The Predictive Value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for Chemotherapy Response in Advanced Breast Cancer

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ABSTRACT

Purpose: The purpose was to evaluate the utility of some bcl-2 family proteins fas and fasL as predictive indicators for chemotherapy response in advanced breast cancer.

Experimental Design: Between October 1994 and October 1997, 283 patients with advanced breast cancer were included in a multicenter randomized study comparing doceatxel (D) to sequential methotrexate and 5-fluorouracil (MF) after anthracycline failure. The response rates (complete response + partial response) were 42 and 21% in the D and MF arms, respectively (P < 0.001). In 126 patients, histological blocks of primary tumors were available for immunohistochemical analysis of bax, bcl-2, bcl-xL, bag-1, fas and fasL.

Results: Of the investigated factors, bag-1 correlated positively with bax, bcl-2, and fasL, and fasL correlated positively with fas and bax. None of these apoptosis-related factors was associated with a response to chemotherapy either in the whole patient population or in the D or MF arms. Interestingly, low bcl-2 expression was associated with shorter time to progression (P = 0.02) and shorter overall survival (OS; P = 0.001). High fasL expression showed a trend toward shorter OS. In multivariate backwards stepwise Cox analysis, in which histological grade and estrogen receptor status (ER) were also included, bcl-2 (P = 0.01) and fasL (P = 0.005) remained highly significantly associated with OS, whereas histological grade and ER lost their significance.

Conclusions: None of the investigated apoptosis-related factors of primary tumor could predict the later response to either D or MF treatment. However, fasL and bcl-2 were strong prognostic factors. Patients who had tumors with high fasL and low bcl-2 expression had the shortest OS.

INTRODUCTION

During the last decade, it has become more obvious that regardless of distinct mechanisms of action, most anticancer agents ultimately kill cancer cells primarily by inducing apoptosis. Drug-induced cell death is understood as a checkpoint response to DNA or other damage. Thus, the efficacy of anticancer treatments does not only depend on the DNA damage or other damage they cause but also depends on the cellular ability to detect and respond to such damage. In preclinical models, mutations in mediators of apoptosis can produce treatment-resistant tumors (1, 2). In the clinic, it is unclear whether monitoring expression of apoptosis-related proteins provides additional information on how patients will respond to treatment.

Mitochondrial and cell-surface death receptor-mediated apoptosis are the two principal pathways leading to programmed cell death (3). The mitochondrial pathway is thought to play a major role in response to cancer treatments and is mediated by the bcl-2 family proteins. More than 20 members of this family have been described thus far in humans. A positive ratio between pro- and antiapoptotic bcl-2 family members leads to cytochrome c release from mitochondria, which triggers the final execution of cell death by the caspase cascade. bcl-2 and bcl-xL are antiapoptotic bcl-2 family proteins, whereas bax is a proapoptotic bcl-2 family protein. bag-1 is a multifunctional protein that blocks apoptosis and interacts with several types of proteins including the bcl-2 family (4).

The fas receptor belongs to the family of tumor necrosis factor-related death receptors, and fasL is its corresponding ligand. Binding of a death ligand to its corresponding death receptor activates the other major apoptotic pathway. Down-regulation of fas has been shown in some carcinomas including breast cancer (5), whereas fasL is sometimes overexpressed in many human tumors including breast cancer (6, 7). Some preclinical studies suggest that classic anticancer agents also require the death receptor fas and its corresponding ligand (fasL) to induce cell death (8, 9).

Currently, no tumor biological factor is available for clinical use for predicting chemotherapy response in breast cancer, in contrast to the ER status, which predicts the response to hormonal treatment. In addition to metastatic breast cancer, such factors could also be used in the choice of neoadjuvant or adjuvant treatment. The purpose of the current study was to...
Table 1 Characteristics of the primary tumors at the time of diagnosis and treatment-related characteristics of the 126 investigated patients

<table>
<thead>
<tr>
<th>Factor and subgroups</th>
<th>No. of patients (%)</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>116 (92%)</td>
<td>5 (0–95)</td>
</tr>
<tr>
<td>Lobular</td>
<td>9 (7%)</td>
<td>5 (0–100)</td>
</tr>
<tr>
<td>Medullary</td>
<td>1 (1%)</td>
<td>50 (0–100)</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>59 (47%)</td>
<td>60 (0–100)</td>
</tr>
<tr>
<td>Negative</td>
<td>55 (44%)</td>
<td>5 (0–100)</td>
</tr>
<tr>
<td>Unknown</td>
<td>12 (10%)</td>
<td>20 (0–100)</td>
</tr>
<tr>
<td>bax (n = 115)</td>
<td></td>
<td>1.6 years (0–23)</td>
</tr>
<tr>
<td>bcl-2</td>
<td>5 (0–95)</td>
<td></td>
</tr>
<tr>
<td>bcl-xL</td>
<td>50 (0–100)</td>
<td></td>
</tr>
<tr>
<td>bag-1</td>
<td>60 (0–100)</td>
<td></td>
</tr>
<tr>
<td>fas</td>
<td>5 (0–100)</td>
<td></td>
</tr>
<tr>
<td>fasL</td>
<td>20 (0–100)</td>
<td></td>
</tr>
<tr>
<td>DFI*</td>
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</table>

Table 2 The significance of the correlation (Spearman) between the investigated tumor biological factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>bcl-2</th>
<th>bcl-xL</th>
<th>bag-1</th>
<th>fasR</th>
<th>fasL</th>
</tr>
</thead>
<tbody>
<tr>
<td>bax</td>
<td>0.78</td>
<td>0.46</td>
<td>0.035</td>
<td>0.057</td>
<td>0.007</td>
</tr>
<tr>
<td>bcl-2</td>
<td>0.33</td>
<td>0.001</td>
<td>0.12</td>
<td>0.47</td>
<td>0.39</td>
</tr>
<tr>
<td>bcl-xL</td>
<td>0.40</td>
<td>0.32</td>
<td>0.47</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>bag-1</td>
<td>0.91</td>
<td>0.03</td>
<td>0.03</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>fasR</td>
<td>0.91</td>
<td>0.03</td>
<td>0.03</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>fasL</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
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</tbody>
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and complete responses were confirmed within 4–6 weeks after first identification of objective response. Response evaluation was performed according to WHO recommendations (11). The main patient and tumor characteristics of the 126 analyzed patients are seen in Table 1.

Immunohistochemical Assays. All of the tissues had been fixed in 4% buffered formalin, processed, and embedded in paraffin according to the normal schedule used in the laboratory. From each block, 5-µm-thick sections were cut on coated slides and dried overnight at 37°C. The sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. Sections to be stained with antibodies against bcl-2, bax, bcl-xL, and bag-1 were pretreated by boiling them for 20 min in citrate buffer (pH 6.0); those to be stained with anti-fas and anti-fasL were pretreated by digestion in 0.5% trypsin (pH 7.2) at 37°C for 30 min. Immunohistochemical stainings were performed by using commercial Elite ABC kits (Vectastain; Vector Laboratories, Burlingame, CA). Blocking serum was applied for 15 min followed by overnight incubation with the diluted primary antibody: bcl-2, 1:200 (clone 124; DAKO); bax, 1:100 (clone 2D2; Zymed); bcl-xL, 1:50 (clone 2H12; Zymed); bag-1, 1:200 [monoclonal mouse (12)]; fas, 1:250 (C20 rabbit polyclonal antibody raised against amino acids 316–335 mapping at the COOH-terminus of the human FAS precursor; Santa Cruz Biotechnology); and fasL, 1:100 (Q20 rabbit polyclonal antibody raised against a peptide corresponding to amino acids 2–21 mapping at the NH2-terminus of fasL of human origin; Santa Cruz Biotechnology). The sections were then incubated with the biotinylated secondary antibody and the peroxidase-labeled ABC solution (Vectastain) for 30 min each. All of the dilutions were made in PBS (pH 7.2), and all of the incubations were performed in humid chambers at room temperature. Between each step in the staining procedures (except before incubation with the primary antibody), the slides were rinsed three times in PBS. Bound peroxidase was visualized in all of the slides with a 3-amino-9-ethyl-carbazole solution [0.2 mg/ml in 0.05 M acetate buffer containing 0.03% perhydroxyl (pH 5.0); AEC; Sigma Chemical Co.] at room temperature for 15 min. Finally, the sections were lightly counterstained in Mayer’s hematoxylin and mounted in Aquamount Mountant (BDH Ltd, Poole, United Kingdom). For each antibody, a known positive case of breast cancer was included in every staining batch as a positive control.

Cells were considered positive only when a distinct cellular micropunctate pattern of staining was seen, except for bag-1, for which nuclear staining was also accepted. The percentage of immunoreactive cells was evaluated as the amount of positive

evaluate the utility of some bcl-2 family proteins fas and fasL as predictive indicators for chemotherapy response in advanced breast cancer.

MATERIALS AND METHODS

Patients and Therapy. Paraffin-embedded blocks of the primary tumor were available for 126 of 283 patients who took part in a randomized multicenter trial comparing docetaxel to MF3 in advanced breast cancer (10). To enter the trial, patients were required to have histologically proven primary breast cancer that had progressed during or after first-line anthracycline treatment for advanced disease or relapsed within 12 months after discontinuation of adjuvant anthracycline therapy. The patients were required to be between 18 and 70 years old with a performance score ≤2 and have no more than one previous chemotherapy regimen for advanced disease (multiple endocrine treatments and radiotherapy were allowed). Only patients with measurable metastatic lesions or nonmeasurable but evaluable metastatic lesions were eligible. Response evaluation was done after every third course, at treatment discontinuation, and every 3 months during follow-up. Partial response

The abbreviations used are: MF, sequential methotrexate and 5-fluorouracil (treatment); TTP, time to progression; OS, overall survival; ER, estrogen receptor; NK, natural killer.
For the statistical analysis of response prediction, we divided were calculated for the investigated tumor biological factors. Median values were used stained sections were scored by two investigators (J. S., E. S.), tumor cells per all of the tumor cells on the section. All of the graded previously in a randomized two-arm multicenter study in which docetaxel was compared with MF in advanced breast cancer after anthracycline failure (10). The results of that study indicated that the response rate was higher (46 versus 22%; \( P < 0.001 \)) and TTP was longer (6.3 versus 3.0 months; \( P < 0.001 \)) in patients who received docetaxel. From

tumor cells per all of the tumor cells on the section. All of the stained sections were scored by two investigators (J. S., E. S.), who were blinded to the clinical data. Median values were used as the cutpoints for low and high expression.

**Statistical Methods.** Spearman correlation coefficients were calculated for the investigated tumor biological factors. For the statistical analysis of response prediction, we divided clinical response into two categories: response [complete response (CR) + partial response (PR)] and nonresponse [stable disease (SD) and progression (PD)]. Associations between tumor biological factors and response to treatment were tested with logistic regression model with the tested tumor biological factors regarded as continuous variables. The univariate and multivariate analysis of TTP and OS were done by the Cox regression model. All of the analyses were performed in the whole immunohistochemical study population (\( n = 126 \)) as well as in the docetaxel and MF arms separately. In the analyses of response and TTP in the whole patient population, in addition to the investigated tumor biological factors, given treatment was also included, because, in the whole randomized trial population (\( n = 283 \)), the treatment arm had significant impact on treatment outcome. Three patients in the docetaxel group had a nonevaluable response and were excluded from the analyses of response.

### RESULTS

The patient materials used were derived from archival tumor specimens available from patients who had been enrolled previously in a randomized two-arm multicenter study in which docetaxel was compared with MF in advanced breast cancer after anthracycline failure (10). The results of that study indicated that the response rate was higher (46 versus 22%; \( P < 0.001 \)) and TTP was longer (6.3 versus 3.0 months; \( P < 0.001 \)) in patients who received docetaxel. From
this original study with 283 patients, 126 specimens of primary tumors were available for immunostaining with bcl-2, bax, bcl-xL, bag-1, fas, and fasL. Of these 126 cases, 67 were in the docetaxel arm and 59 in the MF arm (Table 1). The response rate to docetaxel and MF in this subgroup of 126 patients was 51 and 24%, respectively.

Of the 126 investigated tumors, the median value for bax staining was 5%; for bcl-2, 5%; for bcl-xL, 50%; for bag-1, 60%; for fas, 5%; and for fasL, 20% (Table 1). The correlation between the investigated tumor factors is shown in Table 2. Of the investigated factors, bag-1 correlated positively with bax, bcl-2, and fasL, and fasL correlated positively with fas and bax. bcl-2 expression correlated positively with ER. None of the other apoptosis-related tumor biological factors were associated with ER (data not shown).

None of these apoptosis-related factors was associated with response to chemotherapy either in the whole patient population or in the docetaxel or MF arms (Tables 3–5). Interestingly, low bcl-2 expression was associated with shorter TTP ($P = 0.02$) and shorter OS ($P = 0.001$; Table 6). High fasL expression showed a trend toward shorter OS. In multivariate backwards stepwise Cox analysis, in which histological grade and ER were also included, bcl-2 ($P = 0.01$) and fasL ($P = 0.005$) remained highly significantly associated with OS, whereas histological grade and ER lost their significance (Table 6). Patients with high bcl-2 expression and low fasL expression had the best OS (Fig. 1). fasL staining was very often seen in peripheral areas of the tumors as seen in Fig. 2. None of the other investigated apoptosis-related factors was associated with TTP or OS (data not shown).
DISCUSSION

Because drugs with distinct primary targets end up in apoptotic cell death, mutations in apoptotic pathways could produce multidrug resistance. In preclinical studies, bcl-2 or bcl-xL overexpression has been demonstrated to suppress apoptosis (13), whereas induction of bax has been reported to restore sensitivity to drug- and radiation-induced apoptosis (14). However, to date, there are only limited and conflicting clinical data to support any clear connection between bcl-2 family proteins and response to chemotherapy in hematological malignancies or solid tumors (15, 16).

In the current study, bax, bcl-2, bcl-xL, bag-1, fasR, and fasL were not predictive factors for chemotherapy response in advanced breast cancer. Previously in breast cancer, the majority of studies have shown no relationship between bcl-2 expression and response to chemotherapy (17–21), although in one study, bcl-2 overexpression predicted a worse response to chemotherapy (22). Interestingly, the first clinical study on the predictive value of bax demonstrated that patients having primary tumors with reduced levels of bax showed worse response rates to chemotherapy, especially in the subgroup of patients treated with a lower-dose-intensity regimen of chemotherapy (17). No association between bax expression and response to chemotherapy was seen in the current study or in another study in the neoadjuvant setting (20). Notably, these two studies did not investigate dose intensity on the contrary to the study by Rajewski et al. (17). This suggests that bax may still be a useful marker in selecting patients for more intense treatment schedules instead of weekly treatment schedules, although the findings should be confirmed in other studies with various dose intensities.

bcl-2 expression was associated with a better prognosis in the current study, which is in line with previous studies. Theoretically, this seems paradoxical because, in preclinical models, bcl-2 overexpression provides a survival advantage. One possible explanation for this phenomenon is that bcl-2 positive tumors often have ERs and a more favorable prognosis. Indeed, estrogen has been shown to be a positive regulator of bcl-2 expression in breast cancer cell lines (23). Furthermore, in the present study, bcl-2 expression correlated positively with ER positivity.

Bag-1 expression has previously been associated either with better (24) or with worse (25) prognosis in early breast cancer, but bag-1 was not any prognostic indicator in the current study. This may be explained by a different patient population, i.e., the patients in the current study represented metastatic disease. Moreover, in the current study, we did not analyze nuclear and cytoplasmic expression of bag-1 separately. Notably, in the previous studies, the cytosolic pattern of staining indicated a better prognosis (24), and the nuclear pattern of staining a worse prognosis (25).

To the best of our knowledge, the present study is the first one to assess the clinical utility of fas or fasL as predictive indicators for chemotherapy response. Neither of them predicted a response to chemotherapy. Interestingly, fasL was a strong prognostic factor: in the multivariate analysis it remained statistically the most significant indicator of OS, whereas ER and histological grade lost their significance. Patients whose tumors had both high bcl-2 expression and low fasL expression survived significantly longer than others. This is in line with a previous study in which a ratio of fasL:fas mRNA transcripts of >1 has been found to be an indicator of shorter disease-free survival in breast cancer patients (26). fasL may thus appear as a novel prognostic indicator for breast cancer, although these preliminary findings need to be confirmed in studies with more patients with early-stage disease.

Initially the fas/fasL system was identified as eliminating unwanted cells by apoptosis, such as the peripheral deletion of activated mature T cells at the end of an immune response or the killing of targets by CTLs and by NK cells (27). Up-regulation of fasL expression has been found in various human tumors, possibly promoting the escape of tumor cells from an attack by the cellular immune system. Theoretically, a tumor could do this by binding its fasL to a fas receptor of a NK cell or a CTL, which would lead to death of the NK cell or the CTL. This may be the biological rationale for our finding that tumors typically express fasL in their peripheral invasive areas.

Emerging clinical evidence has not thus far demonstrated any clear connection between defective apoptotic pathways and treatment failure. The heterogeneous results may be attributable to the following reasons. Firstly, because of the multiplicity and redundancy of apoptotic pathways, defects at one site may lead to the activity of another pathway. Secondly, the genetics of human tumors is so variable that simple comparisons may be inadequate to find biologically relevant correlations. It is probable that whole apoptotic pathways instead of single mediators or proteins should be investigated; the recent applications of microarrays and proteomics may help in this respect. Finally, much work is still needed to understand when and how these multiple apoptotic pathways contribute to chemosensitivity.

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REFERENCES

7. Mullauer, L., Mosberger, I., Grusch, M., Rudas, M., and Chott, A. Fas ligand is expressed in normal breast epithelial cells and is frequently
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