bcl-2 but not p53 Expression Is Associated with Resistance to Chemotherapy in Advanced Breast Cancer

Andrea Bonetti, Marta Zaninelli, Roberto Leone, Gian Luigi Cetto, Giuseppe Pelosi, Sonia Biolo, Alessandra Menghi, Erminia Manfrin, Franco Bonetti, and Quirino Piubello


ABSTRACT

Programmed cell death is an important determinant of the response to chemotherapy. Among the factors controlling this process, a significant role is played by bcl-2 and p53, the expression of which, together with estrogen receptor content and tumor proliferative activity, was investigated by means of immunohistochemistry in 55 advanced breast cancer patients (median age, 60 years; range, 25–71 years). Analysis of bcl-2 expression identified two groups of patients with a significant difference in response rate. A total of 17 patients (31%) responded to chemotherapy (5 had a complete response and 12 had a partial response): 14 of 32 (44%) bcl-2-negative patients (<40% stained cells) and only 3 of 23 (13%) bcl-2-positive patients (≥40% of stained cells; P = 0.019 by Fisher’s exact test). The two groups were well balanced in terms of age, performance status, disease-free survival, menopausal status, and type of chemotherapy. bcl-2-negative tumors showed a tendency toward a higher p53 expression and proliferation rate, whereas an excess of bone as the dominant disease site was evident among the bcl-2-positive ones. However, the only variable to result significantly different between the two groups was estrogen receptor expression (P = 0.004). A multivariate logistic regression model showed that bcl-2 maintained its power of discriminating two groups with a different probability of responding to chemotherapy, although the greatest contribution was given by dominant disease site and type of chemotherapy. In conclusion, the results of this study suggest a possible role for bcl-2 in predicting resistance to chemotherapy.

INTRODUCTION

Tissue homeostasis depends on the maintenance of a strict balance between cell proliferation and PCD, a distinct mode of cell death that is responsible for the deletion of cells in normal tissue and in some pathological contexts, such as tumors. Under physiological conditions, apoptosis is a complex phenomenon regulated by various genes encoding proteins that either induce or inhibit cell death. The extent of apoptosis in tumors can be enhanced by the administration of almost any kind of chemotherapeutic drug, but loss of the genes required to complete PCD or overactivation of those that block the process may make tumors resistant to common anticancer agents.

Among the genes involved in the apoptotic pathway, an important role is played by bcl-2, which was originally discovered at the t(14;18)(q32;q21) breakpoint in follicular lymphomas. Bcl-2 encodes a Mr 26,000 protein that has been localized to the mitochondrial membrane, the nuclear membrane, and the endoplasmic reticulum, and it has been shown that the expression of bcl-2 can prevent the PCD induced by most chemotherapeutic drugs.

The p53 tumor suppressor gene is the most commonly mutated gene in human cancer. The wild-type p53 product has antitransforming and antiproliferative activity and, in some cases, promotes PCD. A number of DNA-damaging agents that trigger PCD also induce p53 expression. On the other hand, mutant p53 can inhibit PCD, although it does not seem to have the same protective effect as bcl-2. The mutation of the p53 gene leads to prolonged protein half-life and its accumulation in the nuclei, thus making it possible to detect by means of immunohistochemistry.

As a result of the experimental data linking bcl-2 and p53 overexpression to resistance to most chemotherapeutic agents, it seemed rational to investigate their possible resistance-inducing role in the clinical setting.

PATIENTS AND METHODS

Patients. This retrospective study involved 55 patients with histologically documented advanced breast cancer treated with chemotherapy for metastatic disease. The patients were considered eligible if they had measurable disease and ineligible if the only manifestation of disease was a malignant effusion, a previously irradiated lesion, brain metastasis, or nuclide scan evidence. Clinical staging was based upon a complete history, physical examination, a routine biochemical profile, a complete blood cell count, and the results of imaging procedures for all.

The abbreviations used are: PCD, programmed cell death; CMF, 600 mg/m2 cyclophosphamide + 40 mg/m2 methotrexate + 600 mg/m2 5-fluorouracil; TBS, Tris-buffered saline; ER, estrogen receptor; CI, confidence interval; CR, complete response; PR, partial response.

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patients before the beginning and after three cycles of chemotherapy. Response was evaluated according to the standard WHO criteria (23).

The evaluated chemotherapy was the first for metastatic disease. The standard protocols were: CMF on day 1 ($n = 34$ patients); and $500 \text{mg/m}^2$ cyclophosphamide + $50 \text{mg/m}^2$ doxorubicin (or $70 \text{mg/m}^2$ epirubicin) + $750 \text{mg/m}^2$ 5-fluorouracil on day 1 ($n = 21$ patients). All of the drugs were given i.v. in cycles separated by a 3–4-week interval.

**Immunohistochemical Assay.** Sections of each paraffin-embedded block used for the initial diagnosis of cancer were mounted on adhesive-coated slides, heated to $60^\circ C$ for 10 min, cooled, deparaffinized, and hydrated by means of three changes of xylene and graded alcohol. The slides were then placed in buffer citrate and heated in a microwave oven for three 5-min cycles at $600 \text{W}$. At the end of the procedure, the slides were left at room temperature for 30 min and then rinsed in TBS buffer. Endogenous peroxidase activity was inactivated by means of incubation for 30 min in 1% hydrogen peroxide. After rinsing in distilled water and TBS, the slides were incubated with blocking serum in TBS for 20 min to suppress the nonspecific binding of immunoglobulin. Specific immunostaining was evaluated by means of overnight incubation with the appropriate mouse monoclonal antibody [clone 124 for bcl-2 (DBA Italy) in a 1:40 dilution; and clone Pab 1801 for p53 (Oncogene Science) in a 1:20 dilution]. After TBS rinsing, a secondary biotinylated antimouse antibody (Vector) was applied for 30 min, and the slides were then rinsed with TBS, incubated with streptavidin in a 1:40 dilution; and clone Pab 1801 for p53 (Oncogene Science) (26) and ERICA monoclonal kits (Abbott, Chicago, IL; Ref. 25), respectively. The details of the methods have been published elsewhere (26). All of the pathobiological features were separately evaluated by two observers who were blinded to the clinical outcome of the patients. At least 1000 cells were counted, and the results were expressed as the percentage of positive cells.

**Statistical Analysis.** Crude and stratified analyses of the between-group differences were performed using $x^2$ statistics or Fisher’s exact test. A multivariate analysis based on unconditional logistic regression (27) was made to investigate the prognostic role of bcl-2 levels and the other explanatory covariates in relation to the response rate measured as a dichotomous variable. The overall survival estimates were obtained using the Kaplan-Meier method, and the significance of the differences in survival between the two groups was measured by means of Gehan’s Wilcoxon test.

**RESULTS**

This retrospective study involved 73 consecutive breast cancer patients who had had a relapse between January 1, 1989, and December 31, 1991. Eighteen patients were not eligible for several reasons (bilateral breast tumors, early progression, grade 3–4 Eastern Cooperative Oncology Group performance status, lack of archived material, lost to follow-up, and chemotherapy administered other than CMF or anthracycline-based regimens). The remaining 55 patients satisfied the eligibility criteria described previously. The stains for bcl-2 were obtained either on the primary tumors (44 patients; 80%) or at the relapse site (11 patients; 20%). The distribution of bcl-2 at the two sites was similar, with 27 of 44 (61%) primary tumors and 5 of 11 (45%) metastases presenting a low bcl-2 expression. Oncoprotein p53 expression was evaluated in a subgroup of 43 patients. The degree of proliferative activity measured by means of Ki-67 antibody and ER immunohistochemical assays had already been determined on the fresh material collected at the time of cancer diagnosis in the majority of patients. The tumors were considered highly proliferative and ER positive when >25% and >10%, respectively, of tumor cells reacted with the appropriate antibodies.

The response to chemotherapy was correlated with bcl-2 expression using different cutoff levels. The 40% value resulted the lowest cutoff separating two groups with a significant difference in response rate. When lower cutoff were used, the statistical significance was lost, although a higher response rate in bcl-2-negative tumors was still observed. The use of the highest cutoff (≥80%) was associated with an improvement of the ability of predicting the resistance to chemotherapy, with only 1 of 20 bcl-2-positive patients responding to chemotherapy ($P = 0.001$).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics according to bcl-2 status</th>
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<tr>
<td></td>
<td>Low bcl-2 ($n = 32$)</td>
</tr>
<tr>
<td><strong>Performance status</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>17 (53)</td>
</tr>
<tr>
<td>1–2</td>
<td>15 (47)</td>
</tr>
<tr>
<td><strong>Menopausal status</strong></td>
<td></td>
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<tr>
<td>Pre- or peri menopause</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Postmenopause</td>
<td>26 (81)</td>
</tr>
<tr>
<td><strong>p53</strong> ($n = 43$)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>10 (42)</td>
</tr>
<tr>
<td>High</td>
<td>14 (58)</td>
</tr>
<tr>
<td><strong>ER</strong> ($n = 45$)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13 (50)</td>
</tr>
<tr>
<td>Positive</td>
<td>13 (50)</td>
</tr>
<tr>
<td><strong>Ki-67 value</strong> ($n = 45$)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>14 (54)</td>
</tr>
<tr>
<td>High</td>
<td>12 (46)</td>
</tr>
<tr>
<td><strong>Dominant disease site</strong></td>
<td></td>
</tr>
<tr>
<td>Soft tissue</td>
<td>10 (31)</td>
</tr>
<tr>
<td>Viscera</td>
<td>15 (47)</td>
</tr>
<tr>
<td>Bone</td>
<td>7 (22)</td>
</tr>
<tr>
<td><strong>Type of first-line chemotherapy</strong></td>
<td></td>
</tr>
<tr>
<td>CMF</td>
<td>19 (59)</td>
</tr>
<tr>
<td>Anthracycline-based</td>
<td>13 (41)</td>
</tr>
<tr>
<td><strong>Adjuvant therapy</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>17 (53)</td>
</tr>
<tr>
<td>CMF</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Anthracycline-based</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Hormonal</td>
<td>8 (25)</td>
</tr>
</tbody>
</table>

a DFS, disease-free survival.
The overall response rate was 31% (95% CI, 19–43%); 14 of 32 bcl-2-negative patients (<40% stained cells) responded (44%; 4 CRs and 10 PRs; 95% CI, 35–53%), compared to only 3 of 23 bcl-2-positive patients (13%; 1 CR and 2 PRs; 95% CI, 0–26%; \( P = 0.019 \) by Fisher's exact test). The two groups (Table 1) were well balanced in terms of age, performance status, disease-free survival, menopausal status, and type of first-line chemotherapy. bcl-2-negative tumors showed a tendency toward a higher p53 expression and proliferation rate, whereas an excess of bone as dominant disease site was evident among the bcl-2-positive tumors. However, the only variable to result significantly different between the two groups was ER expression (\( P = 0.004 \)).

The overall survival of the two groups is shown in Fig. 1. Despite the higher response rate observed in the group with bcl-2-negative tumors, median survival is better in the group of bcl-2-positive patients (23 versus 13 months); the difference in survival approaches statistical significance as measured by Gehan's Wilcoxon test (\( P = 0.08 \)).

Besides bcl-2 expression, we investigated the distribution of responses among the following variables: presence of ER, dominant disease site, Ki-67 levels, type of administered chemotherapy (anthracycline-based versus CMF), and p53 expression (Fig. 2). Univariate analysis revealed a significant association between the response rate and dominant disease site and the type of first-line chemotherapy; a better response rate, although not statistically significant, was also observed among ER-negative tumors. Because some of the variables (p53, Ki-67, and ER) could not be determined in the population as a whole, the association of bcl-2 with the response to chemotherapy was determined in each subgroup, which confirmed a significant difference between bcl-2-negative and -positive tumors. The variables identified by univariate analysis were subsequently evaluated using a multivariate logistic regression model, which showed that bcl-2 maintained its power of discriminating two groups with a different probability of responding to chemotherapy, although the greatest contribution was given by dominant disease site and the type of chemotherapy (Table 2).

**DISCUSSION**

There are no biological markers to indicate the likelihood of a response to systemic chemotherapy in advanced breast carcinoma, and so the patients to be treated in this way are usually selected by means of a process of exclusion (28). The toxicity and costs of chemotherapy are not negligible, but responses are usually only observed in a small proportion of patients, very few of whom achieve a CR. There is, therefore, a great need to identify one or more markers that can predict response or resistance to chemotherapy.

Our study shows that bcl-2 expression is associated with a very low probability of chemotherapeutic response, whereas p53 status failed to discriminate patients with a different likelihood of response. The immunohistochemically determined absence of the predictive value of p53 expression may be due to a number of reasons. Although experimental data have demonstrated the importance of normal p53 function in determining sensitivity to radiation or specific chemotherapeutic agents in Burkitt's lymphoma and lymphoblastoid cell lines (29, 30) and ovarian (31) and breast (32) cancer cell lines, studies with colorectal (33) and head and neck carcinoma (34) cell lines have failed to demonstrate any difference in radiosensitivity between cells containing or lacking p53 functions. Furthermore, there are theoretical reasons for arguing that cells with a defective p53 may be incapable of recognizing damaged DNA and of slowing down to allow the repair, thereby becoming more sensitive to chemotherapy. This point of view is supported by recently published experimental data (35).

Finally, positive p53 staining does not necessarily mean that the protein is functionally inactive, because the concentra-
tion of the protein increases during the G1 and S phases of the cell cycle in response to DNA damage and by means of its binding to other cell proteins, such as mdm-2 (36). The relationship between p53 and the response to chemotherapy in the clinical setting has only been marginally investigated. An association between immunohistochemically assayed p53 levels and chemotherapeutic response has only been demonstrated in patients with non-small cell lung (37) or ovarian cancer (38) receiving cisplatin-based chemotherapy; it has not been found in patients with breast cancer treated with neoadjuvant chemoendocrine therapy (39, 40).

It has been shown that bcl-2 is frequently expressed in breast cancer and is associated with clinicopathological parameters that usually indicate a good prognosis in both node-negative and node-positive patients (41–45). However, its role in determining in vivo chemoresistance has only been rarely investigated. In a study of 119 patients with advanced breast cancer, no difference in response rates was observed between the patients with low or high bcl-2 expression (46); the same study showed that a significantly lower response rate was associated with a low expression of the bcl-2-related bax protein. A randomized trial (47) of the use of perioperative chemotherapy in patients with node-negative breast cancer concluded that the reduction in the disease-free survival hazard ratios in the chemotherapy arm was similar in the two groups of patients with bcl-2-negative or -positive tumors (0.55 and 0.61, respectively), suggesting that bcl-2 expression plays no role in response to chemotherapy. Finally, in a study of neoadjuvant chemoendocrine therapy in primary breast cancer (39), the response to the treatment was higher in bcl-2-positive tumors than in the negative counterparts (85% versus 61%, respectively; $P = 0.08$). This result is probably due to the somewhat confounding impact of the endocrine treatment because it has been reported that high bcl-2 expression predicts a better response to tamoxifen in advanced breast cancer (48). In conclusion, because the published papers differ in terms of the investigated population, the type and the duration of treatment, the use of hormones together with chemotherapy, and the cutoff used to separate bcl-2-negative from -positive tumors, it is difficult to compare these results with ours and to draw firm conclusions.

Besides bcl-2, the other variables identifying two groups of patients with significantly different response rates were dominant disease site and the type of chemotherapy. The differential probability of response according to the dominant disease site (with the highest probability being observed in soft tissue and the lowest in bone) is a very well-known clinical observation (49) that is confirmed by the results of our study. The better
response rate observed in the patients treated with an anthracycline-based regimen, in comparison with those treated using a nonanthracycline regimen, could be the result of a selection bias because the patients in better shape received the more aggressive treatment. However, the importance of the addition of doxorubicin is demonstrated in a number of randomized studies (49). Finally, the European Organization for Research and Treatment of Cancer Breast Cancer Cooperative Group retrospective evaluation of outcomes in 1054 metastatic breast cancer patients recruited to randomized chemotherapy clinical trials indicated administration of an anthracycline and performance status as the only variables associated with long-term survival (50).

In a previously published study of 76 patients with advanced breast cancer (26), the difference in the response rate between those whose tumors showed a high or low rate of proliferation (48% versus 21%) was statistically significant ($P = 0.03$). However, although we observed a difference in response rates relating to tumors proliferative activity (44% in the high proliferative versus 24% in the low proliferative group), this was not statistically significant, possibly because of our smaller sample size.

The predictive role of bcl-2 was confirmed in a multivariate logistic regression model that, however, showed that dominant disease site and the type of chemotherapy had a greater weight. Nevertheless, it must be pointed out that the limited sample size and the small number of events flaw the analysis.

The better response rate observed in bcl-2-negative tumors did not lead to a better survival curve, an observation that has implications for the interpretation of the results of some retrospective studies (43, 44) evaluating bcl-2 expression in patients treated with adjuvant chemo or hormonal therapy, in which the better survival related to bcl-2-positive tumors was thought to be the effect of the adjuvant treatment. The expression of bcl-2 does have a positive impact on survival (which is probably related to “less aggressive behavior”), but this has absolutely no relationship with sensitivity to chemotherapy.

In conclusion, the results of this study suggest that bcl-2 may play a role in predicting resistance to chemotherapy. A combination of the analysis of bcl-2 content with clinical observations (e.g., bone lesions) and perhaps other variables (e.g., P-glycoprotein expression) may make it possible, to avoid chemotherapy in a subgroup of patients with advanced breast cancer.

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