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Reduced Expression of Proapoptotic Gene *BAX* Is Associated with Poor Response Rates to Combination Chemotherapy and Shorter Survival in Women with Metastatic Breast Adenocarcinoma[1]

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ABSTRACT

Bax is a homologue of Bcl-2 that promotes apoptosis. Bax protein levels were assessed by immunohistochemical methods in primary tumors derived from 119 women with metastatic breast cancer. These patients had received combination chemotherapy either with a once a month dosage schedule or in 4 weekly divided doses. The BAX immunostaining results were retrospectively compared with overall survival, time to tumor progression (TTP), and response, as well as several laboratory markers. Normal breast epithelium and *in situ* carcinomas immunostained positively for Bax. Marked reductions in Bax immunostaining were observed in 40 (34%) of 119 evaluable tumors. Reduced Bax correlated with shorter overall survival (median, 8.1 *versus* 15.7 months; $P = 0.04$), faster TTP (median, 2.0 *versus* 6.3 months; $P = 0.009$), and failure to respond (complete response, partial responses; 6% *versus* 42%, $P = 0.01$) in the subgroup of patients who received divided dose therapy. Reduced Bax immunostaining was not significant in the monthly dose group. When the two groups were combined, however, reduced Bax was significantly correlated in univariate analysis with failure to respond (21 *versus* 43% achieving complete response or partial response; $P = 0.02$), faster TTP (median, 3.7 *versus* 9.0 months; $P = 0.02$), and shorter survival (median, 10.7 *versus* 17.1 months; $P = 0.04$). Bax immunostaining was not significantly correlated with tumor histology, S-phase fraction, aneuploidy, p53 *HER2*, or cathepsin D, but was positively associated with Bcl-2 ($P = 0.005$). In multivariate analysis (Bax, tumor grade, and treatment group), reduced Bax was strongly associated with faster TTP ($P \cong 0.009$) and shorter survival ($P \cong 0.001$). Although highly preliminary, the finding suggest that loss of Bax immunostaining represents a novel prognostic indicator of poor response to chemotherapy and shorter survival in women with metastatic breast cancer, and raise the possibility that the subgroup of women with Bax-negative tumors may benefit from more aggressive therapy.

INTRODUCTION

Cell death plays an important role in normal tissue homeostasis where it offsets new cell production caused by cell division with commensurate rates of cell loss in most tissues with self-renewal capacity (reviewed in Ref. 1). Cells succumbing to this mechanism of cell death often undergo characteristic morphological and biochemical changes, collectively referred to as apoptosis (2). Deregulation of the biochemical pathways that control physiological cell death can contribute to neoplastic cell expansion by preventing or delaying normal cell turnover. In addition to their role in the causation of cancer, defects in the cell death pathway can also figure prominently during attempts to treat cancer. This is because essentially all cytotoxic anticancer drugs commonly used in the treatment of human malignancies, as well as radiation, ultimately kill cancer cells primarily by inducing apoptosis and thus rely at least in part on the same biochemical mechanisms involved in physiological cell death control (1, 3, 4). Consequently, genetic defects that prevent or delay normal cell turnover can also render tumor cells relatively more resistant to the cytotoxic effects of chemotherapeutic drugs.

A large body of experimental evidence suggests that one of the critical regulators of apoptosis is the protein encoded by the *BCL-2* gene. The *BCL-2* gene was first identified because of its involvement in the t(14;18) chromosomal translocations found in many B-cell lymphomas (5), but also becomes overexpressed in a wide variety of human cancers through other poorly defined mechanisms (for examples, see Refs. 6–13). Although its biochemical mechanism remains enigmatic, the Bcl-2 protein appears to control a distal step in what may represent a final common pathway for apoptotic cell death (reviewed in Ref. 1). Gene transfer experiments have demonstrated that elevated levels of Bcl-2 protein render tumor cell lines markedly more resistant to a wide variety of chemotherapeutic drugs as well as radiation (14–19). High levels of Bcl-2 protein production and *BCL-2* gene activation have also been associated with poor response to therapy, shorter disease-free survival, and overall survival in at least some subgroups of patients with lymphomas, leukemias, and prostate cancer (7, 20–22).

Recently, a family of genes whose encoded proteins share amino acid sequence homology with Bcl-2 have been identified, some of which function as blockers of cell death and others of which are promoters of apoptosis. Among these, the protein encoded by the *BAX* gene has emerged as a central regulator within this multigene family. The Bax protein is a promoter of cell death. The actions of Bax appear to be neutralized when heterodimerized with Bcl-2 and some other members of the Bcl-2 protein family that function as suppressors of cell death such as Bcl-X_L and Mcl-1 (23–27). It has been proposed that the relative sensitivity of cells to apoptotic stimuli is governed by the ratio of Bax:Bcl-2 and other antiapoptotic Bcl-2 family proteins (23). Consequently, reductions in *BAX* expression should be tantamount to elevations in *BCL-2* expression, and could provide an alternative means of affording tumor cells with a selective survival advantage during oncogenesis and of promoting chemoresistance. In this regard, it has also been shown that *BAX* expression can be directly induced by the tumor suppressor p53 (28–30) and that radiation induces *BAX* expression in sensitive, but not resistant, tumor cell lines that undergo apoptosis in response to radiation (31). Thus, Bax may play an important role in the apoptotic elimination of tumor cells after exposure to DNA-damaging drugs and radiation.

As a first attempt to explore the regulation of *BAX* in human tumors and its prognostic significance, we have immunohistochemically analyzed Bax protein levels in a group of 121 women with metastatic adenocarcinoma of the breast. The results indicate that marked reductions in Bax expression occur in approximately one third of advanced

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breast cancers and suggest that tumors with reduced Bax may respond poorly to chemotherapy.

MATERIALS AND METHODS

Patients and Therapy. The patients, inclusion criteria, and treatment modalities involved in this studied have been described in detail previously (32). Briefly, all patients were women who presented to hospitals in Finland with measurable or assessable metastatic adenocarcinoma of the breast and a performance index of 2 or less, and who had not received previous anthracycline chemotherapy. After obtaining informed consent, patients were randomly assigned to receive chemotherapy (cyclophosphamide, fluorouracil, epirubicin) either once every 4 weeks or once a week. Total monthly doses in the two groups were identical. From an initial 173 patients enrolled, 2 were excluded because of concomitant primary tumors of nonbreast origin and 1 because of misdiagnosis of metastatic disease (32). Archival primary tumor material in the from of paraffin blocks was available from 130 of these 170 patients for immunohistochemical analysis. In 9 specimens, no invasive cancer was found in the block, leaving 121 cases. All 121 of these patients were evaluable for survival and TTP[3], but 15 were inevaluable for treatment response because of: simultaneous radiotherapy (n = 6), premature cessation of therapy (n = 3), nonrandomization (n = 2), simultaneous endocrine therapy (n = 2), or surgical excision of the only evaluable lesion (n = 2).

Preparation of Anti-Bax Antibodies and Immunostaining. Polyclonal antisera were prepared in rabbits using a synthetic peptide (NH_2-cys-pro-glu-leu-ala-leu-asp-pro-val-pro-gln-asp-ala-ser-thr-lys-lys-leu-ser-glu-COOH; amide) corresponding to amino acids 43–61 of the human Bax protein, with a cysteine appended to the NH_2 terminus to facilitate conjugation to maleimide-activated carrier proteins (keyhole limpet hemocyanin and ovalbumin), exactly as described in detail previously (6, 33–35). The specificity of this antiserum for human Bax protein was confirmed by immunoprecipitation, immunoblot, and immunocytochemical methods as described (6, 33–35), as well as by comparisons with preimmune serum and by competition experiments. The preparation and characterization of antisera specific for amino acids 41–54 of the human Bcl-2 protein have been described previously (6, 34, 35).

Formalin-fixed, paraffin-embedded tissue sections were immunostained for Bax or Bcl-2 exactly as described in previous publications (33–35) using a microwave antigen retrieval method and biotinylated goat antirabbit IgG followed by a avidin-biotin complex reagent (Vector Laboratories, Inc.) with horseradish peroxidase. Colorimetric detection of bound antibody was achieved with diaminobenzidine, followed by counterstaining with hematoxylin. The Bax antiserum was used at 1:1,500 (v/v) dilution.

All slides were independently reviewed by at least two investigators (K. F. and either S. K. and/or M. K.) and scored on the basis of the infiltrating component of the histology. Tumors were scored as Bax negative if all or most of the infiltrating tumor cells were unstained (≤10% Bax positive) and as Bax positive if approximately >10% were immunostained. With the exception of two cases, all specimens in which the infiltrating tumor cells were completely negative for Bax contained areas of adjacent Bax-positive carcinoma *in situ*, residual normal breast epithelium, stromal fibroblasts, or infiltrating lymphocytes, thus serving as an internal control to verify adequate preservation of the specimen. Those two Bax-negative specimens were therefore excluded from the analysis. The same scoring approach was also used for evaluation of Bcl-2 immunostaining data, resulting in successful staining of 119 or 121 cases. This method for slide scoring had a >97% concordance among different examiners, and proved to be more reproducible than other methods based on assessments of intensity of immunostaining or attempts to determine percentage of Bax-positive cells on a 4-point scale. In cases where two reviews were in disagreement, a third reviewer (J. C. R.) scored the slides in a blinded fashion and the majority opinion was used for subsequent data analysis.

Statistical Methods. Overall survival and TTP were estimated using the Kaplan-Meier method, and correlations with Bax immunostaining results were calculated according to the Mantel-Cox test. Median follow-up for the 11 patients still surviving was 34.7 (range, 22.4–66.1) months. No patients were lost to follow-up (n = 19). In multivariate calculations performed by proportional hazard regression (Cox) analysis, one patient with medullary carcinoma of the breast was not included because the tumor grade could not be scored (n = 118). TTP was defined as first evidence of new lesions or progression of existing ones. The statistical significance of differences in response were assessed using the χ^2 test, where a positive response represented patients who achieved either a partial response or complete response. Responses to treatment were evaluated according to International Union Against Cancer rules (36), and have been described in detail for these particular patients (32). The significance of differences between the Bax-positive and Bax-negative groups with regard to pretreatment characteristics of the patients (menopausal status, disease sites, ascites, and number of previous cytotoxic regimens) and to other laboratory markers (p53, *HER2*, ER, PR, cathepsin D, S-phase fraction, aneuploidy, and Bcl-2) were determined using the χ^2 test in dichotomized variables. The statistical significance of differences in age, disease-free interval, and histological tumor grade were calculated using the Mann-Whitney U test.

RESULTS

The patient materials used here for evaluation of *BAX* expression were derived from archival primary tumor specimens available from women who had been enrolled previously in a two-arm study that was designed to compare the efficacy and toxicity of delivering combination chemotherapy in either 4 weekly divided doses or once a month, with the total monthly dose of drug and treatment duration being the same for the two groups (32). The results of that study indicated that the response rate was higher in the group who received therapy once a month (47% *versus* 30%; P = 0.02). The subgroup of patients treated once a month also enjoyed longer TTP (median, 9.2 *versus* 5.4 months; P = 0.005) and overall survival (median, 21.2 *versus* 11.8 months; P = 0.01), but experienced significantly more side effects such as hematological toxicity, alopecia, nausea, and vomiting. The pretreatment entry characteristics were not significantly different for these two treatment arms, with regard to patient age, menopausal status, disease sites, number of metastatic sites, previous cytotoxic treatment, and ER^- and PR^- status of tumors.

From this original study, 119 tumor specimens were successfully immunostained for Bax. Many cases contained either some residual non-neoplastic breast epithelium or carcinoma *in situ*, in addition to the infiltrating tumor cells. Normal breast epithelium exhibited intense immunostaining for Bax, 38 (97%) of 39 cases (see Fig. 1*A*). Carcinoma *in situ* was also immunostained for Bax in 41 (98%) of 42 cases. Thus, non-neoplastic epithelium and carcinoma *in situ* lesions are almost uniformly Bax positive. In contrast, Bax immunostaining of the infiltrating tumor cells was often heterogeneous, with some tumor cells containing Bax immunoreactivity at normal or near-normal intensity and others exhibiting faint or no Bax immunostaining. Although the percentage of Bax-positive cells was highly variable within the infiltrating component of tumors, the intensity of immunostaining was homogeneous in that tumor cells typically contained either strong Bax immunoreactivity, typical of normal breast epithelium and *in situ* disease, or alternatively contained no or only faint Bax immunostaining. For this reason, the specimens were scored accordingly to the approximate percentage of Bax-positive tumor cells. For the purposes of this study, Bax-negative cases were arbitrarily defined as those in which the vast majority of the infiltrating tumor cells were unstained for Bax (<10% Bax immunostained), with Bax-positive cases representing all others (*i.e.*, ≥10%). The rationale here was that complete or nearly complete loss of Bax might identify a subgroup of tumors with particularly poor responses to therapy, assuming that the presence of the proapoptotic protein Bax would help to promote cell death in chemotherapy-damaged tumor cells. Fig. 1, *C* and *D*, shows examples of a mostly Bax-negative and a mostly Bax-positive tumor, respectively.

The specificity of the antipeptide antiserum for detection of the

[3] The abbreviations used are: TTP, time to tumor progression; ER, estrogen receptor; PR, progesterone receptor.

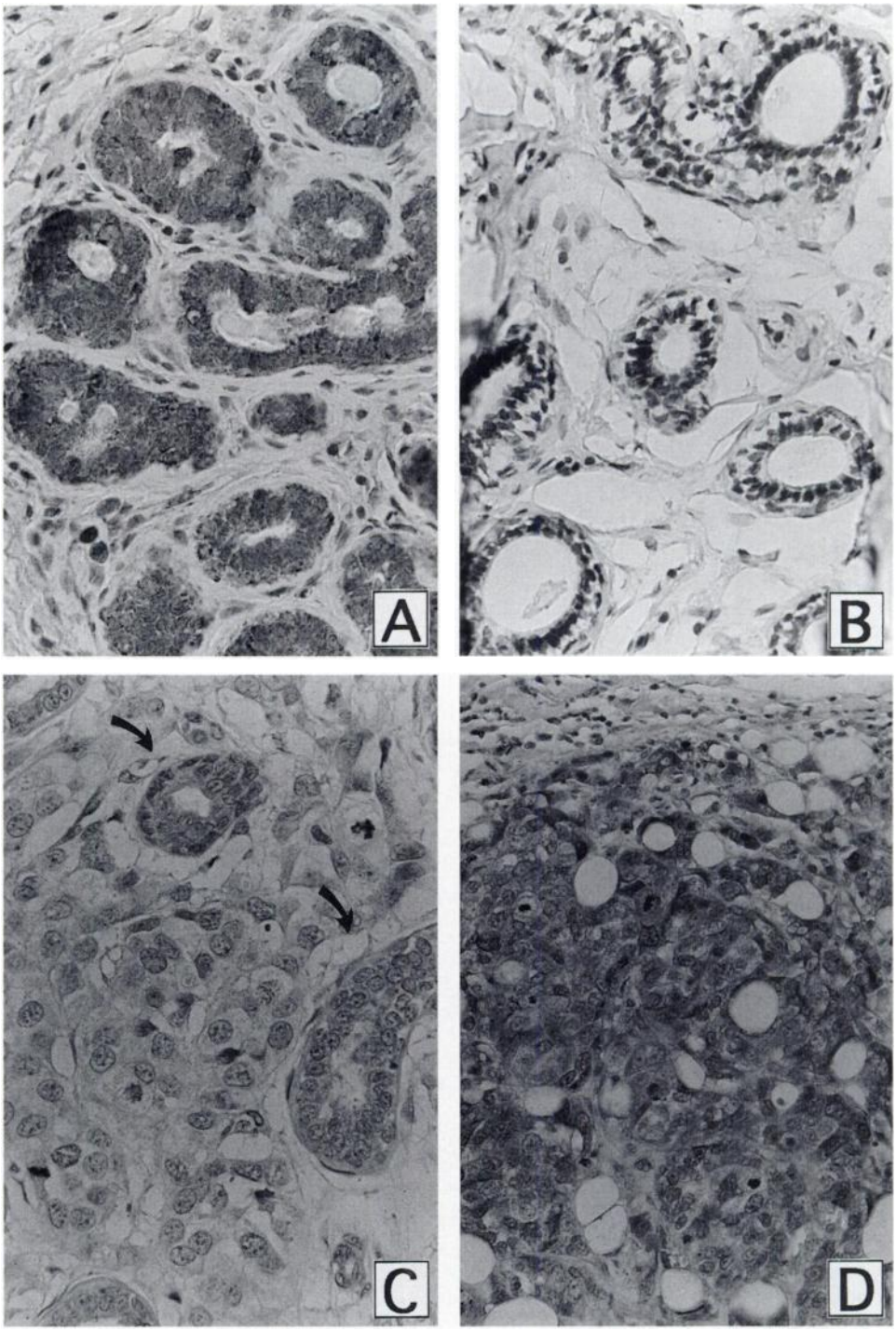

Fig. 1. Representative photomicrographs of Bax immunostaining results. Tissue sections were immunostained using anti-Bax antiserum and a diaminobenzidine based colorimetric detection system. Nuclei were counterstained with hematoxylin. *A* and *B*, normal breast immunostained in absence and presence of competing Bax peptide, respectively. Note strong immunostaining in normal breast epithelium in *A* but prevention of immunostaining in *B* by preadsorption of antibody with Bax peptide. *C*, example of Bax-negative tumor. Infiltrating tumor cells are negative for Bax, but adjacent residual non-neoplastic breast epithelium is Bax positive (*arrows*). *D*, example of Bax-positive tumor, showing strong cytosolic Bax immunoreactivity in infiltrating tumor cells.

human Bax protein was confirmed by several criteria. In immunoblot assays, for example, the antiserum revealed a single M_r 21,000 band typical of the most common form of Bax protein, p21-Bax-α. Fig. 2 shows examples of immunoblot results for the human breast cancer line MCF-7 and normal human breast tissue, as well as peripheral blood lymphocytes from a patient with chronic lymphocytic leukemia, all of which contained the p21-Bax protein. Immunoblot analysis of a variety of mouse tissues and murine cell lines indicated that this antipeptide antiserum does not react with the mouse Bax protein, thus providing an additional specificity control (Fig. 2, *Lane 3*). Further conformation of the specificity of this antiserum for detection of the Bax protein was achieved by gene transfection studies in which human cDNA encoding p21-Bax-α was expressed in the human leukemia line Jurkat. Comparisons of the resulting *BAX*-transfected and control-transfected (NEO) cell lines by immunoblot assay revealed the presence of a single M_r 21,000 band, the intensity of which was much stronger in the *BAX* transfectants relative to control transfectants (Fig. 2, *Lanes 4* and *5*). The reactivity of this antibody reagent with p21-Bax protein was also confirmed by immunoprecipitation analysis of [^{35}S]L-methionine-labeled cell lines (data not shown). Moreover, the subcellular localization of the Bax immunoreactivity in immunostained tumor cells was typical of Bcl-2 family proteins (33–35, 37), with primarily cytosolic immunoreactivity in a granular or punctate pattern, suggestive of association with intracytoplasmic

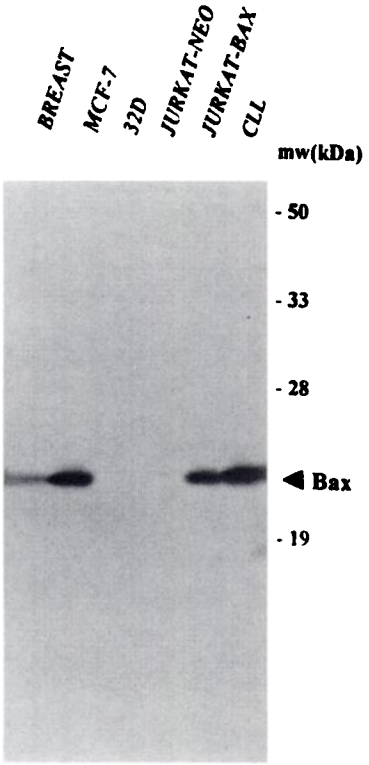

Fig. 2. Immunoblot analysis of anti-Bax antiserum specificity. Detergent lysates were prepared from cells and tissues and normalized for total protein content (50 μg/lane). Data shown represent SDS-PAGE/immunoblot data using antihuman Bax antiserum and diaminobenzine-based colorimetric detection system. Samples included (*left to right*): normal human breast tissue obtained from autopsy; MCF-7 human breast cancer line; 32D murine hempoietic cell line; Jurkat-Neo and Jurkat-Bax, a human T-cell leukemia line that had been stably transfected with negative control (Neo) or human bax expression plasmid (Bax); and peripheral blood lymphocytes from a patient with chronic lymphocytic leukemia (*CLL*). Molecular weight (*mw*) markers, kDa. *Arrow*, position of the M_r 21,000 Bax protein.

organelles, and with staining of perinuclear membranes and nuclear envelope.

Of the 119 primary breast tumor specimens successfully immunostained, 40 (34%) had reduced *BAX* expression in >90% of the cells comprising the invasive component. Table 1 compares the Bax-negative subgroups of specimens with regard to the pretreatment characteristics of the patients from whom the tumors were derived, response rate, tumor histology, and other laboratory markers. There were no statistically significant differences in patient age, menopausal status, disease sites (lymph node, lung, pleural, liver, skin, soft tissue, and visceral involvement), presence of ascites, tumor histological grade, or previous treatment between the Bax-negative and Bax-positive subgroups. The Bax-negative and Bax-positive groups also were not statistically different with regard to ER, PR, p53, *HER2*, cathepsin D, S-phase fraction, or aneuploidy. Positive Bax immunostaining, however, was strongly correlated with Bcl-2-positivity ($P = 0.005$) and also correlated with bone involvement ($P = 0.008$).

Comparisons of the response rates between the Bax-negative and Bax-positive subgroups revealed a statistically significant difference, with patients having Bax-negative tumors tending to achieve less frequently a partial response or complete response than those with Bax-positive tumors [8/39 (21%) *versus* 28/65 (43%); $P = 0.02$; Table 2]. Entry of Bax-negative patients onto the two treatment arms (weekly *versus* monthly dosing) was not significantly different [16/49 (33%) *versus* 23/55 (42%)], excluding bias in treatment approach as an explanation for the observed differences in response rates between patients with Bax-negative and Bax-positive tumors. However, when the two treatment subgroups were examined independently, Bax status correlated with the response rate in the weekly dose group of patients but not the monthly dose group. In the weekly dose group, for example, only 1 (6%) of 16 patients with Bax-negative tumors responded compared to 14 (42%) of 33 of Bax-positive cases ($P = 0.01$), (Table 2). In contrast, in the monthly treatment group, 7 (30%) of 23 patients with Bax-negative tumors responded compared to 14 (44%) of 32 Bax-positive cases ($P = 0.32$). Thus, the significance of Bax status for response rates may be dependent on the dosing schedule used.

Comparison of TTP for all patients (monthly and weekly dosing) revealed that patients with Bax-negative tumors experienced a significantly shorter TTP than those with Bax-positive tumors (median, 3.7 *versus* 9.0 months; $P = 0.02$) (Fig. 3*A*). When the weekly dose group was examined separately, a more striking difference in the Bax-negative and Bax-positive groups was observed, with median TTP being 2.0 months for the former and 6.3 months for the latter (*$P = 0.009$; Fig. 3*B). In contrast, Bax status did not reach statistical significance with regard to TTP in the monthly dose group of patients,

Table 1 *Correlation of Bax immunostaining with other patient and tumor characteristics*

Variable	BAX⁻	BAX⁺	P^a
Postmenopausal	20/39 (51%)	33/78 (42%)	0.36
Age (median yr)	56	53	0.64[b]
Disease-free interval (median, mo)	12	18	0.41
Weekly treatment 1[c]	16/40 (40%)	39/79 (49%)	0.33
Prior treatment[d]	6/40 (15%)	12/79 (15%)	0.98
Nodes (%)	10/40 (25%)	21/79 (27%)	0.85
Skin (%)	10/40 (25%)	10/79 (13%)	0.09
Bone (%)	14/40 (35%)	48/79 (61%)	0.008
Pleura (%)	8/40 (20%)	21/79 (27%)	0.43
Lung (%)	13/40 (33%)	18/79 (23%)	0.25
Liver (%)	14/40 (35%)	26/79 (33%)	0.82
Ascites (%)	3/40 (8%)	4/79 (5%)	0.59
Histological grade, high[e]	12/39 (31%)	17/79 (22%)	0.09
S-phase, high[f]	18/33 (55%)	24/46 (52%)	0.84
Aneuploidy	24/33 (73%)	26/46 (57%)	0.14
ER⁺	16/39 (41%)	37/73 (51%)	0.33
PR⁺	13/38 (34%)	29/73 (40%)	0.57
p53⁺[g]	6/40 (15%)	14/79 (18%)	0.71
Bcl-2⁺	12/40 (30%)	44/79 (56%)	0.005
HER2, high[h]	7/40 (18%)	20/79 (25%)	0.30
Cathepsin D⁺[i]	34/40 (85%)	61/77 (79%)	0.23

[a] *P* values were calculated using the χ^2 method for all correlations with Bax status, except *HER2*, histology grade, cathepsin-D, and disease-free intervals and age where a Mann-Whitney *U* test was used.

[b] Median age at time of entry into study is shown for the Bax-negative and Bax positive subgroups. *P* value calculated using the Mann-Whitney *U* test.

[c] Number of patients that received weekly divided dose therapy, as opposed to once monthly dosing.

[d] Eighteen patients had received prior chemotherapy, all with the cyclophosphamide methotrexate-fluorouacil regimen. Of these patients, 17 were treated with adjuvant therapy and 1 for documented metastatic disease.

[e] Grade 3 histology was defined as "high."

[f] Above median (stratified for ploidy; Ref. 44).

[g] Immunostaining was performed using the D07 mAb. Positive cases represent ≥10% of infiltrating tumor cells with nuclear immunostaining.

[h] Immunostaining performed with NCL-CB11 mAb (Novocastra Labs) (*HER2* high, ≥50% positive).

[i] Immunostaining was performed using mAb I-C-II (Triton Diagnostics, Inc.) and scored as positive >10% immunostained tumor cells.

Table 2 *Loss of Bax is associated with poor response rates to combination chemotherapy in women with metastatic adenocarcinoma of the breast*

Treatment Group	*n*	Bax status	Responder	% Complete response or partial response	P^a
All	104	Negative	8/39	21	0.02
		Positive	28/65	43	
Weekly	49	Negative	1/16	6	0.01
		Positive	14/33	42	
Monthly	55	Negative	7/23	30	0.32
		Positive	14/32	44	

[a] Statistical significance was calculated using the Mann-Whitney *U* test.

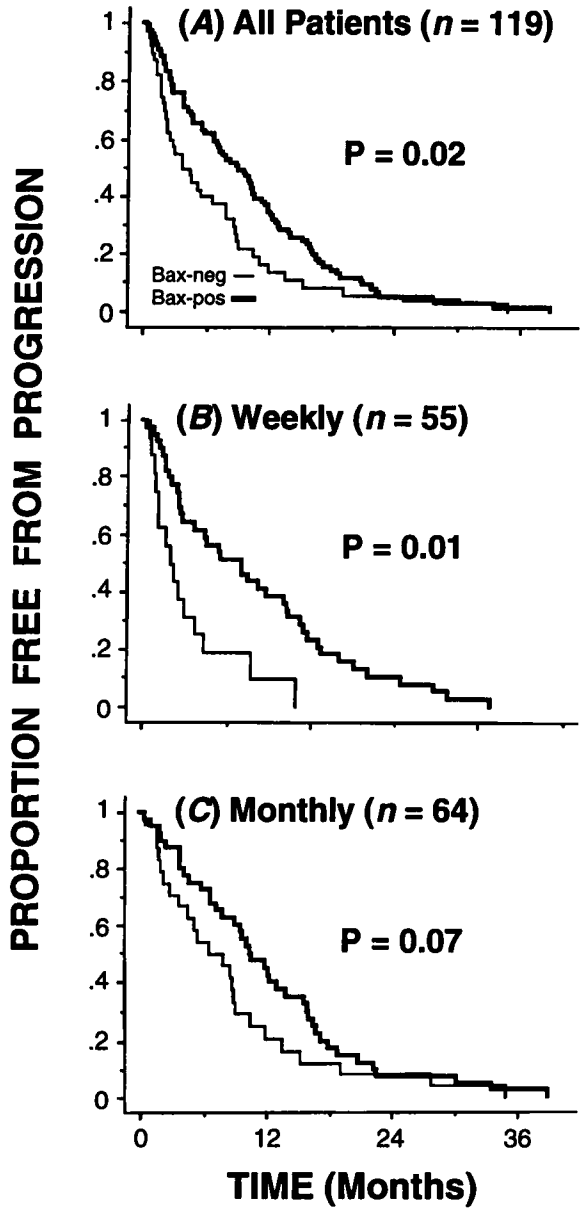

Fig. 3. Kaplan-Meier analysis of TTP in Bax-negative and Bax-positive groups. Kaplan-Meier estimates are shown for TTP in the Bax-negative and Bax-positive tumor groups for all patients (*A*); patients who received weekly divided dose therapy (*B*); and patients who received single monthly dose therapy (*C*). *P* values are indicated and were calculated using log rank analysis.

where the median TTP for the Bax-negative patients was 6.6 months compared to 10.6 months for the Bax-positive patients (P = 0.07; Fig. 3*C*).

Bax status was also statistically significant for overall survival. When all patients were combined (n = 119), median survival for patients with Bax-negative tumors was 10.7 months compared to 17.1 months for the Bax-positive tumors (P = 0.04; Fig. 4*A*). When overall survival was examined for the patients enrolled in the weekly dose arm of the study (n = 55), again patients with Bax-negative tumors died sooner than those with Bax-positive tumors (median 8.1 *versus* 15.7 months; P = 0.04; Fig. 4*B*). In the monthly dose group (n = 64), Bax status was not statistically associated with overall survival (median, 16.0 months for Bax negative versus 25.1 months for Bax positive; P = 0.09; Fig. 4*C*).

Among the various other laboratory markers analyzed for these tumors, as well as the various clinical characteristics of the patients (*e.g.*, disease sites, age, and menopausal status), only tumor grade, treatment (monthly *versus* weekly), S-phase fraction, and Bcl-2 immunostaining were also significantly correlated with either TTP or overall survival. The median TTP for patients with Bcl-2-negative tumors was 6.0 months compared to 7.4 months for patients with Bcl-2-positive tumors (P = 0.01). Moreover, differences in TTP between the Bcl-2-negative and Bcl-2-positive groups were more striking when the patients who received their chemotherapy in 4 weekly divided doses were examined separately (median TTP, 2.6 months for Bcl-2-negative *versus* 5.5 months for Bcl-2-positive; P = 0.01). Similar to the Bax immunostaining results, however, Bcl-2 status was not statistically significant for the women who received their therapy as a single monthly dose (median TTP, 8.6 months for Bcl-2-negative *versus* 9.6 months for Bcl-2-positive; P = 0.08). Histological grade (P < 0.001) and S-phase fraction (P = 0.03), however,

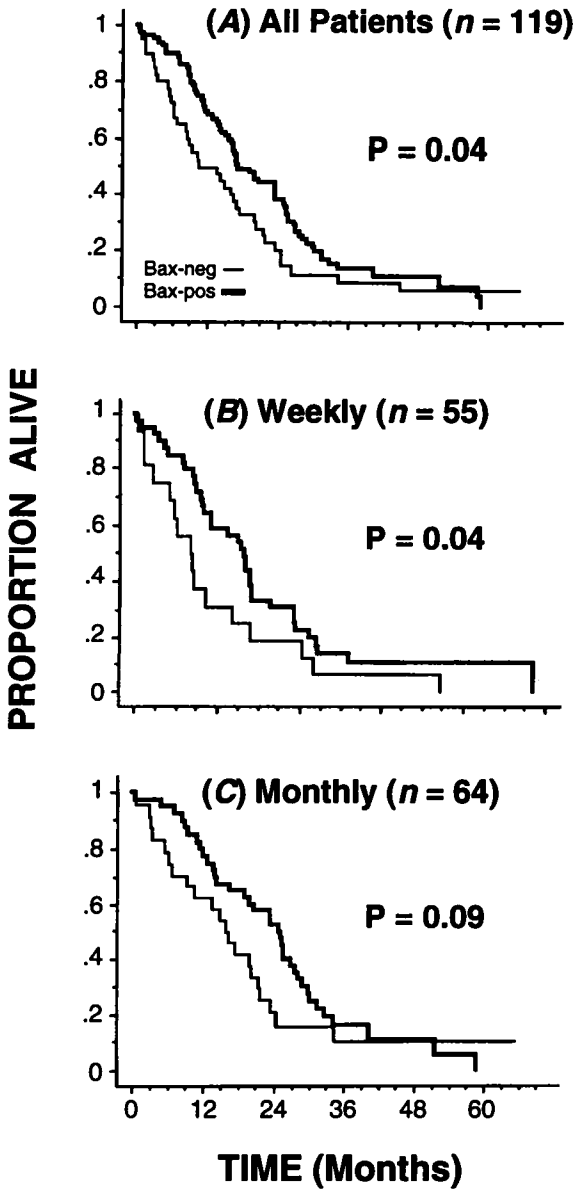

Fig. 4. Kaplan-Meier analysis of overall survival in Bax-negative and Bax-positive groups. Kaplan-Meier estimates are shown for overall survival in the Bax-negative and Bax-positive tumor groups for all patients (*A*); patients who received weekly divided dose therapy (*B*); and patients who received single monthly dose therapy (*C*). *P* values are indicated and were calculated using proportional hazards (Cox) regression method.

did correlate with TTP in the combined group of patients. Unlike Bax immuonstaining, Bcl-2 status was not of prognostic significance for response rate or overall survival, regardless of whether the entire group of patients was examined together or as separate subgroups (monthly *versus* weekly dosing) according to how chemotherapy was delivered (data not shown).

Multivariate analysis was performed using proportional hazard (Cox) regression analysis to determine the significance of Bax immunostaining when evaluated in combination with the treatment approach (weekly *versus* monthly), the only other variable that displayed significance for survival in univariate analysis ($P = 0.01$, log rank analysis). Based on this multivariate analysis, Bax remained significantly associated with overall survival ($P = 0.01$; $n = 119$). When additional variables thought to be of prognostic utility in some groups of patients were included with the Bax status in the Cox analysis (treatment, grade, and S-phase fraction), again Bax remained highly significant (data not shown). When all variables that had displayed significance for TTP in univariate analysis (Bax, tumor grade, treatment, S-phase fraction, and Bcl-2 status) were combined for multivariate calculations, again Bax was highly significant ($P = 0.02$; $n = 77$).

DISCUSSION

Bax, Bcl-2, and their homologues regulate a distal step in an evolutionarily conserved pathway for apoptotic cell death (reviewed in Ref. 1). Through *in vitro* studies where the levels of Bcl-2 protein were experimentally increased or decreased, we have previously shown that increases in the ratio of the antiapoptotic protein Bcl-2 relative to the proapoptotic protein Bax result in markedly enhanced resistance of tumor cell lines to the cytotoxic effects of essentially all currently available chemotherapeutic drugs, as well as radiation (14–19, 38). These observations prompted us to examine the prognostic significance of Bax and Bcl-2 in a group of women with metastatic adenocarcinoma of the breast for whom long-term follow-up data were available, as well as several other laboratory studies for comparisons with Bax and Bcl-2.

Normal breast epithelium and carcinoma *in situ* contained Bax immunoreactivity. In the group of 119 tumor specimens successfully analyzed here from women with metastatic breast cancer, 34% had complete or nearly complete (<10% cells positive) loss of Bax immunostaining within the tumors cells infiltrating through the stroma. These cells are presumably representative of the metastatic disease within these women, thus raising the question of whether reductions in Bax could represent a mechanism that contributes to acquisition of a metastatic phenotype, presumably by decreasing the tendency of these epithelial tumor cells to undergo apoptosis when they lose attachment with extracellular matrix proteins (39).

Reductions in Bax correlated significantly with the response rate, consistent with expectations that diminished Bax protein levels should render cancer cells relatively more resistant to induction of apoptosis by chemotherapeutic drugs. Contrary to expectations about the regulation of chemosensitivity by the ratio of Bax:Bcl-2, many Bax-negative tumors were also Bcl-2 negative ($P = 0.005$). The coordinate expression of Bcl-2 and Bax in many advanced breast cancers does not reflect an artifact in the immunostaining procedure caused by Bcl-2:Bax heterodimerization masking the epitopes recognized by our antibodies, since normal breast epithelium, carcinoma *in situ*, and some invasive cancers immunostained intensely for both Bcl-2 and Bax. Furthermore, in immunoprecipitation experiments performed using these same antibodies, we have observed coprecipitation of Bcl-2 with Bax and vice versa.[4] Several previous studies have demonstrated a correlation between loss of Bcl-2 and poor clinical outcome in women with breast cancer (11–13), an unexpected finding given the documented association *in vitro* between high levels of Bcl-2 protein and chemoresistance (14–19). The data presented here showing marked reductions in Bax expression in many tumors that also lack Bcl-2, however, suggest that Bcl-2-negative tumors may have progressed to a point where they are less dependent on Bcl-2 for their survival and imply that one of the reasons for this reduced dependence may be because of diminished Bax. Based on our previous studies, however, it is also clear that Bcl-2 is not the only antiapoptotic member of the Bcl-2 protein family normally found in breast epithelium (34, 40). Therefore, despite the absence of Bcl-2, reductions in Bax still may be biologically significant because the ratio of Bax to these other antiapoptotic Bcl-2 family proteins such as Bcl-X_L and Mcl-1 presumably has changed in tumors where loss of Bax occurred.

Previous *in vitro* studies of the effects of gene transfer-mediated elevations and antisense-mediated reductions in Bcl-2 protein levels on chemoresponses of tumor cell lines have shown that for most anticancer drugs, elevations in the Bcl-2:Bax ratio do not prevent drug-induced apoptosis but rather shift the dose-response curve to the right so that higher concentrations of drugs are required to achieve an equivalent percentage of tumor cell kill (14–19, 38, 41). This observation may explain the finding here that reduced Bax was not of statistical significance for women who received their chemotherapy once a month as opposed to those who received their treatment in four divided weekly doses. In the absence of Bax, theoretically the concentrations of drugs needed to induce a response are higher. We would speculate, therefore, that administering the chemotherapy as a single monthly dose allows drugs to surpass a critical threshold concentration that Bax-negative tumor cells can tolerate, and consequently results in essentially equivalent responses in the Bax-negative and Bax-positive groups. In this regard, it is important to note that gene transfer experiments (23) indicate that Bax is a regulator, not an effector, of the programmed cell death pathway, and thus it should be possible to induce apoptosis even in the absence of Bax provided that the apoptotic stimulus is strong enough.

In addition to response rate, loss of Bax was significantly associated with faster TTP and shorter overall survival in the weekly dose group as well as when all patients were considered in combination. Bax status was not statistically significant, however, for the group of women who were treated with the more toxic once a month dosing approach. Although highly preliminary because of the number of patients involved, reduced Bax, therefore, appears to be particularly important as a prognostic indicator for those women who received divided dose therapy, probably for the reasons discussed above regarding attaining concentrations of cytotoxic drugs necessary to negate the influence that loss of Bax has on chemoresponses. Although it is difficult to know how generally these observations can be applied to other groups of women with metastatic breast cancer, the implication of these data is that at last some women with Bax-negative tumors might benefit from more aggressive therapeutic intervention.

The mechanisms responsible for the reduced *BAX* expression in a subgroup of advanced breast cancers are currently unknown. Previously, we demonstrated that the *BAX* gene promoter contains four consensus binding site sequences for the tumor suppressor p53 and showed that p53 can transactivate the *BAX* gene promoter (29). However, our analysis of transgenic mice that contained homozygous disruptions in their *p53* genes (p53 "knockouts") indicated that p53-

[4] Unpublished data.

independent mechanisms for maintaining *BAX* gene expression also exist in some tissues (28). In this regard, no correlation was seen here between p53 immunostaining and Bax immunostaining, suggesting that p53 is not a dominant regulator of *BAX* expression in breast cancers. However, since radiation has been shown to induce p53-dependent elevations in *BAX* gene expression in some tumor lines (31), we cannot exclude the possibility of a role for p53 in transiently inducing expression of *BAX* in breast tumors where DNA damage has been induced by chemotherapeutic drugs or ionizing radiation. An additional caveat about the p53 immunostaining results is that it tends to represent a minimum estimate of tumors with loss of functional p53, since deletion of both alleles results in a negative result.

In addition to p53, Bax immunostaining did not correlate with several other laboratory markers, including ER, PR, and *HER2/NEU*, arguing against any mechanistic connections between alterations in the expression of these other genes and *BAX*. In contrast, positive Bcl-2 immunostaining did correlate with ER and PR positivity ($P = 0.003$ and 0.004, respectively). In this regard, we have obtained evidence that estrogen is a positive regulator of *BCL-2* gene expression in human breast cancer lines (42), thus suggesting a mechanism for the association of loss of Bcl-2-positivity with loss of ERs. Since loss of estrogen dependence also defines a transition point in the progression of breast tumors to an autonomous growth phase generally characterized by more aggressive disease, the implication is that these ER^- tumors have acquired genetic changes that rendered them relatively less dependent on Bcl-2 for their survival. In many cases, one of these additional alterations appears to be concomitant loss of Bax, but correlations of Bax with the presence or absence of hormone receptors did not reach statistical significance.

At present very little is available in terms of reliable prognostic indicators of response to chemotherapy and clinical outcome for women with metastatic breast cancer (reviewed in Ref. 43). Although this study involved an ethnically and racially homogeneous patient population, preliminary analysis of Bax in more diverse patient populations derived from urban centers in the United States also suggests that loss of Bax occurs in about one third of advanced adenocarcinomas of the breast.[5] To the extent that these initial data obtained from a relatively small group of Finnish women prove to be more broadly applicable where correlations of Bax status with patient response rates, TTP, and overall survival are concerned, the findings presented here raise the possibility that women with metastatic breast cancer whose tumors are Bax negative might benefit from more aggressive therapy. Therefore, this hypothesis should be tested both by analysis of larger groups of more diverse patient populations and by prospective, randomized clinical trials in which the Bax status is correlated with patient survival.

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