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

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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 differentially 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 and absence of estrogen receptors (ER) 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>Cases expressing Bcl-2 (%)</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
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<tbody>
<tr>
<td>Low TLI</td>
<td>1.5</td>
<td>2.5*</td>
<td>12.0*</td>
<td></td>
</tr>
<tr>
<td>Intermediate TLI</td>
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<td>3.0</td>
<td>5.0</td>
<td></td>
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<tr>
<td>High TLI</td>
<td>2.5</td>
<td>4.0</td>
<td>3.5</td>
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<tr>
<td>Intermediate TLI</td>
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<td>3.4</td>
<td>3.4</td>
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</tr>
<tr>
<td>High TLI</td>
<td>4.5**</td>
<td>4.0</td>
<td>0.5</td>
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<tr>
<td>High TLI</td>
<td>12.9**</td>
<td>1.5</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

* tend to be ER+ tumors, ** tend to be ER- tumors.

TLI, thymidine labelling index.
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

<table>
<thead>
<tr>
<th>Authors</th>
<th>Stage</th>
<th>No. of cases</th>
<th>Follow-up (years)</th>
<th>Univariate</th>
<th>Multivariate</th>
<th>Univariate</th>
<th>Multivariate</th>
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<td>Van Slooten et al. (1996)</td>
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<td>Charpin et al. (1998)</td>
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<td>82</td>
<td>10</td>
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<td>Lipponen et al. (1995)</td>
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<td>140</td>
<td>10</td>
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<td>No</td>
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<td>—</td>
<td>—</td>
<td>Yes</td>
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</tbody>
</table>

In parentheses, 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>
<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>
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<td>(Yes)</td>
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<td>III-IV</td>
<td>46</td>
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</table>

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

1ER+ tumors; 2Elderly patients; 3Neoadjuvant treatment.
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>
</thead>
<tbody>
<tr>
<td>Van Slooten et al. (1996)</td>
<td>I</td>
<td>441</td>
<td>FAC vs CTR(^4)</td>
<td>4</td>
<td>Yes(^7)</td>
<td>No</td>
<td>—</td>
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<td>Veronese et al. (1998)</td>
<td>II-III(_A) (^2)</td>
<td>80</td>
<td>CMF</td>
<td>5</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
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<tr>
<td>Gasparini et al. (1995)</td>
<td>II-III(_A) (^3)</td>
<td>99</td>
<td>CMF</td>
<td>6</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
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<td>II-III(_A)</td>
<td>82</td>
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<td>7</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>II</td>
<td>61</td>
<td>Not specified</td>
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<td>No</td>
<td>Yes</td>
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</tr>
<tr>
<td>Krajewski et al. (1995)</td>
<td>IV</td>
<td>119</td>
<td>FEC</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>No</td>
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<tr>
<td>Frassoldati et al. (1997)</td>
<td>II-III(_B)</td>
<td>29</td>
<td>A(^5), CMF(^6)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>No</td>
</tr>
<tr>
<td>Ellis et al. (1998)</td>
<td>II-III</td>
<td>40</td>
<td>FEC(^6)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Collecchi et al. (1998)</td>
<td>II(_B)</td>
<td>70</td>
<td>FEC(^6)</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^1\) Premenopausal patients; \(^2\) ER\(^+\) tumors; \(^3\) p53\(^+\) tumors; \(^4\) randomized, controlled (CTR) trial; \(^5\) A = doxorubicin alone; \(^6\) neoadjuvant treatment; \(^7\) the 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 tumor size, morphologic features, grade, proliferative rate, p53 expression and steroid receptors (Silvestrini et al. 1994, Hellemans et al. 1995, Lipponen et al. 1995, Charpin et al. 1998) or at a longer follow-up (Joensuu et al. 1994).

In our pilot study on 283 node-negative breast cancer patients (Silvestrini et al. 1994), those with Bcl-2-overexpressing tumors had probabilities of relapse and death about twice and three times lower than patients with tumors weakly or not expressing Bcl-2, respectively. However, Bcl-2 failed to maintain its prognostic role for 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 tumor size, morphologic features, grade, proliferative rate, p53 expression and steroid receptors (Silvestrini et al. 1994, Hellemans et al. 1995, Lipponen et al. 1995, Charpin et al. 1998) or at a longer follow-up (Joensuu et al. 1994).

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**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. 1996, 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
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 (Krajewski et al. 1995, Frassoldati et al. 1997, Collecchi et al. 1998, Ellis et al. 1998), whereas a favorable outcome in terms of relapse-free or overall survival was generally observed for patients with Bcl-2-positive tumors (Gasparini et al. 1995, Krajewski et al. 1995, 1997, Lipponen et al. 1995, Veronese et al. 1998). In the only randomized trial reported in the literature in which Bcl-2 expression was determined (Van Slooten et al. 1996), comparing perioperative 5-fluorouracil, doxorubicin and cyclophosphamide (FAC) with surgery alone, a treatment benefit was observed for Bcl-2-positive but also for Bcl-2-negative tumors. Such data raise the question of whether clinical outcome following chemotherapy is related to the expression of Bcl-2, i.e. to a likely differentiated behavior of the tumor in which a growth-stalling effect of Bcl-2 could delay regrowth of the surviving clones, as already reported for follicular center lymphoma cells (Strasser et al. 1997), or to the lack of Bcl-2 expression and thus to a possible susceptibility to apoptosis.

In our experience at the Istituto Nazionale Tumori of Milan, we initially analyzed the role of Bcl-2 and Bax in the context of the clinical protocols of neoadjuvant chemotherapy active since 1988, and in particular in the first trial in which the primary treatment administered to patients with large but operable tumors consisted of three or four cycles of CMF or of anthracycline- or mitoxantrone-containing combinations. Biologic markers, and thus also Bcl-2 and Bax, were determined before starting treatment, at the time of diagnosis on incisional biopsy, and the determinations were repeated 3 months later, at the end of primary chemotherapy, on surgical specimens (Daidone et al. 1995). As regards changes induced in Bcl-2 and Bax profiles by primary treatment, Bcl-2 expression seemed to be marginally modulated, since no change between pre-and post-treatment values was observed in about 70% of the cases, whereas Bax expression remained almost unchanged in non-expressing or weakly expressing tumors, but decreased in over-expressing tumors. Such findings, which at least for Bcl-2 are in contrast with other published results (Collecchi et al. 1998, Ellis et al. 1998), could be obviously affected, among other factors, by the time schedule used in the protocol for biomarker determination.

The predictivity on clinical outcome, in terms of objective clinical response and relapse-free survival, was analyzed as a function of pre- and post-treatment values of Bcl-2 and Bax and of their changes. Neither Bcl-2 nor Bax expression, nor their changes, appeared to be predictors 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.

**Acknowledgements**

We thank Ms B Canova and Ms B Johnston for editorial assistance, and Dr P Boracchi for statistical support, stimulating discussion and input for multiple correspondence analysis. Supported in part by grants from the Italian Health Ministry and from Consiglio Nazionale delle Ricerche.

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