Expression of Bax in relation to Bcl-2 and other predictive parameters in breast cancer

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Summary

Background: It has been suggested that modification of the physiological susceptibility to induction of programmed cell death in transformed cells contributes to the pathogenesis of cancer. One of the major regulators of cell death is the bcl-2 family. In breast cancer, altered expression of Bcl-2 has been described. Distribution of its counterpart Bax and the differential expression pattern have still to be evaluated.

Patients and methods: Bax expression was investigated by immunohistochemistry in 122 primary breast cancers. Results were correlated with expression of Bcl-2 and other variables of predictive value.

Results: There was a positive association between Bax expression and histological grading, (over)expression of c-erbB-1 and -2 and proliferative activity. The correlation was most significant in cases where no concomitant Bcl-2 expression could be detected. In the same subgroup an inverse correlation with positivity for estrogen (and progesterone) receptors was observed. The presence of Bax was not significantly associated with either tumor type and size, nodal status or expression of c-erbB-3.

Conclusion: Expression of Bax was coupled with negative histopathological features, especially when expression of Bcl-2 was downregulated concomitantly. It may appear that alterations of the differential Bax/Bcl-2 expression pattern take part in deregulation of proliferation and loss of differentiation, thus playing an important role in malignant progression.

Key words: Bax, Bcl-2, breast cancer, prognostic factors

Abbreviations: ER – estrogen receptor; PR – progesterone receptor; DCIS – ductal carcinoma in situ

Introduction

Alterations in the machinery of programmed cell death, which under physiological circumstances eliminates most of transformed or otherwise unwanted cells, are suggested to play a crucial role in tumorigenesis. One of the most important regulators of the death machinery is the still growing bcl-2 family. Besides bcl-2, known as an inhibitor of cell death, it includes bax, bad, bak, bcl-XL, mcl-1 and A-1, encoding for molecules with either agonistic or antagonistic action to bcl-2 [1-5]. The corresponding proteins are highly conserved in two regions, the Bcl-2-homologous domains BH1 and BH2, which are required for the formation of homo- or heterodimers [6-8]. Heterodimerisation, especially between Bcl-2 and its most powerful antagonist Bax, is suggested to be the essential modifier of the actual Bcl-2 effect and to determine a cell's commitment to either apoptosis or survival [1].

Until now, most of the studies about the presumed involvement of the bcl-2 family in tumor progression have focused on the expression pattern of Bcl-2 alone. In breast cancer, the presence of Bcl-2 has been shown to be related to features of differentiation and good prognosis [9]. An inverse correlation with histological grading, proliferative activity, mutations of p53 and c-erbB-2 expression has been demonstrated [10-13]. Bcl-2 immunoreactivity was positively correlated with c-erbB-3 expression [14], hormone receptor positivity [10-16] and the probability of response to endocrine therapy [17, 18]. Considering its apoptosis-inhibiting effect and the presumed enhanced survival of Bcl-2-positive transformed cells, these results are surprising. Interaction of Bcl-2 with other family members such as Bax may account for this discrepancy, but there are still very few comparative data about the in vivo distribution of Bax and Bcl-2.

In mice, Krajewski and coworkers [19] found a complex pattern of Bax production that paralleled the expression of Bcl-2 in some tissues, such as lactating breast, and was reciprocal in others, such as lymph nodes and colon. Bax-mRNA could be detected in normal cell lines and tissues of the human breast. Malignant cell lines and primary cancers also showed bax expression, but to a lesser extent [20].

On the basis of these results, it may be speculated that deregulation of the physiological Bcl-2/Bax balance contributes to the pathogenesis and progression of breast cancer. To further elucidate this question, Bax protein expression was studied by immunocytochemis-
try in 122 primary adenocarcinomas of the human breast. The expression pattern of Bax was evaluated in relation to Bcl-2 and other histopathological variables, such as nodal status, histological grading and tumor type, hormone receptor expression, positivity for members of the type I growth factor receptor family (c-erbB-1 to -3) and proliferative activity.

**Patients and methods**

**Patients**

Results were derived retrospectively from a consecutive series of 122 primary breast cancer specimens analysed at the Department of Pathology II at the University Hospital of Göttingen. For postsurgical staging the international Tumor-Node-Metastasis classification was applied. Histological typing and grading were performed according to the classification of Bloom and Richardson [21].

**Immunohistochemistry**

Staining for Bax was performed on 1–2 μm paraffin sections which were dewaxed in xylene and rehydrated through graded alcohols. Immunostaining with the primary antibody (polyclonal rabbit anti-Bax N-20, Santa Cruz, CA, U.S.A., 1:200) was followed by application of two bridging antibodies (mouse anti-rabbit, rabbit anti-mouse, DAKO, Germany, 1:50). The primary signal was enhanced by the alkaline phosphatase-antialkaline phosphatase method (APAAP-complex, DAKO, 1:100). Stain was developed with new fuchsin substrate.

According to the manufacturer, the primary antibody was raised against a peptide corresponding to amino acids 11–30 of human Bax, a region where homology with other family members is weak. It picks up a 21 kD protein in Western blots, showing no cross-reactivity with Bcl-2 and Bcl-X. We additionally confirmed the lack of cross-reaction with Bcl-2 by demonstration of the typical reciprocal staining pattern in control lymph nodes.

For detection of Bcl-2 and assessment of proliferative activity, immunostaining with the moAbs anti-Bcl-2–124 (DAKO) and MIB1 (Dianova, Hamburg, Germany) against the Ki-67 antigen was used as described before [14]. Immunohistochemistry for c-erbB-1 and -2 was performed according to the protocol of Meden et al. [22]. c-erbB-3 was stained with the antibody RTJ1, kindly provided by W.J. Gullick, (London, U.K.) according to the same protocol. Hormone receptors were detected by immunostaining of frozen tissue sections following the method of Marx et al. [23].

All slides were evaluated in a blinded fashion without knowledge of the clinicopathological data. Staining for Bax was considered positive when the intensity of the colour reaction was comparable to the accompanying positive control, the germ center of a lymph node. Results for Bcl-2 were distributed into four groups comparing the colour intensity with that of interspersed lymphocytes as an internal positive control: negativity, weak (slight staining, mostly focally restricted), positive (moderately strong staining in most of the cells), and strongly positive (strong lymphocyte-like staining in almost all cells). For comparative evaluation all positive groups were taken together. Staining for both ER and PR was classified according to the number of positive cells: negative (<50%) and positive (≥50%). MIB1 staining was considered positive when more than 30% of the cells gave a nuclear signal. c-erbB1–3 positivity was based on detection of a distinct membrane-associated precipitate (with concomitant cytoplasmic staining in the case of c-erbB-3).

**Expression of Bax**

48% (58/122) of the cancers showed a positive cytoplasmic immunoreactivity for Bax. The staining pattern was mostly homogeneous. In contrast to Bcl-2, the epithelia of normal large and small ducts as well as interspersed lymphocytes were Bax-negative. As for the relation between Bax and Bcl-2, four non-statistically related groups of approximately the same size were distinguishable: negativity as well as positivity for both proteins (22% and 27%), isolated presence of Bax with concomitant absence of Bcl-2 (21%) and the presence of Bcl-2 with an absence of Bax (30%).

**Relation to histological parameters and tumor staging**

There was a significant positive correlation between Bax expression and histological grading, which was most accentuated in the group where only Bax was positive without concomitant Bcl-2 expression (Table 1 and Figure 1). The majority of GI tumors was Bax-negative.

**Table 1. Expression of Bax in relation to histological grading.**

<table>
<thead>
<tr>
<th>n=113</th>
<th>Histological grading</th>
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<tbody>
<tr>
<td></td>
<td>G I</td>
</tr>
<tr>
<td>Bax-neg.</td>
<td>9/15 (60%)</td>
</tr>
<tr>
<td>Bax-pos. total</td>
<td>6/15 (40%)</td>
</tr>
<tr>
<td>Bax-pos./Bcl-2-neg.</td>
<td>0/15 (0%)</td>
</tr>
</tbody>
</table>

**Figure 1. Differential expression pattern of Bax and Bcl-2 in relation to various histopathological variables.**
negative (60%) whereas most of the GIII tumors gave a positive signal (62%), two-thirds of these showing isolated Bax-positivity (46%, \( p < 0.001 \)). No relation was seen between histological subtypes and Bax expression, mainly because most of the subgroups were too small to allow valid conclusions: 51% (41/81) of the ductal, 23% (3/13) of the lobular, 40% (2/5) of the mucinous, 29% (2/7) of the tubular, 50% (4/8) of the medullary and 100% (4/4) of the early invasive carcinomas as well as 50% (2/4) of the DCIS cases were Bax-positive. Tumor size was not correlated with Bax expression. Likewise, no significant difference was demonstrated between nodal-negative and -positive cases (47% ~ 27/57 versus 51% = 23/45 Bax-positivity).

**Correlation with ER- and PR-expression**

ER-positive tumors tended to be Bax-negative (60%). The observed inverse correlation was highly significant in the Bax-positive/Bcl-2-negative group (\( p < 0.001 \)). The data are given in Table 2 and Figure 1. A similar but non-significant association was found between Bax expression and PR-positivity (\( p = 0.08 \)), with 24% (22/91) of PR-negative and only 10% (3/31) of the PR-positive tumors being Bax-positive/Bcl-2-negative.

**Bcl-2 expression and proliferative activity**

There was no clearcut difference between Ki-67-positive and -negative tumors regarding either overall positivity or negativity for Bax. However, evaluation of the two Ki-67 subsets in relation to combined Bax- and Bcl-2-immunostaining revealed significantly higher isolated Bax-positivity in Ki-67-positive than in -negative tumors (\( p = 0.006 \), Table 3 and Figure 1).

**Relation to expression of c-erbB-1 to -3**

Expression of c-erbB -2 was positively correlated with Bax-immunoreactivity (Table 4, Figure 1). Again, the difference was most accentuated in the group with isolated Bax-positivity (\( p = 0.006 \)). A similar but not significant tendency was detected between expression of c-erbB-1 and Bax/Bcl-2, with 19% (17/91) of the c-erbB-1-negative tumors being Bax-positive versus 26% (8/31) of the positive ones (\( p = 0.3 \)). No correlation could be established with expression of c-erbB-3.

**Table 4. Expression of Bax in relation to c-erbB-2.**

<table>
<thead>
<tr>
<th>n = 116</th>
<th>c-erbB-2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg.</td>
</tr>
<tr>
<td>Bax-neg.</td>
<td>46/85 (54%)</td>
</tr>
<tr>
<td>Bax-pos. total</td>
<td>39/85 (46%)</td>
</tr>
<tr>
<td>Bax-pos./Bcl-2-neg.</td>
<td>13/85 (15%)</td>
</tr>
</tbody>
</table>

**Discussion**

The role of the bcl-2 family in malignant transformation is still unknown. Previous studies of the expression of the Bcl-2 in breast cancers [9–18] demonstrated that the presence or absence of Bcl-2 in tumor cells differs from the expression pattern in the respective nontransformed cells. As the apoptosis-inhibiting effect of Bcl-2 depends on the interaction with other family members, a conclusive evaluation has to take into account also the concomitant expression of these homologues, especially the main Bcl-2-antagonist Bax.

The present study shows no direct correlation between the distribution of Bax and Bcl-2. Association of overall Bax-positivity with any of the other investigated variables, either histological grading, hormone receptor expression, proliferative activity or positivity for c-erbB-1 and -2, was only weak. However, differences became statistically significant when the results were evaluated in relation to the four subsets of Bax/Bcl-2 expression. There was a strong positive correlation between Bax presence/ Bcl-2 absence and histological grading, expression of c-erbB-2 and proliferative activity. The same association was shown with c-erbB-1 expression. A significant inverse correlation was found between Bax presence/Bcl-2 absence and expression of estrogen receptors, to a lesser extent also with progesterone receptors.

In general, these results are consistent with the data of other investigators. Krajewski and coworkers [19] described a complex relation between Bax and Bcl-2 expression in the mouse, in some cases parallel, in others reciprocal, similar to the present data. Barghou et al. [20] investigated the mRNA expression of bcl-2 and bax in 10 human breast cancer samples. All of them were strongly positive for bcl-2 whereas expression of bax was mostly low or undetectable. In the present study, the strongly Bcl-2-positive tumors usually belonged to the G I-(or G II)-subclass [14], in which the majority of cases were Bax-negative.
Recently, Krajewski et al. [24] reported a positive correlation between Bax and Bcl-2 expression in 119 human breast cancers as well as an association between reduced Bax levels and shorter survival and poor response to therapy. As the data were derived retrospectively from a cohort of metastatic cases, differences in patient selection may account for the differing results. The unusually low rate of Bcl-2-positive tumors (47%) as compared to other studies corroborates this hypothesis.

Taken together, expression of Bax in breast cancer appears to be associated with a low degree of differentiation, high proliferative activity, (over)expression of c-erbB-2 and negativity for estrogen receptors, all of them considered predictors of poor clinical outcome. These data are compatible with the former results about Bcl-2, where the opposite, i.e. correlation with positive prognostic factors, has been shown [9–15, 17, 18].

Considering the progressive diminution of Bcl-2-positive cases in association with increasingly negative histopathological features, it may be suggested that the normally expressed bcl-2 gene is gradually downregulated in the course of malignant progression whereas bax transcription is stable or enhanced. Bcl-2 expression has been demonstrated to be responsive to estrogen [25]. As progression is usually coupled with loss of hormone receptor expression, bcl-2 downregulation may represent a consequence of hormonal insensitivity, while the independently regulated bax remains unaffected.

Another candidate for the deregulation of the Bcl-2/Bax balance is p53. Wild type p53 has been shown to be a transcriptional activator of bax and to repress expression of bcl-2 [26, 27]. Mutations of p53, frequently found in breast cancer, interfere with p53 function [28, 29] and may alter the interaction with bax and bcl-2.

The pathophysiological significance of bax/bcl-2 deregulation remains to be clarified. The surprising fact that expression of Bax, which is supposed to shorten cell survival, is associated with predictors of negative outcome, may be explained by counteraction of the death-promoting effect through other members of the bcl-2 family, such as Bcl-xL [20, 30, 31].

On the other hand, the lack of association between tissue-specific Bax expression and apoptotic rates [19], may indicate an additional role for Bax. Like Bcl-2 [14, 18, 32, 33], it may be involved in the regulation of proliferation and differentiation, which is supported by the observed correlation between Bax expression and proliferative activity.

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References

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