BAX Expression in Hodgkin and Reed-Sternberg Cells of Hodgkin’s Disease: Correlation with Clinical Outcome

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ABSTRACT

Purpose: BAX, a proapoptotic member of the BCL-2 family of proteins, has been detected in Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin’s disease (HD), but its clinical significance is unknown. Therefore, we correlated BAX expression with presenting features and clinical outcome in untreated patients with HD.

Design: Patients with biopsy-proven HD were eligible if they were untreated previously and if pretreatment paraffin-embedded tumor tissue was available. BAX was detected by immunohistochemistry without knowledge of clinical features or outcome. A tumor was considered as positive if any number of HRS cells expressed BAX, but other cutoffs of BAX expression were examined for analysis of clinical outcome.

Results: We identified 260 patients with HD. The median age was 31 years, and 55% were male. HRS cells expressed BAX in 181 of 195 (93%) nodular sclerosis, 47 of 48 (98%) mixed cellularity, 1 case of lymphocyte depletion, all 6 unclassified classical HD, and all 10 lymphocyte predominance tumors. Using a cutoff of 50% positive HRS cells for BAX expression, the 5-year failure-free survival (FFS) for patients with high versus low BAX expression was 83 versus 93%, respectively (P = 0.19 by Log-rank) for 116 patients treated with doxorubicin, bleomycin, vinblastine, and dacarbazine or equivalent regimens; it was 78 versus 79%, respectively, for 79 patients treated with mitoxantrone, vincristine, vinblastine, and prednisone or radiotherapy (P = 0.45 by Log-rank); it was 71 versus 81%, respectively, for 26 patients treated with nitrogen mustard, vincristine, prednisone, and procarbazine (P = 0.6 by Log-rank); and it was 72 versus 82% for 29 patients treated only with radiotherapy (P = 0.57 by Log-rank). The 5-year FFS was not statistically different when we used cutoffs of 20, 30, and 75% for BAX expression.

Conclusion: BAX is often expressed by HRS cells in HD and does not correlate with FFS.

INTRODUCTION

Although the cure rate for HD is high, up to 30% of patients relapse and eventually die of disease or treatment complications (1–3). Various clinical and laboratory features have been used to predict FFS and overall survival and to identify groups of patients with inferior prognosis. These include age; gender; peripheral or mediastinal bulk; stage IV; involvement of bone marrow or inguinal lymph nodes; anemia, leukocyte, and lymphocyte counts; and serum levels of albumin, LDH, β₂-microglobulin (4–11), and interleukin-10 (12–15). However, additional prognostic factors related to the biology of HD are currently under investigation to improve prediction of clinical outcome and provide a rational basis for investigational therapy.

Immunological and molecular studies have shown that most HRS cells of classical HD are derived from germinal center B cells with rearranged immunoglobulin genes bearing crippling mutations (16–19). These mutations cause apoptosis in normal B-lymphocytes. Alterations of apoptotic pathways are involved in oncogenic mechanisms in human cancers (20). The role of BCL-2 family proteins in apoptosis is well established (21, 22). More than 20 proteins have been described that share homology with BH domains of the bcl-2 gene, with either antiapoptotic or proapoptotic function.

BAX is a Mr 21,000 protein with significant homology clustered in the BH1 and BH2 regions of the bcl-2 gene and was the first of the BCL-2 homologues characterized as an important cell death agonist (23). BAX induces apoptotic cell death by its insertion into the mitochondrial membrane, binding to and thus...
inhibiting BCL-2 function and causing release of cytochrome c (24). However, alternative hypotheses have been proposed (24). The balance between BCL-2 and BAX is important for the induction of programmed cell death; when BCL-2 predominates, apoptosis is inhibited, whereas when the levels of BAX are increased, the cell initiates the apoptotic machinery (21).

Previous studies have shown that many members of the BCL-2 family are expressed in HRS cells of HD, including BAX (25–29), and >90% of HD tumors have been reported to be BAX positive (25). However, because the number of HD tumors studied was small and selection criteria and treatment were not specified, the clinical significance of BAX expression remains unknown.

Therefore, we decided to investigate the expression of BAX in previously untreated patients with HD and determine its association with presenting clinical and laboratory features and with clinical outcome. To minimize the effect of heterogeneous therapy, we determined the FFS of uniformly treated groups of patients.

PATIENTS AND METHODS

Patients. Patients were eligible if they had presented from 1984 to 1996 without any prior treatment at the University of Texas M. D. Anderson Cancer Center (Houston, TX), Istituto Tumori Nazionale (Milan, Italy), University of Verona (Verona, Italy), and the National and Kapodistrian University of Athens (Athens, Greece). It was required that the pathological diagnosis be initially established on the basis of tissue biopsy, and pretreatment tumor be available for immunohistochemical determination of BAX expression; and the histological diagnosis be confirmed when slides were reviewed at the time of immunohistochemical analysis, according to criteria defined by the WHO classifications (30). Patients with HIV-1 infection were ineligible.

Staging. All patients had physical examination, chest radiograph, bone marrow biopsy, and computerized tomography of chest, abdomen, and pelvis. When clinically indicated, lymphangiogram, gallium scan, and computerized tomography of the head and neck were also obtained according to the individual practices of the participating institutions. The ratio of mediastinal mass to thoracic diameter was measured at the T4-T5 interspace as described previously (4) and was considered high if mass to thoracic diameter was measured at the T4-T5 interspace as practices of the participating institutions. The ratio of mediastinal head and neck were also obtained according to the individual phangiogram, gallium scan, and computerized tomography of the chest, abdomen, and pelvis. When clinically indicated, lym-

Diagnosis. The diagnosis of HD was established on the basis of tissue biopsy, pretreatment tumor being available for immunohistochemical determination of BAX expression. All statistical calculations were performed using StatView (Abacus Concepts, Inc., Berkeley, CA).

Immunohistochemistry. Immunohistochemical detection of BAX was achieved using heat-induced antigen retrieval and the monoclonal antibody 2D2 (Zymed, South San Francisco, CA) at a dilution of 1:40 as described previously (36). BAX-positive normal prostate tissue was used as an external positive control in each experiment (37). In addition, coexisting small reactive lymphocytes served as internal positive and negative controls in each HD slide.

Routine slides were reviewed at the time of immuno-histochemical analysis for confirmation of diagnosis of HD, according to criteria defined by the WHO classification (30). In every case, HRS cells were shown to be positive for CD15 and/or CD30 in the same tissue blocks assessed for BAX. BAX immunostains were reviewed without knowledge of clinical features or outcome. Slides were considered evaluable if all concurrent internal and external controls stained appropriately. Any cytoplasmic BAX staining of the malignant HRS cells was considered positive. At least 100 HRS cells in representative fields were manually counted in each tumor to determine the percentage of BAX-positive HRS cells. For the purpose of the statistical analysis of clinical outcome and based on the distribution of BAX-positive HRS cells among tumors, we used a 50% cutoff to define high versus low expression of BAX. However, other cutoffs for BAX expression were also used for correlation with clinical outcome.

Statistical Analysis. FFS was measured according to the method of Kaplan and Meier (38), from the beginning of treatment to primary treatment failure, relapse, or last follow-up. Patients who died during treatment without evidence of progressive disease, or after the end of therapy without prior evidence of relapse, were censored. The statistical significance of differences in FFS between groups of patients was estimated by the Log-rank test (39). The comparisons between BAX expression and clinical or laboratory parameters were based on x2 and Fisher’s exact tests. Nonparametric Mann-Whitney test was used to evaluate the correlation between patient age and BAX expression. All statistical calculations were performed using StatView (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Study Group. We identified 1765 untreated patients with HD who presented to the participating institutions between 1984
The present study group included 260 patients. Most presenting clinical and laboratory features of patients with known (260 patients) versus unknown (1505 patients) BAX expression are similar, as shown in Table 1. Therefore, we consider the analyzed group to be representative of the whole patient population. For these 260 patients, HD was classified as NS in 195, mixed cellularity in 48, lymphocyte depletion in 1 patient, classical HD not otherwise specified in 6, and lymphocyte predominance HD in 10 patients (Table 1). Treatment was ABVD or an equivalent regimen in 116, NOVP followed by radiotherapy in 79, MOPP in 26, and only radiotherapy in 29 patients.

**BAX Expression.** BAX was expressed by any HRS cells in 245 of 260 (94%) tumors (Table 2) and was detected in the cytoplasm of HRS cells but with variable staining intensity (Fig. 1). The percentage of HRS expressing BAX varied considerably between tumors, as shown in Fig. 2. The mean percentage of BAX-positive HRS cells was 56 ± 29 but ranged from 0 to 97%.

For the purpose of a first statistical analysis and based on the histogram of BAX-positive HRS cells (Fig. 2), we considered BAX expression to be high if 50% of HRS expressed BAX. BAX immunoreactivity in 50% of HRS cells was more frequent in females (P = 0.003, Fisher’s exact test) and patients younger than 45 years (P = 0.05, Fisher’s exact test) in this study but was not statistically associated with any other presenting clinical and laboratory features, including stage, B-symptoms, bone marrow involvement, bulky disease, or laboratory parameters.

**FFS Analysis.** After a median follow-up of 85 months for the survivors, 16 of the 116 patients treated with ABVD or an equivalent regimen failed. Using a 50% cutoff for high versus low BAX expression by HRS cells, the 5-year FFS for patients with high versus low BAX expression was 83 versus 93% (P = 0.19 by

### Table 1 Presenting clinical and laboratory characteristics of patients with HD

<table>
<thead>
<tr>
<th></th>
<th>BAX expression known</th>
<th>BAX expression unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>All patients</td>
<td>260</td>
<td>100</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>34</td>
<td>NA*</td>
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<tr>
<td>Range</td>
<td>6–74</td>
<td>NA</td>
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<tr>
<td>Male sex</td>
<td>144</td>
<td>55</td>
</tr>
<tr>
<td>B-symptoms</td>
<td>100/258</td>
<td>39</td>
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<td>Histology</td>
<td></td>
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<td>Classical HD</td>
<td></td>
<td></td>
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<tr>
<td>Nodular sclerosis</td>
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<td>75</td>
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<tr>
<td>Mixed cellularity</td>
<td>48</td>
<td>18</td>
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<tr>
<td>Lymphocyte depletion</td>
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<td>Unclassified</td>
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<td>2</td>
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<tr>
<td>Lymphocyte predominance</td>
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<td>4</td>
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<td>Ann Arbor stage</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>II</td>
<td>125</td>
<td>48</td>
</tr>
<tr>
<td>III</td>
<td>60</td>
<td>23</td>
</tr>
<tr>
<td>IV</td>
<td>33</td>
<td>13</td>
</tr>
<tr>
<td>High-serum LDH</td>
<td>93/254</td>
<td>37</td>
</tr>
<tr>
<td>High-serum β₂-microglobulin</td>
<td>31/187</td>
<td>17</td>
</tr>
<tr>
<td>Inguinal or iliac node involvement</td>
<td>49/260</td>
<td>19</td>
</tr>
<tr>
<td>Mediastinal mass ratio &gt; 0.45</td>
<td>28/220</td>
<td>13</td>
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<tr>
<td>Low serum albumin</td>
<td>25/250</td>
<td>10</td>
</tr>
<tr>
<td>Anemia</td>
<td>97/260</td>
<td>37</td>
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</table>

* NA, not applicable.
* Evaluated by Mann-Whitney test. Fisher’s exact test was used for all other comparisons.

### Table 2 BAX expression in HRS cells of HD according to histologic subtype

<table>
<thead>
<tr>
<th>Histology</th>
<th>Patients No.</th>
<th>BAX-positive patients</th>
<th>% of HRS cells expressing BAX</th>
<th>Mean ± SD*</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All HD patients</td>
<td>260</td>
<td>245</td>
<td>94</td>
<td>56 ± 29</td>
<td>0–97</td>
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<tr>
<td>Classical HD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td>195</td>
<td>181</td>
<td>93</td>
<td>56 ± 30</td>
<td>0–97</td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td>48</td>
<td>47</td>
<td>98</td>
<td>57 ± 26</td>
<td>0–96</td>
</tr>
<tr>
<td>Lymphocyte depletion</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Unclassified</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>64 ± 29</td>
<td>28–92</td>
</tr>
<tr>
<td>Lymphocyte predominance</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>69 ± 22</td>
<td>20–93</td>
</tr>
</tbody>
</table>

* SD, standard deviation.
Log-rank; Fig. 3). When the analysis was restricted to the 57 patients with Ann Arbor stage I or II or the 59 patients with Ann Arbor stage III or IV, the 5-year FFS also did not differ significantly for tumors with high versus low BAX expression.

Twenty of 79 patients with Ann Arbor stage I-III who were treated with NOVP and radiotherapy failed. The 5-year FFS was 78% versus 79% for those with high versus low BAX expression, respectively (P = 0.45 by Log-rank). Seven of the 26 patients treated with MOPP failed. The 5-year FFS for MOPP-treated patients with any stage of disease did not significantly differ between patients with high versus low BAX expression (71% versus 81%, P = 0.6 by Log-rank). Seven of 29 patients treated only with radiotherapy failed. The 5-year FFS for patients with high versus low BAX expression was 72% versus 82% (P = 0.57 by Log-rank).

**Cutoffs for BAX Expression.** Because the frequency of BAX expression appeared to be a continuous variable and because it is not intuitive which cutoff for BAX expression may promote chemotherapy-induced apoptosis, four different cutoffs for percentage of BAX-positive HRS cells were investigated: 20, 30, 50, and 75%. The selection of these cutoffs was based on the distribution of BAX-positive HRS cells (histogram) in the cases of classical HD of the present series (Fig. 2). Analysis using a cutoff of any positive HRS cells was not done, as only 15 (6%) HD tumors had BAX-negative HRS cells. As shown in Table 3, there was no statistically significant difference in FFS between patients with high BAX versus low BAX expression.

**DISCUSSION**

We report that BAX is frequently expressed in HRS cells of HD, but the levels of its expression are not associated with statistically different clinical outcome among uniformly treated patients. This finding is based on analysis of 260 patients from an international database, including previously untreated patients with biopsy-proven HD. Thus, we eliminated most selection biases toward presenting features and clinical outcome, which might arise by entry of patients who are accrued at relapse. Statistical analysis showed that the presenting clinical and laboratory characteristics of patients with known and unknown BAX status were similar. Therefore, we considered the study population to be representative of the entire patient population.

BAX was detected in HRS cells of 94% of patients with HD, if we consider the presence for any BAX-positive HRS cells as a criterion for positivity. These results are in concordance with those of Brousset et al. (25), who has reported that HRS cells in 92% of tumors expressed BAX (25). In that study, 28% of HD tumors had >75% of BAX-positive HRS cells, compared with 34% in our series (Table 4). We also report the distribution of the percentage of
BAX-positive HRS cells, thus providing more detailed information for BAX expression by HRS cells of HD. The frequency of BAX expression among HRS cells of individual tumors was comparable for all histological subtypes (Table 2).

High expression of BAX (≥50% positive HRS cells) was not associated with major differences in most presenting clinical or laboratory features (Table 3). Female patients had statistically more frequently high BAX expression (P = 0.003). Younger age was also correlated with high BAX expression in HD (P = 0.05). The biological basis of these statistical associations is uncertain. Although female gender and younger age are associated with better prognosis in HD (11), BAX expression by itself was not correlated with superior FFS in this cohort of homogeneously treated patients. In contrast, patients with high BAX expression had slightly inferior FFS, but this association was not statistically significant. Furthermore, when different cutoffs were used to distinguish high from low BAX expression, FFS does not change significantly, but the percentage of tumors with high BAX expression declines (Table 3).

The prognostic significance of BAX expression has been investigated previously in DLBCL, with conflicting results (40, 41). Gascoyne et al. (40) detected BAX in >1% of tumor cells in 106 of 111 DLBCL but found that it was not associated with statistically different survival. Among the 85 patients with BCL-2-negative DLBCL, BAX was expressed (>1% of tumor cells) in 78 tumors and was associated with significantly higher relapse-free and overall survival. However, the number of patients with BAX-negative tumors was very small, and multivariate analysis demonstrated that BAX expression added no additional prognostic information (40). Using a 10% cutoff for BAX expression, Bairy et al. (41) showed that BAX-positive DLBCL was statistically more frequent in short-lived compared with long-lived patients (41). Furthermore, in other aggressive lymphoid neoplasms, including acute lymphoblastic leukemia, high BAX expression correlated with lower relapse-free survival (42). In our population and with a 50% cutoff for BAX expression, FFS was slightly inferior for high BAX expression, although the difference was not statistically significant.

These results are contrary to our expectations, because we suspected that high BAX expression would promote chemotherapy-induced apoptosis. Several explanations are possible for these associations with clinical outcome: (a) it is possible that the relative ratio of various proapoptotic and antiapoptotic members of the BCL-2 family, and not just BAX expression, may determine the susceptibility of tumor cells to apoptosis induced by cytotoxic agents; and (b) a conformational change in the BAX molecule is required for its insertion into the mitochondrial membrane and its subsequent binding to BCL-2, which results in release of cytochrome c and in the subsequent initiation of apoptotic cell death cascade (24). Because the detection method used in our study is based on heat-induced antigen retrieval, the conformational change of BAX is destroyed and thus cannot be detected, as it cannot be detected on Western blots after protein denaturation.

We conclude that BAX is expressed by HRS cells in 94% of patients with HD, but the percentage of BAX-positive HRS cells varies greatly among different tumors. Expression of BAX was not associated with different presenting clinical and laboratory features or prognosis among uniformly treated patients with HD.

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