Clinical studies of Bcl-2 and treatment benefit in breast cancer patients

M G Daidone, A Luisi, S Veneroni, E Benini and R Silvestrini

Oncologia Sperimentale C, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milan, Italy

Abstract

Interest in translational studies aimed at investigating the role of biologic markers in predicting clinical outcome of breast cancer patients and, in particular, response to specific treatments, has progressively increased. Among biologic variables presently under investigation, apoptosis markers, in particular Bcl-2 and Bax expression, are receiving much attention for their relationship with the cellular response to genotoxic damage in experimental tumors. Retrospective, independent studies were carried out by several research groups on about 5000 patients with breast cancer at different stages and with an adequate follow-up. The outcome of separate analyses as a function of treatment generally demonstrated that Bcl-2 overexpression, which correlates with biologic features of a differentiated phenotype (slow proliferation, high steroid receptor levels, absence of p53 and c-erbB-2 expression), is associated with a favorable outcome. Such a finding is mainly evident following surgery as well as endocrine treatment. Conversely, no or weak Bcl-2 expression, alone or in association with bax overexpression, appears indicative of a radiation response, and preliminary emerging evidence supports the involvement of such an association of apoptosis-related markers even as predictors of long-term response to neoadjuvant cytotoxic treatment. Although the findings of an involvement of Bcl-2 and Bax as determinants of treatment response should be confirmed within the context of randomized clinical trials, they indicate a combined consideration of proteins that negatively and positively regulate apoptosis in translational studies on the effect of chemical and physical agents at a cellular level.

Introduction

A biologic event which has been described in experimental systems to be under the control of several genes is the activation of programmed cell death by apoptosis. The survival threshold for a cell is determined by the balance between cell-death suppressor and cell-death promoter signals provided by external factors or stimuli as well as by intracellular molecules. The Bcl-2 family of genes has a central role in the control of the program. Since the identification 15 years ago of the Bcl-2 gene (Tsujimoto et al. 1984), whose product acts as an anti-apoptotic molecule, a group of genes with sequence homology to Bcl-2 but with a different role in regulating apoptosis has been described (Strasset et al. 1997). In fact, the Bcl-2 gene family encodes proteins that inhibit apoptosis induced by damage or trophic factor withdrawal (Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1) but also proteins that can promote or accelerate apoptosis (Bax, Bcl-xS, Bad, Bak, Bik/Nbk, Bid, Bag-1). In homeostatic conditions, the relative balance of expression between pro- and anti-apoptotic molecules (which is under the control of several factors including p53) and of their dimerization status determines cell survival or death. In normal and pathologic situations, all these proteins are differently expressed in human tissues of different embryologic derivation and in different stages of histologic differentiation, and they may prevent or trigger apoptosis via cell-type specific signaling pathways.

The recent availability of reagents able to detect the expression of Bcl-2 and related genes has substantially contributed to the understanding of some of the genetically controlled mechanisms that regulate active cell death and to the investigation of the role of pro- and anti-apoptotic proteins in determining the cellular response to cytotoxic drugs, hormonal agents and radiation. A number of studies have been carried out in normal epithelial cells and in carcinomas, and breast tissue has provided an interesting subject of investigation. The mammary gland
is subjected to growth or involution in response to hormonal stimuli. Bcl-2 expression has been shown to inversely parallel cell turnover or the modeling of tissues by apoptosis (Pezzella & Gatter 1995), whereas Bax may have a critical, although not necessarily exclusive, role in promoting programmed cell death (Streuli et al. 1997).

In breast carcinomas, the expression of Bcl-2 has been generally associated with the presence of markers of differentiation, such as high levels of steroid receptors, low proliferative rate and weak or absent p53 accumulation. The complex pattern of interrelations among variables related to hormonal dependence, proliferative potential and apoptosis was investigated on a substantial series of primary tumors (Silvestrini et al. 1994), and the multiple correspondence analysis with multivariate techniques allowed us to classify clinical breast cancers into three subsets characterized by different biologic profiles (Table 1). To obtain a graphical representation of the structure, experimental points were projected in a space defined by three factorial axes based on biologic differences (Fig. 1). The first axis (F1) separates putatively favorable from unfavorable cell conditions; the second (F2), different modulations of Bcl-2 and p53 expression; and the third (F3), different modulation of tumor cell proliferation and p53 expression.

Tumors with low p53 expression frequently showed high levels of Bcl-2 and were part of subset 1, whereas tumors with high p53 expression frequently showed low levels of Bcl-2 and also the absence of estrogen receptors (ER) and were in subset 2. The contribution of cell proliferation, although marginal, was more evident for subset 1, in which a low proliferative rate was associated with low p53 or high Bcl-2 expression. A lower association among biologic variables was observed for tumors with an intermediate proliferative rate and Bcl-2 and p53

**Table 1** Association among Bcl-2 expression, p53 accumulation and proliferative rate

<table>
<thead>
<tr>
<th>p53 expression</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low TLI</td>
<td>1.5</td>
<td>2.5*</td>
<td>12.0*</td>
</tr>
<tr>
<td>Intermediate TLI</td>
<td>2.5</td>
<td>9.3</td>
<td>3.5</td>
</tr>
<tr>
<td>High TLI</td>
<td>2.5</td>
<td>4.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Corresponds to ER+ tumors,* **corresponds to ER- tumors.

TLI, thymidine labelling index.

**Figure 1** Projection of the first three factorial axes displaying clouds and interrelations among biologic variables. Open circles: p53, Bcl-2 and [3H]thymidine labeling index (TLI) values corresponding to the first tertile of frequency distribution (low values). Open squares: p53, Bcl-2 and TLI values corresponding to the second tertile of frequency distribution (intermediate values). Filled circles: p53, Bcl-2 and TLI values corresponding to the third tertile of frequency distribution (high values). Distances from the plane (F1, F2) are represented by vertical segments; lines above the plane are continuous, lines below the plane are broken.
expression, which were represented mainly in subset 3. Subsets 1 and 3 did not appear to be characterized by a different frequency of ER-positive tumors.

**Bcl-2 expression and translational studies**

In the past 5 years, a number of studies have analyzed the relationship between Bcl-2 expression and patient outcome in clinical breast cancer. In most of the studies, Bcl-2 overexpression was associated with a favorable prognosis in patients with node-negative and/or node-positive tumors subjected to local-regional, hormonal or cytotoxic therapies. However, the association with treatment response can be derived with extreme caution from published results, since it was only occasionally investigated within the context of randomized controlled trials.

Taking into consideration such criticisms and the complex pattern of interrelations among Bcl-2 and biologic variables that play a role in breast cancer prognosis and that might be associated with treatment response, we investigated the relevance of Bcl-2 in substantial series of breast cancers at different stages, with an adequate follow-up that allowed us to analyze total or specific relapse-free survival, overall survival and objective clinical response according to the clinical situation. Patients had been submitted to several treatments - local-regional, hormonal or cytotoxic - and the translational studies were in most of the cases retrospective - pilot and/or confirmatory. However, Bcl-2 expression was also investigated within the context of randomized controlled trials.

**Table 3 Bcl-2 expression and clinical outcome in breast cancer patients treated with endocrine therapy**

<table>
<thead>
<tr>
<th>Authors</th>
<th>Stage</th>
<th>No. of cases</th>
<th>Follow-up (years)</th>
<th>Relapse-free survival/ freedom from progression</th>
<th>Overall survival</th>
<th>Objective clinical response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hurlimann et al.</td>
<td>I-IV</td>
<td>75</td>
<td>5</td>
<td>No —</td>
<td>No —</td>
<td>—</td>
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<td>Silvestrini et al.</td>
<td>II-III</td>
<td>240</td>
<td>5</td>
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<tr>
<td>Veronesi et al.</td>
<td>II-III</td>
<td>66</td>
<td>5</td>
<td>Yes —</td>
<td>Yes —</td>
<td>—</td>
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<tr>
<td>Kobayashi et al.</td>
<td>I-III</td>
<td>142</td>
<td>6</td>
<td>Yes —</td>
<td>—</td>
<td>—</td>
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<td>Gasparini et al.</td>
<td>II-III</td>
<td>81</td>
<td>6</td>
<td>Yes (Yes)</td>
<td>(Yes) No</td>
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<tr>
<td>Helleman et al.</td>
<td>II-III</td>
<td>107</td>
<td>9</td>
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<tr>
<td>Elledge et al.</td>
<td>IV</td>
<td>205</td>
<td>5</td>
<td>Yes Yes</td>
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<td>Keen et al.</td>
<td>I-III</td>
<td>51</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Gee et al.</td>
<td>III-IV</td>
<td>46</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
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</tbody>
</table>

In parenthesis, outcome better but not significantly different for patients with Bcl-2+ compared with patients with Bcl-2- tumors.

1p53+ tumors; 2premenopausal patients; 3G2 tumors.
**Table 4** Bcl-2 expression and clinical outcome in breast cancer patients treated with chemotherapy

<table>
<thead>
<tr>
<th>Authors</th>
<th>Stage</th>
<th>No. of cases</th>
<th>Treatment</th>
<th>Follow-up (years)</th>
<th>Univariate</th>
<th>Multivariate</th>
<th>Univariate</th>
<th>Multivariate</th>
<th>Objective clinical response</th>
</tr>
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<tr>
<td>Van Slooten <em>et al.</em> (1996)</td>
<td>I¹</td>
<td>441</td>
<td>FAC vs CTR⁴</td>
<td>4</td>
<td>Yes⁷</td>
<td>No</td>
<td>—</td>
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<tr>
<td>Veronese <em>et al.</em> (1998)</td>
<td>II-IIIₐ²</td>
<td>80</td>
<td>CMF</td>
<td>5</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Gasparini <em>et al.</em> (1995)</td>
<td>II-IIIₐ³</td>
<td>99</td>
<td>CMF</td>
<td>6</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Krajewski <em>et al.</em> (1997)</td>
<td>II-IIIₐ</td>
<td>82</td>
<td>Not specified</td>
<td>7</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>Lipponen <em>et al.</em> (1995)</td>
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<td>61</td>
<td>Not specified</td>
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<td>Yes</td>
<td>—</td>
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<tr>
<td>Krajewski <em>et al.</em> (1995)</td>
<td>IV</td>
<td>119</td>
<td>FEC</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
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<td>Frassoldi <em>et al.</em> (1997)</td>
<td>II-III₈</td>
<td>29</td>
<td>A⁵, CMF⁶</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Ellis <em>et al.</em> (1998)</td>
<td>II</td>
<td>40</td>
<td>FEC⁶</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Collecchi <em>et al.</em> (1998)</td>
<td>III₇</td>
<td>70</td>
<td>FEC⁶</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>No</td>
</tr>
</tbody>
</table>

¹Premenopausal patients; ²ER⁺ tumors; ³p53⁺ tumors; ⁴randomized, controlled (CTR) trial; ⁵A= doxorubicin alone; ⁶neoadjuvant treatment; ⁷the advantage was also observed for patients with Bcl-2⁺ tumors.
determined within the context of randomized trials of local-regional treatment or in pilot studies of neoadjuvant chemotherapy. In view of the availability of numerous archival specimens, we evaluated the expression of Bcl-2, and in limited subsets of cases even of Bax, by immunocytochemistry and by scoring and determining the fraction of positive cells at a cytoplasmic level after assessing the presence of a satisfactory concordance with Western blotting results on the same set of clinical tumors.

Relationship between Bcl-2 expression and prognosis

In all but two of the published studies on a total of over 1200 patients (Table 2), mostly on patients with stage I tumors subjected only to local-regional treatment until relapse (Joensuu et al. 1994, Silvestrini et al. 1994, Hellemans et al. 1995, Lipponen et al. 1995, Barbareschi et al. 1996, Van Slooten et al. 1996, Kapranos et al. 1997, Krajewski et al. 1997, Charpin et al. 1998, Veronese et al. 1998), a general advantage in terms of relapse-free and overall survival was reported for patients with Bcl-2-overexpressing tumors. However, such an advantage was generally lost in multivariate analyses including information provided by p53. Similar results were obtained even in a confirmatory study on more than 1000 patients with node-negative tumors in which, in addition to Bcl-2 expression, the prognostic contribution of cell proliferation, steroid receptors and p53 and Bax expression were also investigated. In the new series of patients with node-negative tumors in which, in addition to Bcl-2 expression, the prognostic contribution of cell proliferation, steroid receptors and p53 and Bax expression were also investigated. In the new series of

Relationship between Bcl-2 expression and response to endocrine therapy

Hormonal treatment can induce apoptosis in breast cancer cell lines and can modulate Bcl-2 expression in clinical tumors following neoadjuvant therapy, although with opposite patterns according to the two published studies (Johnston et al. 1994, Keen et al. 1997). Since the therapeutic effect of hormones may be mediated by activation of the apoptotic program, its suppression or delay by Bcl-2 might result in treatment failure. However, as regards such a hypothesis, in almost all the published reports dealing with a total of over 1000 patients (Table 3), the expression of Bcl-2 surprisingly appears as an indicator of a favorable outcome following endocrine treatment in patients with limited disease (Gasparini et al. 1995, Hurlimann et al. 1995, Silvestrini et al. 1995, Hellemans et al. 1995, Kobayashi et al. 1997, Veronese et al. 1998) and as a predictor of treatment response in patients with advanced disease (Gee et al. 1994, Elledge et al. 1997, Keen et al. 1997). In addition, within ER-positive tumors, the integration of clinically relevant information provided by biologic variables that proved to be independent in multivariate analysis (cell proliferation, progesterone receptors (PgR), p53 and Bcl-2 expression) allowed us to separate patients with ER-positive tumors into different risk groups. Bcl-2 overexpression contributed, along with a low proliferation, high PgR levels and weak or absent p53 expression, to the identification of the most favorable subsets (Silvestrini et al. 1996). Such findings provide further insight into the hypothesis that Bcl-2 expression should be viewed as a differentiation marker or a surrogate marker for other molecular or biologic processes related to hormone sensitivity rather than a predictor of response to hormonal treatment.

Relationship between Bcl-2 expression and response to radiotherapy

In breast cancer, the benefit of postoperative radiotherapy in improving local control following breast-conserving surgery is well documented. Few studies have investigated the role of biologic markers on radiation response (Jansson et al. 1995, Silvestrini et al. 1997). However, an accurate identification of biologic features, correlated with the reduction of local recurrence following radiotherapy, could avoid overtreatment in patients not destined to relapse regardless of radiation treatment or, conversely, to relapse despite irradiation.

In about 600 patients with node-negative tumors smaller than 2 cm and entering clinical protocols of conservative surgery (quadrantectomy) with or without radiotherapy at the Istituto Nazionale Tumori of Milan, we analyzed the role of Bcl-2 and Bax expression in predicting local relapse. The risk of local recurrence was comparatively evaluated in an exploratory analysis as a function of the administration of radiotherapy within subsets of Bcl-2-negative or -positive and Bax-positive or...
-negative tumors. The benefit of radiotherapy (with a 6-fold reduction of recurrence) was limited to patients with Bcl-2-negative (hazard ratio (HR) for local recurrence, 5.5 (95% CI, 2.2-13.4) for surgery alone versus surgery plus radiotherapy) or Bax-positive tumors (HR for local recurrence rate, 6.5 (95% CI, 2.5-17.1) for surgery alone versus surgery plus radiotherapy), i.e. with tumors showing features favoring apoptosis and thus exhibiting a possible trend of susceptibility to radiation. Conversely, the recurrence rate for patients with Bcl-2-positive or Bax-negative tumors was similar for the two local-regional approaches, regardless of administration of radiotherapy.

When considered singly, Bcl-2 and Bax identified as putatively responsive to radiotherapy about 50% of the cases. In combination, identification with the two markers increased to 70%, and the combined consideration of the two apoptosis-related markers showed that the only category apparently not benefiting from radiotherapy was the subset of patients whose tumors did not exhibit a putative susceptibility to apoptosis, i.e. those Bcl-2-positive and Bax-negative. Such findings, in agreement with preclinical (Sakakura et al. 1996) and clinical evidence on some (Harima et al. 1996) but not on other tumor types (Wilson et al. 1996), provide support for the role of factors that regulate the apoptotic pathway in determining cell response to radiation. They also highlight the importance of considering multiple, and possibly opposite, regulators of apoptosis within the context of translational studies on predictors of treatment response.

### Relationship between Bcl-2 expression and response to chemotherapy

Evidence from experimental systems and clinical tumors, including leukemia, neuroblastoma and small cell lung cancer, supports an involvement of Bcl-2 expression in determining resistance to anti-cancer agents. However, in retrospective studies on clinical breast cancer subjected to different treatment regimens (cyclophosphamide and 5-fluorouracil, plus methotrexate (CMF) or epirubicin (FEC), or doxorubicin alone) (Table 4), Bcl-2 expression did not appear to provide information on objective clinical response in terms of tumor reduction, which was achieved in over 80% of the cases. As regards relapse-free survival, in an exploratory analysis separately carried out on subsets of tumors homogeneous for biopathologic stage and treatment (high-risk, node-negative plus one to three node-positive tumors completing the treatment with postsurgical adjuvant chemotherapy), a weak or absent Bcl-2 expression or expression of the proapoptotic gene, Bax, was associated with a favorable outcome following chemotherapy. In a combined consideration of such biologic profiles, patients with tumors Bcl-2-positive and Bax-negative, i.e. in a condition of preventing or delaying apoptosis, had a probability of relapse-free survival about three times lower than those whose tumors exhibited the opposite biologic profile, i.e. absence of Bcl-2 and presence of Bax, which together favor the apoptotic process. An intermediate relapse-free survival was observed for patients...
whose tumors expressed only one of the apoptosis-promoting factors.

Conclusions

In our experience with about 2500 patients, Bcl-2 overexpression, which correlates with biologic features of a differentiated phenotype (low proliferative rate, high steroid receptor levels, absence of p53 and c-erbB-2 expression), appears to be associated with a favorable clinical outcome following radical surgery (although with a weak prognostic capability), as well as following endocrine treatment. Such findings are in agreement with the experience of other research groups and have been validated in confirmatory studies. They are consistent with the hypothesis of a complex interaction between Bcl-2, tumor development and biologic or clinical progression. The association between Bcl-2 expression and clinical outcome following endocrine treatment could be due to an identification of indolent, well-differentiated tumors rather than to a direct involvement of the anti-apoptotic marker in determining sensitivity to hormonal treatment. The finding that Bax expression does not improve clinical predictivity of Bcl-2 alone following tamoxifen treatment (unpublished results) is in keeping with such a hypothesis. Conversely, Bcl-2 appears indicative of radiation response, and such a finding, which is supported and reinforced even by the relevance of Bax expression in predicting local control following radiotherapy, is consistent with the hypothesis of an involvement of pro- and anti-apoptotic proteins in determining the fate of tumor cells exposed to radiation.

The role of Bcl-2 expression on clinical outcome following chemotherapy is still under investigation and validation, since available data are in some instances contrasting, and the interpretation of treatment benefit as a function of biomarkers is difficult in the absence of randomized, controlled trials. In our experience, derived from the analysis of Bcl-2 and Bax expression in the context of a neoadjuvant chemotherapy trial (which represents an ideal model to evaluate the clinical value of biomarkers (Dowsett 1998)), we observed a long-term treatment benefit for patients whose tumors showed features favouring apoptosis (no or weak Bcl-2 expression and Bax overexpression) which, in experimental tumors, appear as determinants of cellular response to genotoxic damage. Although the findings, as well as those obtained following radiation, should be confirmed through independent and, possibly, randomized clinical trials, they are in favor of the combined consideration of proteins that regulate (by promoting or contrasting) apoptosis in translational studies on the effect of cytotoxic drugs, hormonal agents and radiation at a cellular level.

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