemotherapy for breast cancer has become popular [1–7]. The only demonstrated benefit in terms of treatment effects is the achievement of tumour shrinkage sufficient to allow breast saving surgery in some of the patients. To date, no prolongation of disease-free survival has been shown from this approach [8–10]. Theoretically, the use of preoperative chemotherapy may eliminate systemic micrometastases, thus preventing the development of drug-resistant cells [11]. The major advantage from primary systemic treatment is the test of tumour response *in vivo*, obtaining the necessary information for improved tailoring of further adjuvant treatments given postoperatively. The assessment of factors which are

Correspondence to M. Colleoni, e-mail: mcol@ieo.it
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associated with response or resistance to primary therapies is an important step in the development of such sequential treatments.

In the few studies with proper follow-up, it has been observed that patients with a major response after primary chemotherapy have a better relapse-free survival than non-responders [4, 8, 12, 13]. Moreover, some investigators have found that not only the response but also the type and degree of response predict overall outcome in terms of disease freedom and overall survival [1–3]. Some baseline biological features have been described as useful markers for sensitivity to treatment. These include oestrogen (ER) and progesterone receptors (PgR) [14], the tumour suppresser gene product p53 [14–16], c-erb-B2 amplification and overexpression [14, 16], bcl-2 expression [14] and cell proliferation markers such as Mib1 [17, 18]. Moreover, some investigators have reported on changes of markers like Mib1 to be an important prognostic feature in assessing response to preoperative treatment [18].

Recently, a trend in higher response to neoadjuvant chemoendocrine therapy was observed in patients with tumours positive for ER, PgR and bcl-2, and a statistically significant higher response rate was reported for c-erbB-2 negative tumours [14]. Based on these considerations, the aim of the present study was to evaluate retrospectively the prognostic value of baseline biological parameters in patients submitted to a programme of primary chemotherapy, or a sequence of chemotherapy and radiation to the breast.

PATIENTS AND METHODS

Patients

Patients with biopsy-proven T_2–T_3, N_{0-2} breast cancer observed at the European Institute of Oncology from January 1995 to December 1997 were considered eligible for the study. Other inclusion criteria were: non-metastatic tumours; < 75 years of age; largest tumour diameter > 2.5 cm; Eastern Cooperative Oncology Group (ECOG) performance status 0–1; white blood cell (WBC) count > 4000 mm^3 and platelet count > 100,000 mm^3; serum creatinine < 1.2 mg/dl; bilirubin < 3 mg/dl; aspartate and alanine aminotransferase < 2.5 the upper limit. Patients with evidence of cardiac disease (congestive heart failure, history of myocardial infraction within the previous 3 months), severe vascular disease or uncontrolled concomitant infections were excluded.

Treatment

Group A was treated with chemotherapy followed by radiotherapy accordingly to the following schedule: doxorubicin 60 mg/m^2 day 1 plus cyclophosphamide 600 mg/m^2 day 1, cycles repeated every 21 days 3 times (AC regimen); group B was treated with the following schedule: 5-fluorouracil 350 mg/m^2 days 1, 2, 3, folinic acid 100 mg/m^2 days 1, 2, 3 and vinorelbine 20 mg/m^2 day 1 and 3 (FLN regimen). Tumour response was evaluated after each cycle, and surgery was planned in patients with clinically progressing disease. Response after each cycle was evaluated by clinical measurement of the two largest diameters. After three cycles, patients also had mammography to assess response. Based upon logistics, patients also had a complementary ultrasound breast examination. In case of stable disease patients were submitted to surgery. In case of partial remission or complete remissions patients were given three more cycles followed by radiotherapy 50 Gy plus 10 Gy boost (group A) or 3 more cycles of chemotherapy (group B) and then were submitted to surgery. The AC regimen was selected because significant activity has been demonstrated in the preoperative setting [4, 8, 19]. The FLN regimen was administered on the basis of the activity shown in a phase II trial carried out at our institution on metastatic breast cancer [20].

Radiation treatment consisted of high-voltage radiotherapy administered to the involved breast 3–4 weeks after the last course of chemotherapy at the dose of 50 Gy using two opposite tangential fields and with a 10 Gy boost applied to the residual tumour mass (group A only). Surgery consisted of classic quadrantectomy as described by Veronesi and associates [6] and axillary node dissection, which was always performed via a separate incision. In cases with a diameter > 2.5 cm and/or when breast saving surgery was not feasible, mastectomy was planned.

Responses were evaluated according to both radiological (breast ultrasound plus Rx mammography) and clinical evaluation and graded according to standard WHO criteria. A complete response was defined as the disappearance of all parameters of disease by two observations not less than 4 weeks apart. A partial response was defined as a 50% or more reduction in the products of the perpendicular diameters of the lesion without any evidence of new lesions. Stabilisation of disease was defined as a less than 50% reduction or less than 25% increase in the products of the perpendicular diameters of the lesion without any evidence of new lesions. Progressive disease was defined as a > 25% increase or the appearance of new lesions. Side-effects were scored according to WHO criteria.

Immunohistochemistry

Duplicate immunostaining experiments for the localisation of ER, PgR, p53, BCL-2, c-erbB-2 and Mib1 were performed on consecutive serial paraffin sections of the tru-cut biopsy before treatment and of the residual tumour obtained at surgery. Immunostaining for ER, PgR and Mib1 was performed with an automated immunostainer (Dako-Biotech Tech Mate™ 500, Glostrup, Denmark) using an avidin-biotinylated peroxidase staining method. The remaining immunoreactions were performed using the ABC staining method as already reported [19]. Briefly, the slides were dewaxed, rehydrated, and immersed in boiling citrate buffer (pH 6), in a microwave at 650 W, two changes for 5 min each. Slides were then allowed to cool at room temperature for 20 min and treated with 3% hydrogen peroxide in distilled water to inhibit endogenous peroxidase activity. After washing, the slides were subsequently incubated with: (i) specific monoclonal antibodies, overnight, at 4°C; (ii) 1:200 dilution of a biotinylated rabbit antiserum to mouse immunoglobulin, for 30 min and (iii) 1:100 dilution of the streptavidin-biotinylated peroxidase complex, 30 min. Peroxidase activity was detected using diaminobenzidine as the chromogenic substrate. The primary monoclonal antibodies, their source and working dilution are detailed in Table 1.

Scoring of immunocytochemical results

The stained slides were evaluated independently by two of the authors. Only nuclear immunoreactivity was evaluated for ER, PgR, p53, Mib1, whilst cytoplasmic and membrane immunoreactivity were considered for Bcl2 and c-erbB-2, respectively. For ER, PgR, Mib1 the percentage of immunoreactive cells in at least 2000 neoplastic cells was recorded.

Table 1. *Monoclonal antibody used for immunohistochemical analysis*

Reagent	Clone designation	Working dilution	Source
Oestrogen receptor	1D5	1/50	Dako, Denmark
Progesterone receptor	1A6	1/20	Novocastra Lab. Ltd, U.K.
Mib1	MIB1	1/200	Coulter-Immunotech, U.S.A.
bcl-2	124	1/80	Dako, Denmark
p53	DO7	1/2000	Dako, Denmark
c-erbB-2	TAB250	1/200	Triton, California, U.S.A.

For the remaining immunoreactions (p53, Bcl2 and cerbB2), the tumours were classified into four groups, according to the percentage of immunoreactive neoplastic cells, as follows: negative tumours: less than 1% of immunoreactive cells; +: 1–10% immunoreactive cells; ++: 10–25% immunoreactive cells; +++: 25–50% immunoreactive cells; ++++: more than 50% immunoreactive cells.

The threshold for p53, c-erb-B2 and Bcl2 positivity was 10%; for ER and PgR positivity 10% and for Mib1 positivity 20%. The choice for immunocytochemical threshold values was based on results from previous studies. In particular, a 10% cut-off for the expression of p-53 was validated by other authors with regard to clinical end-points [21] and a 10% positivity is the threshold selected for c-erb-B2 over-expression when monoclonal anti-HER2 antibody treatment is being considered [22]. Previous studies demonstrated that a 10% threshold for hormone receptor [14] correlates with clinical outcome. Evaluation of Mib1 staining was based on the percentage of positively staining nuclei. The median percentage value observed at our Institution (20%) was used to divide values into high and low. Bcl-2 immunostaining has not been prospectively validated with regards to clinical end-points and a 10% cut-off point was arbitrarily selected.

Statistical analysis

Fishers's exact test was used to evaluate the association between the positive and negative biomarkers and clinical response.

Table 2. *Patient characteristics*

	FLN regimen	AC regimen	All patients
Entered/assessable	33/33	40/40	73/73
Median age, (range)	52 (36–70) years	43 (30–68) years	49 (30–70) years
Premenopausal/ postmenopausal	19/14	26/14	45/28
ECOG performance status 0–1	33	40	73
Tumour stage			
T_2*	33	35	68
T_3	–	5	5
Tumour size			
≤3.5 cm	28	29	57
> 3.5 cm < 4.5 cm	4	4	8
≥4.5 cm	1	7	8

*At baseline.

Table 3. *Baseline and post-treatment markers in* 73 *evaluable*

	Positive (%)		Negative (%)	
	Pre	Post	Pre	Post
ER	49 (67)	39 (53)	24 (33)	34 (47)
PgR	29 (40)	18 (25)	44 (60)	55 (75)
p53	14 (19)	17 (23)	59 (81)	56 (77)
Mib1	37 (51)	18 (25)	36 (49)	55 (75)
c-erbB-2	7 (10)	8 (10)	66 (90)	65 (89)
bcl-2	46 (63)	54 (74)	27 (37)	19 (26)

RESULTS

73 patients were included in the study and their tumours assessed by immunostaining. Their characteristics are shown in Table 2. Most had a T_2 lesion with 45 of 73 having the primary measure < 3.5 cm in diameter.

After three courses of chemotherapy with the AC regimen, 13 out of the 40 patients (33%) had stable disease and were offered surgery. Of the remaining 27 responding patients, 6 had a radiological and clinical complete remission and 21 had a partial response (response rate 68%; 95% confidence interval, 51–81%). After 3 courses of chemotherapy with the FLN regimen, 17 out of the 33 patients (51%) had stable disease and were offered surgery, and 16 had a partial response (response rate 49%; 95% confidence interval, 31–66%). Overall, the response rate was 59% (95% confidence interval 47–70%). Only 5 non-responding patients in the FLN group and 7 non-responding patients in the AC group required mastectomy. All other patients received quadrantectomy.

Pre- and post-treatment biological features are shown in Table 3. A decline in expression of ER, PgR and Mib1 was observed. The staining for p53, c-erbB2 and bcl2 remained unchanged.

The correlation between baseline features and response is shown in Table 4. A significant difference in response was observed for patients with p53-positive tumours (12 of 14 responding) compared with those with p53 negative tumours (31 of 59 responded) ($P = 0.04$). The expression of PgR also correlated with response to chemotherapy. In fact, 32 of 44 tumours negative for PgR responded compared with 11 of 29 tumours positive for PgR ($P = 0.05$). The expression of the Mib1 was investigated according to either baseline degree of staining (low versus high), and according to the degree of change in staining between pre- and post-chemotherapy (Table 5). A significant difference in response was observed for patients with elevated baseline Mib1 values (28 of 37 responding) compared with those with low values (15 of 36 responded) ($P = 0.05$). Also patients who presented a significant decrease in the value (> 50%) had a significantly higher response rate (80% versus 44% $P = 0.04$). A decrease

Table 4. *Response according to baseline features*

	Positive	Responsive pts (%)	Negative	Responsive pts (%)	*P* value
p53	14	12 (86)	59	31 (53)	0.04
c-erbB2	7	6 (86)	66	37 (56)	0.03
bcl-2	46	29 (63)	27	14 (52)	1.0
ER	49	28 (27)	24	15 (63)	1.0
PgR	29	11 (38)	44	32 (73)	0.05

Table 5. *Response according to Mib*1 *and its modifications*

Mib1	No. of pts	Responsive pts (%)	*P* value
Baseline staining			
High (≥20%)	37	28 (76)	
Low (<20%)	36	15 (42)	0.05
Change in staining*			
Decrease ≥50%	25	20 (80)	
Decrease <50%	48	21 (44)	0.04

*Change, pre- and post-chemotherapy.

> 50% was observed in only 7 of 36 patients with low baseline expression of the marker. Conversely, 18 of 37 patients with elevated baseline Mib1 experienced a significant decrease. 6 of 7 patients with tumours positive for c-erbB-2 responded, as compared with 37 of the 66 patients with negative tumours that had a response. This observation was statistically significant despite the small number of patients with c-erbB-2 positive tumours ($P = 0.03$).

The results observed with Mib-1 were consistent within each of the treatment administered, although the majority of remissions were observed with the AC regimen. The majority of tumours positive for p53 (13 of 14 tumours) and c-erb-B2 (5 of 7 tumours) was observed in the group of patients treated with the AC regimen. However, 1 patient with the p53 positive tumour in the group treated with the FLN regimen had a response as did patients positive for c-erbB-2. No statistical difference in response rates was observed according to the level of ER and no significant difference in response was observed between patients with bcl-2-positive and bcl-2 negative tumours.

DISCUSSION

Preoperative chemotherapy might be beneficial for patients with breast cancer in several ways besides allowing breast conservation surgery in some of the patients. The information on response to a chemotherapy regimen may be used for tailoring further postoperative treatments. In this way, it might also result in additional eradication of occult micrometastases leading to an overall benefit in terms of disease freedom and prolonged survival. Finally, the response to the treatment may be used as a prognostic marker. The detection of preoperative biological features and the patterns of their change after exposure to treatment might thus serve as a surrogate for prediction of response.

Until now the only demonstrated benefit from preoperative chemotherapy is the improvement in surgical breast conservation [7], whereas no definite advantage on survival has yet been shown [8]. Limited data have been published on the correlation between preoperative markers, response to treatment and patient outcome. Preliminary experience has indicated a potential role as predictive factors for the changes of various histopathological patterns induced by treatment. However, assessment of such features is arduous due to heterogeneity of methods used in the various studies.

The results of our trial indicated that tumour cells changed significantly after exposure to chemotherapy or chemotherapy and radiotherapy. As shown in Table 2 the degree of positive staining for hormone receptors and Mib1 were reduced after primary chemotherapy whilst tumours positive for bcl-2 p53 and c-erbB-2 remained essentially unchanged. A major problem in the pretreatment assessment of predictive features is represented by the limited tissue sample that is achievable with the core-biopsy. In fact the results observed with the trucut might be representative only of a minor part of the tumour and the limited expression of several features (p53, c-erbB-2, PgR) may be related to this fact. Conversely, the low rate of positivity of PgR and c-erbB-2 after chemotherapy may be influenced by the treatment itself. Moreover, the difference observed between the present study and results from other authors may be also related to the different cut-offs. In fact, Makris and colleagues [14] found a 31% positivity-rate for c-erbB-2 and of 39% positivity for p-53 but a rate of positive staining tumour cell nuclei between 1/100 and 1/10 was considered as positive. Conversely, other authors reported results similar to ours. Chollet and colleagues [13] reported only a 24% rate of positivity for PgR expression at baseline and MacGrogan and colleagues [16] showed a 27% of positivity for p53.

In the present study we found a significant difference in response to primary chemotherapy in highly proliferating tumours with a Mib1 index over 20%. This observation is in line with previous published studies. In particular, *in vitro* studies have shown an increased sensitivity of highly proliferating tumours to chemotherapeutic agents in breast carcinoma cell lines [23, 24] and a significant correlation between response to preoperative chemotherapy and elevated baseline Mib1 was recently published [16]. Others who tested Mib1 expression tumours challenged with preoperative chemotherapy [17, 18] found a significant correlation between response, high baseline expression and low expression post-chemotherapy. In the present study we observed a significant correlation between Mib1 reduction and objective response to chemotherapy. In fact 80% (20/25) of the patients that had a substantial decrease in the marker, had a response. It is noteworthy that all 6 complete remissions had a high baseline Mib1 and a very low value after treatment (≤8%). These data indicate the possibility of predicting response by monitoring changes in the proliferative fraction during chemotherapy, thus avoiding unnecessary chemotherapy and submitting immediately to surgery patients that may not benefit from the treatment.

A second relevant result of the current study is the correlation between response and altered p53. Normal p53 tumour suppressor gene function is known to be correlated with apoptosis. Its normal function is required for an efficient activation of apoptosis following irradiation or treatment with chemotherapy [25, 26]. In particular, Lowe and colleagues [25] tested the effect of doxorubicin and other cytotoxics to induce p53-dependent cell death. They found that lack of p53 in cells predicted their resistance to all treatments. It has also been hypothesised that the cytotoxic effects of chemotherapeutic agents could be enhanced by mutated *p53* which is no longer able to repair drug-induced DNA damage. In fact, *p53* has been shown to stimulate indirectly a DNA repair mechanism through activation of other effector genes such as *GADD45*, *p21* and *PCNA* [27]. Data on p53 overexpression and response to treatment are, however, conflicting. Several reports have failed to detect a correlation between the marker and response to neoadjuvant chemotherapy [14, 16]. Other investigators have found a correlation between *p53* mutation or overexpression and chemoresistance [28, 29]. In particular, Rush and colleagues [28], using a similar methodology as the one adopted in our study, found a negative correlation between p53 expression

and response to cisplatin-based chemotherapy in non-small-cell lung cancer. Other studies have shown similar results [30]. Allred and colleagues indicated that the overexpression of this gene product could indicate a great responsiveness to chemotherapy in node-positive breast cancer [31]. The evaluation of p53 through immunohistochemistry has been based on the assumption that accumulation of the protein indicated mutation of the *p53* gene. However, occasionally, *p53* mutation and expression are not consistently present, and the precise significance of such phenomenon is unknown [32]. Although a definitive conclusion on this issue might require a larger sample size, we hypothesise that the assessment of p53 is an important feature of efficacy of treatment.

Only 7 patients were positive for c-erbB-2 and 6 of them responded to chemotherapy, whilst 37 of the 66 patients negative for c-erbB-2 achieved a response. The difference was statistically significant ($P = 0.03$). Although the limited number of positive tumours (5 in the group of patients treated with the AC regimen and 2 in the group treated with the FLN regimen) prevents a firm conclusion, these data are interesting and deserve further investigation. In fact, the Cancer and Acute Leukemia Group B (CALGB) investigators found that higher-doses of a doxorubicin-containing combination were more effective than lower-doses in patients with c-erbB-2-positive tumours [33]. Conversely, the International Breast Cancer Study Group (IBCSG) observed that CMF adjuvant chemotherapy was more effective for c-erbB-2-negative compared with c-erbB-2-positive tumours [34].

In our experience negative staining for progesterone receptors correlated with response to therapy. *In vitro* studies have shown an increased sensitivity to cytotoxics agents, especially doxorubicin, of ER-negative tumour cell lines [35]. Conversely, clinical studies showed conflicting data about the relationship between finding a large number of cells expressing hormone receptors and response to chemotherapy. In the neoadjuvant setting, MacGrogan and colleagues [16] found a significant chemosensitivity for ER-negative tumours but not for PgR-negative tumours, whereas Makris and colleagues [14] found no significant correlation between hormone receptor expression and response to chemoendocrine therapy. This latter observation may be due to the combined effect of the two treatment modalities. However, other authors failed to observe a correlation between hormone receptor expression and response to chemotherapy [36].

In conclusion, the results of this study suggest that preoperative assessment of Mib1, c-erbB-2, PgR and p53 may be useful for prediction of response to chemotherapy. Moreover, monitoring of changes in Mib1 during chemotherapy may be of value in predicting response. This study is helpful for tailoring novel research programmes using other regimens of preoperative chemotherapy.

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