

# Prognostic significance of apoptosis regulators in breast cancer

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## Abstract

Dysregulation of normal programmed cell death mechanisms plays an important role in the pathogenesis and progression of breast cancer, as well as in responses of tumors to therapeutic intervention. Overexpression of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-x<sub>L</sub> has been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as Bax promote apoptosis and sensitize tumor cells to various anticancer therapies. Though the mechanisms by which Bcl-2 family proteins regulate apoptosis are diverse, ultimately they govern decision steps that determine whether certain caspase family cell death proteases remain quiescent or become active. To date, approximately 17 cellular homologs of Bcl-2 and at least 15 caspases have been identified in mammals. Other types of proteins may also modulate apoptotic responses through effects on apoptosis-regulatory proteins, such as BAG-1 - a heat shock protein 70 kDa (Hsp70/Hsc70)-binding protein that can modulate stress responses and alter the functions of a variety of proteins involved in cell death and division. In this report, we summarize our attempts thus far to explore the expression of several Bcl-2 family proteins, caspase-3, and BAG-1 in primary breast cancer specimens and breast cancer cell lines. Moreover, we describe some of our preliminary observations concerning the prognostic significance of these apoptosis regulatory proteins in breast cancer patients, contrasting results derived from women with localized disease (with or without node involvement) and metastatic cancer.

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## Bcl-2 family proteins

One of the first cell death-regulating genes to be identified was Bcl-2, an anti-apoptotic gene that appears to block a distal step in an evolutionarily conserved pathway crucial to apoptosis and programmed cell death. The Bcl-2 gene becomes overexpressed in germinal center B-cells as a result of t(14;18) chromosomal translocations that occur in >85% of follicular non-Hodgkin's lymphomas, thereby

contributing to neoplastic cell expansion by preventing normal cell turnover (reviewed in Reed 1994). Elevated levels of Bcl-2 in the absence of translocations have also been described in a variety of solid tumors, such as hormone refractory prostate cancers (Shabaik *et al.* 1994).

Bcl-2 can prevent apoptosis induction by a wide variety of stimuli, including (i) chemotherapeutic drugs and gamma irradiation in cancer cells; (ii) neurotrophic

factor withdrawal from neurons; (iii) cytotoxic cytokines such as tumor necrosis factor- $\alpha$ , Fas-ligand, and transforming growth factor- $\beta$ ; (iv) heat shock; (v) calcium ionophores; and (vi) chemicals that induce oxidative injury (reviewed by Reed 1994, Zamzami *et al.* 1998). The ability to prevent or delay cell death triggered by multiple mechanisms has suggested that the Bcl-2 protein controls a late event in a final common pathway involved in programmed and apoptotic cell death.

Of note with regards to Bcl-2 function are the intracellular locations of the Bcl-2 protein, which predominantly include the nuclear envelope and outer mitochondrial membrane, but also possibly at the nuclear pore complexes and mitochondrial junctional complexes where the inner and outer membranes of these DNA-containing organelles make contact, as well as some parts of the endoplasmic reticulum (Krajewski *et al.* 1993). The COOH-terminal hydrophobic domain of Bcl-2 is important for membrane anchorage, but its deletion does not entirely abrogate Bcl-2 survival function, nor does it completely abolish the ability of Bcl-2 to associate with membranes.

At least 17 Bcl-2 family members have been identified in mammalian cells, with several others found in viruses (reviewed in Reed 1998b). All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) (Oltvai *et al.* 1993, Reed 1994). Most anti-apoptotic members contain all four of these domains. Some pro-apoptotic Bcl-2 family proteins possess only the BH3 domain, which is essential for their function (Zha *et al.* 1996).

Relevant to the function of several members of the Bcl-2 family is their ability to homo- and heterodimerize with each other (Oltvai *et al.* 1993, Sato *et al.* 1994, Sedlak *et al.* 1995). The three-dimensional structure of the Bcl-x<sub>L</sub> protein bound to an inhibitory domain from the pro-apoptotic protein Bak suggests that BH3 domains of pro-apoptotic Bcl-2 family proteins function as peptide ligands that insert into a hydrophobic cleft or pocket on the surface of Bcl-x<sub>L</sub> (Muchmore *et al.* 1996, Aritomi *et al.* 1997). Some pro-apoptotic Bcl-2 family proteins preferentially dimerize with subsets of the pro-survival members. For instance, Bok interacts with Mcl-1 and BHRF1 but not with Bcl-2, Bcl-x<sub>L</sub> or Bcl-W. These different preferences for dimerization partners imply that tissue-specific expression of various Bcl-2 family members can be an important determinant of whether a suitable dimerization partner is or is not present within a given cell.

Some Bcl-2 family proteins also possess dimerization-independent functions. For example, Bcl-x<sub>L</sub> has been reported to bind the CED-4-like domain of Apaf-1, a protein that binds to and activates pro-caspase-9 (Li *et al.* 1997, Chou *et al.* 1998, Pan *et al.* 1998). Bcl-x<sub>L</sub> may

inhibit the association of Apaf-1 with pro-caspase-9 and thereby prevent caspase-9 activation (Zou *et al.* 1997, Hakem *et al.* 1998) or it may form a ternary complex with Apaf-1 and pro-caspase-9 that prevents caspase activation (Pan *et al.* 1998). In addition to possibly regulating Apaf-1, Bcl-2 family proteins are also capable of performing caspase-independent functions that control cell life and death. These caspase-independent functions are probably related to the apparent structural similarity of some types of Bcl-2 family proteins to the pore-forming domains of bacterial toxins (Reed 1997b, Schendel *et al.* 1997). Some pro-apoptotic members of this family such as Bax and Bak, for example, appear to induce alterations in mitochondrial permeability barrier function by inserting into the mitochondrial membrane and triggering release of caspase-activating proteins from these organelles, particularly cytochrome c, which is a necessary cofactor for Apaf-1 (Wolter *et al.* 1997, Eskes *et al.* 1998). Other biochemical functions for Bcl-2 family proteins have also been described, which may be important in some cellular contexts, suggesting that Bcl-2 and several of its homologs are multifunctional proteins (Reed & Krajewski 1998, Wang & Reed 1998).

Interestingly, several anti-apoptotic Bcl-2 family proteins not only block cell death, but also have an inhibitory effect on cell proliferation (Borner 1996, Reed *et al.* 1996). The explanation for this phenomenon remains unknown, but mutagenesis studies indicate that the anti-apoptotic and anti-proliferative actions of Bcl-2 can be dissociated (Reed 1996a, 1998).

## Studies of Bcl-2 family expression in breast cancer

Expression of the Bcl-2 gene is regulated by estrogens in mammary epithelial cells and estrogen receptor (ER)-positive breast cancer cell lines (Johnston *et al.* 1994, Barbareschi *et al.* 1996, Zapata *et al.* 1998). Expression of the Bcl-2 protein has been detected by immunohistochemical methods in ~80% of breast cancers derived from women with primary tumors and having either node positivity or negativity (Gee *et al.* 1994, Krajewski *et al.* 1995a). Bcl-2 immunostaining has been correlated with ER and progesterone receptor (PR) positivity in several independent studies (Leek *et al.* 1994, Silvestrini *et al.* 1994). Surprisingly, statistical analysis revealed that Bcl-2-positive patients had better prognosis and an overall better survival rate, compared with Bcl-2 negative (Joensuu *et al.* 1994, Silvestrini *et al.* 1996, Zhang *et al.* 1998). These studies have included groups of uniformly treated women with node-negative, node-positive, or metastatic disease, confirming an association of Bcl-2 with favorable prognosis even in multivariate analyses

that attempted to correct for other variables (Silvestrini *et al.* 1994, Krajewski *et al.* 1995a, Elledge *et al.* 1998).

Several possible explanations for these seemingly paradoxical results can be envisioned (Reed 1996a). Among these possible explanations are: (i) inhibitory effects of Bcl-2 on cell proliferation; (ii) regulation of Bcl-2 expression by estrogen (Huang *et al.* 1997); and (iii) the presence of Bcl-2 antagonists, which negate its cytoprotective function (Krajewski *et al.* 1995a). Nevertheless, in controlled experiments, gene transfection of Bcl-2 into breast cancer cell lines has uniformly resulted in enhanced resistance to apoptosis (Pratt *et al.* 1998), implying that Bcl-2 is indeed capable of suppressing cell death in these cells, at least under some circumstances.

An inverse relationship between Bcl-2 and p53 immunopositivity in breast cancers has been reported in several studies where immunostaining results were evaluated as dichotomous variables employing arbitrary cut-off percentage points (>0.5% for p53 and >20% for Bcl-2). We therefore undertook a detailed comparison of p53 and Bcl-2 in a collection of 135 breast cancers having between 0.5 and 95% nuclear p53 immunopositivity (Krajewski *et al.* 1997c). Evaluating the data as continuous variables, we confirmed a strong inverse relationship between expression of Bcl-2 and p53 immunopositivity ( $P=0.004$ ), suggesting that mutations in p53 are somehow related to regulation of Bcl-2 gene expression in breast cancers. Consistent with these findings, Haldar *et al.* (1994) reported that transfection of mutant p53 into a p53-wild-type breast cancer cell line suppressed expression of Bcl-2. However, in some types of cells, p53 has the opposite effect on Bcl-2 expression (Liukkonen *et al.* 1997, Pettaway 1998), implying a great deal of tissue specificity in the interaction of p53 with Bcl-2.

### Bax as a tumor suppressor gene

The discovery of inactivating mutations within the Bax gene of tumors has suggested that Bax may function as a tumor suppressor. Rampino *et al.* (1997) observed frame-shift mutations within a homopolymeric stretch of eight consecutive guanines (G8) in more than 50% of colon cancers having the microsatellite mutator phenotype (MMP+). Such mutations were absent in MMP-tumors. A similar situation exists for MMP+ gastric cancers (Yamamoto *et al.* 1997, 1998). Studies of Bax-deficient mice (knock-outs) also support the concept of Bax as a tumor suppressor gene (Knudson *et al.* 1995).

At present, no evidence exists that Bax frame-shift mutations occur in breast cancers. Nevertheless, an apparent loss of Bax expression has been reported in some breast tumors. For example, in collaboration with Blomqvist and colleagues, we performed an immuno-

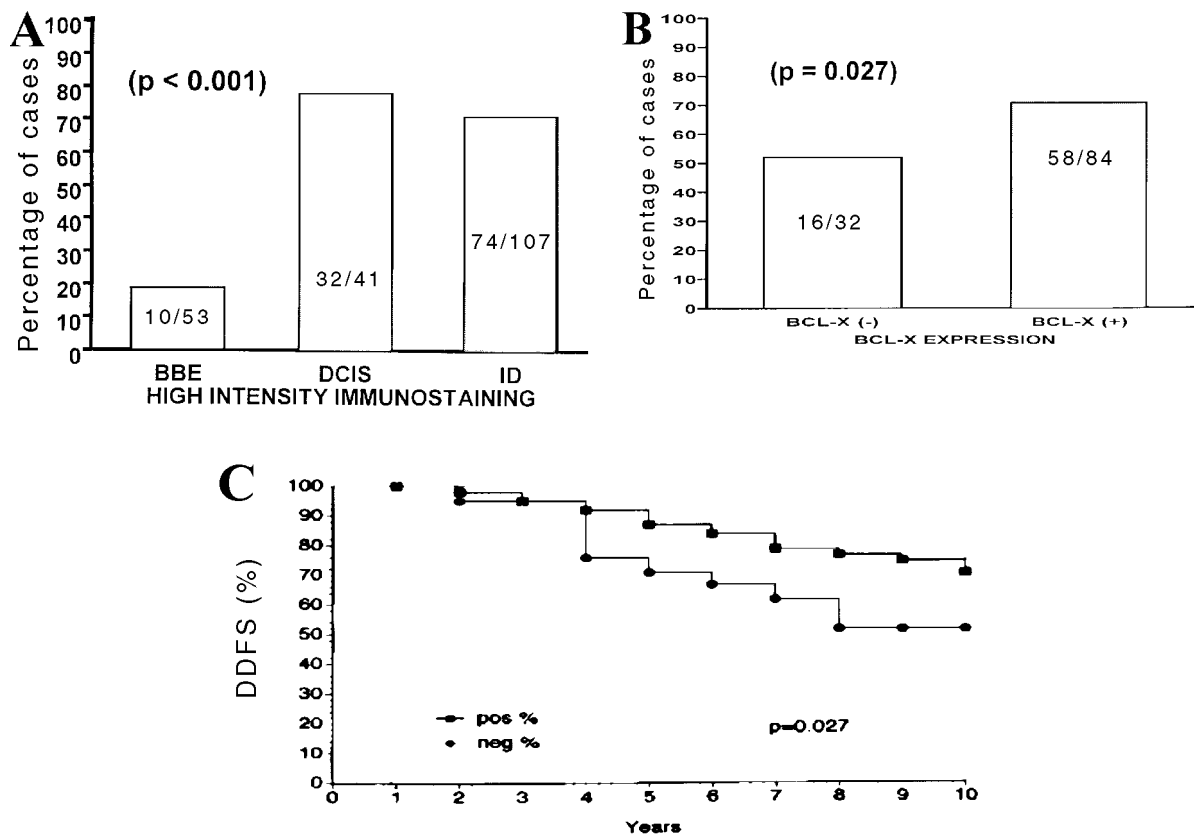
histochemical analysis of Bax expression in primary tumors derived from 119 women with metastatic breast cancer (Krajewski *et al.* 1995a). These studies revealed expression of Bax in normal mammary epithelium and most carcinomas *in situ*. However, apparent loss of Bax expression (<10% immunopositive) was seen in roughly one-third of specimens. Bax immunostaining was not significantly correlated with p53, HER2, cathepsin D or S-phase fraction, but was positively associated with Bcl-2. More importantly, reduced Bax correlated with shorter overall survival (OS), faster time to tumor progression, and failure to respond to therapy. Therapy for these patients with metastatic disease consisted of 5-fluorouracil, epirubicin and cyclophosphamide, administered with either single monthly dosing or in weekly divided doses. The findings suggest that loss of Bax immunostaining represents a prognostic indicator of poor response to therapy and reduced survival in women with metastatic breast cancer who are treated with combination chemotherapy. Moreover, the correlation was strongest for patients who received the divided dose regimen, implying that more intensive chemotherapy may be advisable for women whose tumors are Bax immunonegative (Krajewski *et al.* 1995a, Kapranos *et al.* 1997).

The relationships between Bax and the tumor suppressor p53 in human breast cancer were also examined in this same cohort as well as in the aforementioned study of 135 p53-positive breast cancers (Krajewski *et al.* 1997c). Previous to this study, it had been shown that wild-type p53 can bind directly to the Bax gene promoter and induce its transcriptional activation, at least in some types of permissive cells (Miyashita & Reed 1995). However, in breast cancers, no correlation between the percentages of Bax- and p53-immunopositive tumor cells was observed when examined as either dichotomous or continuous variables (Veronese *et al.* 1998).

In contrast to breast cancer patients with metastatic disease who were treated with chemotherapy, significant correlations of Bax immunostaining and outcome have not been observed for patients with localized disease. Among the studies we have performed on this topic is a case-controlled study of 50 women with node-negative early-stage tumors who experienced local or distant recurrence after lumpectomy and local radiation, compared with 50 similar patients who did not relapse (Turner, personal communication). Thus, the prognostic significance of Bax in breast cancer may be limited to settings where chemotherapy is involved in the treatment of the patients.

### Expression of Bcl-x and Mcl-1 in breast cancers

Bcl-x<sub>L</sub> and Mcl-1 are anti-apoptotic members of the Bcl-2 protein family which have been implicated in cancer



**Figure 1** Summary of the statistical analysis of Bcl-x immunocytochemical expression data. Panel A demonstrates significant upregulation of this apoptosis antagonist in ductal carcinoma *in situ* (DCIS) and/or invasive tumor (ID) versus normal/benign breast epithelium (BBE). Using 20% cut-off in patients with 10 years or longer survival (panel B), the analysis of overall survival (OS) and distant disease free survival I (DDFS) showed positive correlation between Bcl-x positiveness and DDFS (panel C).

chemoresistance (Reed 1997a). The genomic organization, promoter region and chromosomal localization of the mouse Bcl-x gene have been described recently by Grillot *et al.* (1997). The remarkable structural similarity between the Bcl-2 and Bcl-x genes suggests that they have evolved from a common ancestral gene by way of gene duplication. Through alternate splicing, Bcl-x can encode several proteins, including the Bcl-x<sub>L</sub> (long) protein which inhibits apoptosis and a shorter isoform Bcl-x<sub>S</sub> which promotes apoptosis by acting as a trans-dominant inhibitor of Bcl-2 and Bcl-x<sub>L</sub>. *In vivo*, Bcl-x<sub>S</sub> is expressed at high levels in only occasional types of cells, such as developing thymocytes, which are known to undergo a high rate of turnover. By contrast, Bcl-x<sub>L</sub> is ubiquitously expressed (Krajewski *et al.* 1994b, 1997a). The expression of Bcl-x has been described in a variety of normal and pathological tissues. With regards to cancers, Bcl-x expression was observed in low-grade gliomas and neuroblastomas

(Krajewski *et al.* 1997b). Overexpression of Bcl-x<sub>L</sub> in human gastric adenocarcinomas and primary colorectal adenocarcinomas has also been reported (Krajewska *et al.* 1996a,c), as well as increases in Bcl-x immunostaining in advanced prostate cancers (Krajewska *et al.* 1996b).

Mcl-1 is the largest of the known Bcl-2 family proteins. This protein contains several PEST sequences that appear to render the protein relatively short-lived (Bodrug *et al.* 1995). Expression of Mcl-1 has not been explored extensively in cancers to date. However, higher levels of Mcl-1 protein have been correlated with failure to achieve complete remission among patients with B-cell chronic lymphocytic leukemia (Kitada *et al.* 1998).

Recently, we characterized the expression of Bcl-x<sub>L</sub> and Mcl-1 in 116 early-stage breast adenocarcinomas by immunohistochemical assays. Mcl-1 immunostaining data, contrary to Bcl-x findings, were not significant in this cohort of patients. The Bcl-x immunostaining was

**Table 1** Clinical-pathological characteristics of the breast cancer specimens used in the study of Bcl-2 family members and BAG-1

Clinical-pathological data	Cases (No. (%))
Number of patients	116
Median age (years)	52
Intraductal	6 (5.2)
Infiltrating ductal and intraductal	32 (27.6)
Infiltrating ductal cancer	68 (58.6)
Infiltrating lobular cancer	5 (4.3)
Medullary cancer	3 (2.6)
Mucinous (colloid)	1 (0.9)
Adenocarcinoma (NOS)	1 (0.9)
Stage I/II	116 (100)
Mean pathologic size (cm)	1.8
Axillary dissection	63 (53)
Positive lymph nodes	13 (20)
Estrogen receptor positive	50 (67)
Adjuvant chemotherapy	20 (16.4)
Metastatic disease	35 (29)
Median follow-up (years)	14.0
Median survival (years)	9.5

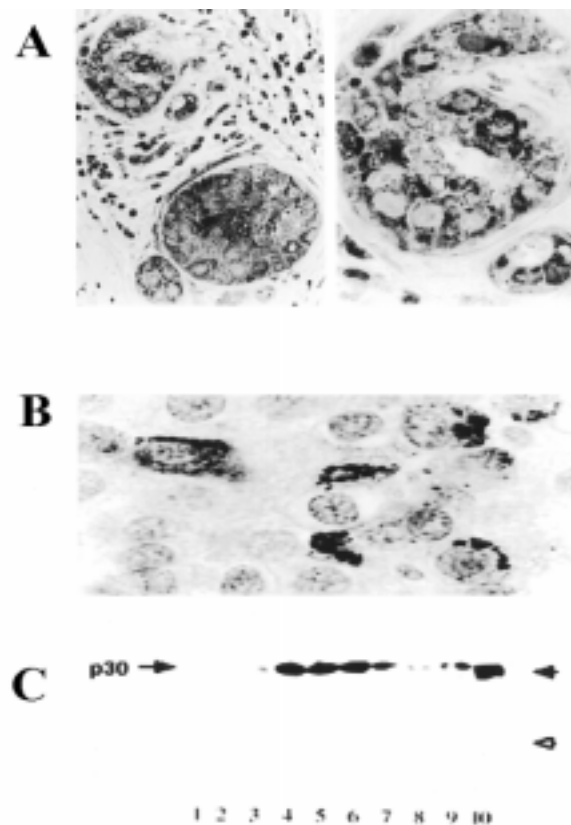
NOS, not otherwise specified.

performed in duplicate using a polyclonal anti-peptide rabbit antiserum that reacts with both Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub> (Krajewski *et al.* 1994a,b, 1995b), and a Bcl-x<sub>L</sub>-specific monoclonal antibody recently generated in our laboratories. The immunostaining results were arbitrarily scored according to intensity as 0=negative; 1=weak; 2=moderate; 3=strong; and 4=very strong. The intensity of the immunostaining was also evaluated in side-by-side comparisons of invasive cancer and carcinoma *in situ* with adjacent areas of normal mammary epithelium, scoring the intensity as greater than, less than, or equal to normal adjacent mammary epithelium (T>N; T<N; T=N). The approximate percentages of immunopositive cells were scored separately for the two components: *in situ* and invasive cancer. The population base for this study comprised 145 patients with stage I or II breast cancer treated with lumpectomy and radiation therapy to the intact breast at Yale-New Haven Hospital between 1973 and 1993. From this population, 116 cases were randomly chosen for analysis (Table 1).

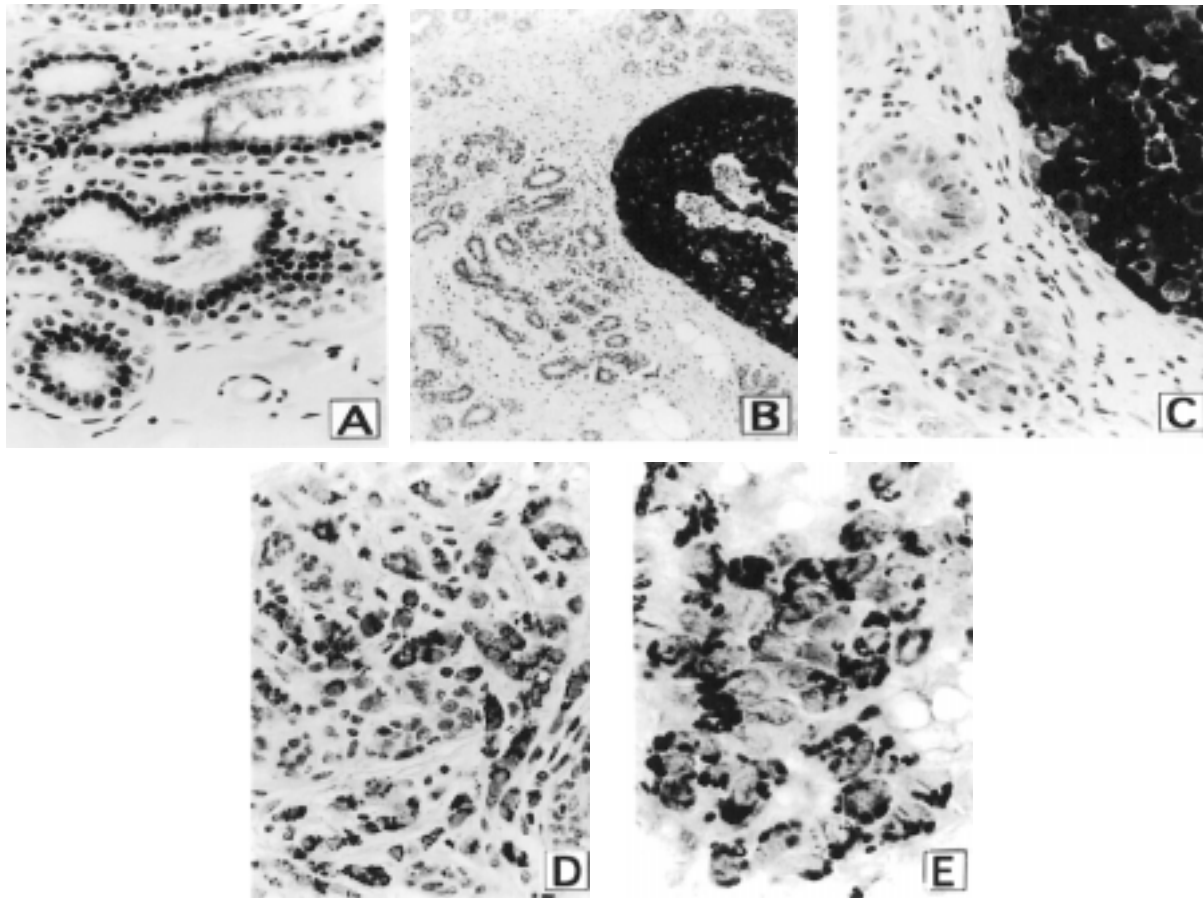
Analysis of Bcl-x<sub>L</sub> expression in normal and malignant breast tissue revealed a marked difference between normal and cancer epithelia: weak expression of Bcl-x<sub>L</sub> was observed in normal epithelial cells, whereas strong immunostaining was found in the malignant cells in ~75% of specimens evaluated ( $P<0.001$ ; Fig. 1). Figure 2 demonstrates examples of Bcl-x immunostaining of *in situ* and invasive ductal carcinoma. Similar results were obtained with both the Bcl-x<sub>L</sub>-specific polyclonal

antiserum (Fig. 2A) and the monoclonal antibody Bcl-x<sub>L</sub>/P45A5 (Fig. 2B), implying that Bcl-x<sub>L</sub> is the isoform of Bcl-x predominantly expressed in breast tissue. This presumption has been confirmed by immunoblot analysis of randomly selected breast surgical biopsies (Fig. 2C) and over ten breast cancer cell lines (Zapata *et al.* 1998).

Similar to prior studies of Bcl-2, immunostaining for Bcl-x<sub>L</sub> was positively correlated with ER ( $P=0.046$ ) and PR ( $P=0.027$ ). In contrast, Bcl-x was not significantly correlated with c-erbB2, p53, cyclin D1 or insulin-like



**Figure 2** Typical examples of high Bcl-x immunoreactivity in ductal carcinoma *in situ* stained with rabbit polyclonal antibody and Avidin-Biotin Peroxidase complex (ABC)/ 3,3'-Diaminobenzidine (DAB) detection method (A:  $\times 200$  and  $\times 400$  respectively). (B) Demonstrates invasive ductal carcinoma with an organellar/mitochondrial pattern in the cytosol of tumor cells with the use of mouse hybridoma clone Bcl-x-L/P45A5 ( $\times 1000$ ). The immunoreactivity in breast tumors was associated exclusively with the long form of Bcl-x protein, as demonstrated in (C) in tissue lysates prepared (lanes 3-9) from surgical biopsies and subjected to SDS-PAGE/immunoblot assay using 0.1% (v/v) rabbit anti-Bcl-x antiserum. Note the absence of Bcl-x<sub>S</sub> and the variable amounts of Bcl-x<sub>L</sub> expression in tumors, compared with recombinant protein loaded in lane 10. The open arrowhead indicates the expected position of Bcl-x<sub>S</sub>.



**Figure 3** Overview of BAG-1 expression patterns in normal/benign breast epithelium (A) and adenocarcinoma. (B-E) In normal mammary epithelium most of the epithelial cells exhibit strong, specific nuclear immunostaining with anti-BAG-1 monoclonal antibody (A:  $\times 200$ ). (B and C) *In situ* lesions were very strongly immunoreactive with both nuclear and cytoplasmic localization of BAG-1 (B  $\times 200$ ; C  $\times 400$ ). (D and E) Invasive carcinoma cells were typically stained in the cytoplasm only, in either diffuse or granular/organellar patterns with no, or rarely positively labeled, nuclei in the entire tumor (negative  $<20\%$ ; D and E  $\times 200$  and  $\times 400$  respectively).

growth factor receptor. Interestingly, contrary to *a priori* expectations, high levels of Bcl- $x_L$  immunostaining were associated with longer distant disease free survival (DDFS) (Fig. 1), at least by univariate analysis. In multivariate analysis, however, Bcl- $x_L$  lost its significance with DDFS. Bcl- $x_L$  was not associated with OS in this cohort of early-stage breast cancers treated with lumpectomy and local radiation. In the future, it will be of interest to evaluate Bcl- $x_L$  in other study populations, particularly patients with metastatic disease treated with chemotherapy.

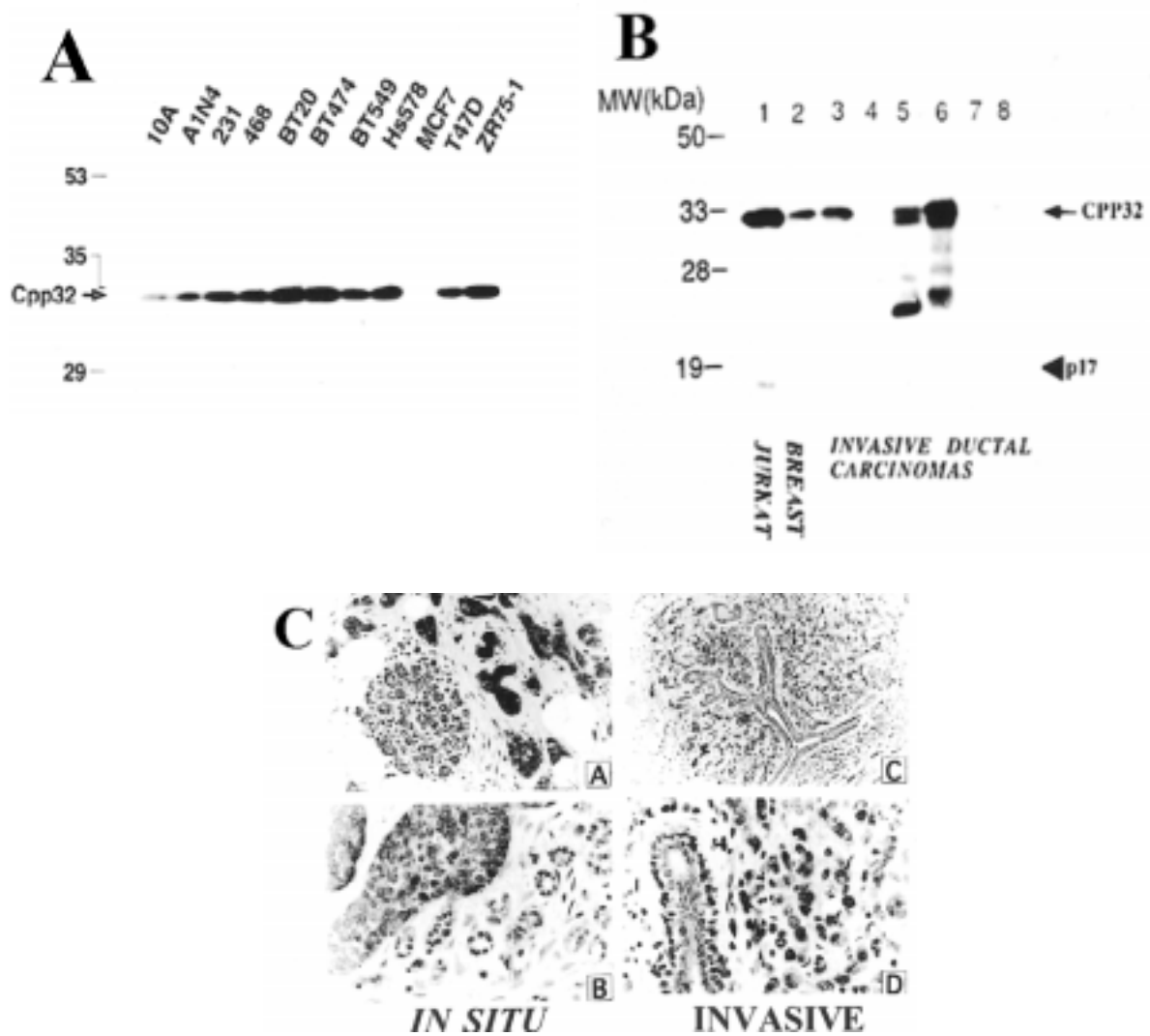
### BAG-1 expression in breast cancers

BAG-1 is a multifunctional protein that blocks apoptosis and interacts with several types of proteins, including

Bcl-2 (Takayama *et al.* 1995), the kinase RAF-1 (Wang *et al.* 1996), certain tyrosine kinase growth factor receptors (Zeiner & Gehring 1995, Bardelli *et al.* 1996), Siah family proteins (Matsuzawa *et al.* 1998), and steroid hormone receptors (Froesch *et al.* 1998), apparently by virtue of its ability to bind and regulate the Hsp70/Hsc70 family of molecular chaperones (Takayama *et al.* 1998). Three isoforms of human BAG-1 protein have been described, including BAG-1, BAG-1M (Rap46/Hap46), and BAG-1L. The BAG-1 protein is primarily cytosolic, whereas BAG-1M and BAG-1L are generally nuclear in their locations (Takayama *et al.* 1998). In addition, cytosolic BAG-1 can be found associated with intracellular membranes and mitochondria in some types of cells. Moreover, transfection of Bcl-2 has been reported to cause

redistribution of BAG-1 to intracellular membranes (Takayama *et al.* 1998). All three of these BAG-1 isoforms bind Hsc70. Overexpression of BAG-1 by gene transfection has been reported to block or delay apoptosis induced by multiple stimuli (Takayama *et al.* 1997). It may be relevant in this regard that overexpression of Hsc70 has recently been shown to prevent apoptosis at a very distal step in the pathway through an unclear mechanism

(Jääteelä *et al.* 1998). BAG-1 can also render some types of cells independent of growth factors for their proliferation and survival in culture (Clevenger *et al.* 1997) and can enhance metastatic spread of tumor cells in animal models. BAG-1L co-immunoprecipitates with androgen receptor (AR) in prostate cancer cells, increasing resistance to anti-androgen drugs and promoting ligand-independent AR activation (Froesch *et*



**Figure 4** Analysis of Cpp32/Caspase 3 immunoreactivity in tissue culture and biopsy specimens. Lysates from representative breast cancer cell lines (A), normal mammary tissue and surgical specimens from primary tumors (B), normalized for total protein content (50 µg) were subjected to SDS-PAGE (12%) immunoblotting. Only the MCF7 cell line on A and three human specimens on B were negative. Among the latter, specimens in lanes 4 and 7 contained only adipose tissue and in lane 8 there was no residual tumor, only normal mammary epithelium confirmed by H&E. Note upregulated levels of Cpp32 proform in most of the invasive breast cancers (lanes 3, 5 and 6) in comparison to normal breast (lanes 2 and 8). These data are in agreement with immunocytochemistry presented on C(A-D), where higher Caspase 3 immunoreactivity was found in both in situ (C(A), x 150; C(B), x 400) and invasive adenocarcinoma (C(C) and C(D), x 100 and x 400 respectively).

*al.* 1998). Though BAG-1M has been reported to bind ER *in vitro* (Zeiner & Gehring 1995), the biological significance of this interaction remains unexplored to date.

Using a monoclonal antibody that we generated against the BAG-1 protein and which recognizes all three of the known BAG-1 isoforms (Takayama *et al.* 1998), we examined the expression and intracellular location of BAG-1 proteins in early-stage breast cancers (Turner *et al.* 1997). Cytosol and nuclei were immunopositive in approximately 70-84% of normal mammary epithelium (Fig. 3A). By contrast, in 41% of carcinomas *in situ* (Fig. 3B and C) and in 93% of invasive cancers (Fig. 3D and E), BAG-1 immunoreactivity was mostly in a cytoplasmic location and of 1 or 2+ intensity or missing. Invasive cancers in 89% were characterized by lack of nuclear staining and variable amounts of cytosolic/organellar immunoreactivity. Interestingly, the presence of BAG-1 in tumor cells positively correlated with Bcl-2 and Bcl-x expression ( $P=0.012$ ). Higher levels of BAG-1 nuclear immunostaining (>20%) correlated with longer OS among this group of patients with early-stage disease ( $P<0.001$ ), suggesting that BAG-1 may serve as a novel prognostic marker in breast cancer.

Why lower levels of BAG-1 should be associated with neoplastic transformation in the breast is unclear. However, one potential explanation may reside in the recent discovery of an entire family of BAG family proteins, BAG-1, BAG-2, BAG-3, BAG-4 and BAG-5 (Takayama *et al.* 1999). Conceivably, the need for BAG-1 may be supplanted by alterations in the expression of other members of this family of Hsp70/Hsc70 molecular chaperone regulators.

## Breast cancer and caspases

To gain insights into the role of caspases in the pathogenesis of breast cancer, we recently analyzed the patterns of caspase-3 immunoreactivity in the normal and malignant breast. The principal intracellular effectors of apoptosis are a family of cysteine proteases with homology to interleukin-1 $\beta$  converting enzyme and the nematode cell death protease, CED-3. These proteases have specificity for aspartic acid in the P1 position, and exist as inactive zymogens in cells, becoming proteolytically activated by cleavage at particular aspartic acid residues, thereby generating active subunits with molecular masses typically of 17-20 kDa and 10-13 kDa (Fig. 4A and B). These subunits assemble into an enzymatically active heterotetramer. To date, at least 15 members of the caspase family of proteases have been identified in mammals. Most of these proteases induce apoptosis when overexpressed in mammalian cells by gene transfer methods. Caspase-3 represents a prominent member of this family of proteases which operates in the distal portion of convergent proteolytic cascades, serving

as an effector of apoptosis by cleaving critical protein substrates required for apoptotic cell death.

We were inspired to evaluate the expression of caspase-3 in breast cancers by our observation that MCF7 breast cancer cells fail to express caspase-3, as determined by immunoblotting, while several other breast cancer lines do contain caspase-3 (Fig. 4A). Using immunocytochemical assays and archival paraffin-embedded primary tumors, we analyzed 56 cases of ductal breast cancer derived from the Ottawa University Hospital. Surprisingly, caspase-3 immunointensity was higher in 86% of the breast cancers, both *in situ* and in infiltrating adenocarcinoma, when compared with the adjacent normal mammary epithelium present in the same specimens ( $P=0.005$ ; Fig. 4C(A-D)). In no case was caspase-3 immunoreactivity absent from breast cancers. Since reduced apoptosis is generally associated with tumorigenesis, our data suggest a scenario in which high levels of caspase-3 pro-form are present, but perhaps fail to become activated to participate in apoptosis. Conceivably, for example, breast cancers could express high levels of caspase-3 inhibitors such as IAP family protein, XIAP, c-IAP-1, or c-IAP-2, thus explaining their ability to tolerate high levels of caspase-3 (Deveraux *et al.* 1997, 1999, Roy *et al.* 1997). It is possible that caspases play other roles in cellular physiology besides cell death induction, and that higher levels of caspase-3 somehow are a net benefit within the context of breast cancer cells.

## Conclusions

Investigations of apoptosis proteins in breast cancers reveal some unexpected results. Expression of Bcl-2, for example, is commonly associated with favorable prognosis in breast cancer. Though the reason for this remains unknown, the anti-proliferative effect of Bcl-2 may be involved. Alternatively, the observation that expression of Bcl-2 is estrogen-inducible in mammary cells (Pratt *et al.* 1998) suggests that the presence of Bcl-2 may represent a fortuitous marker of tumors that have arisen by a less aggressive genetic pathway involving a dependence on steroid hormones. In this regard, though immunohistochemical or ligand-binding assessments of ER and PR in breast cancers are valuable, they do not assess the actual transcriptional activity of these nuclear hormone receptors. Regulation of ER and PR activity is highly complex, influenced by many other proteins in ways that are only partially understood. Expression of Bcl-2 in neoplastic mammary epithelial cells may provide a surrogate marker that reflects the functional status of these or other hormone receptors in mammary cancers. Consequently, Bcl-2 immunopositivity may connote a more treatable form of breast cancer, thus accounting for its association with longer survival among women with



node-negative and node-positive breast cancer as well as in women with metastatic disease.

Compared with Bcl-2, far less is known about the expression of other apoptosis proteins in breast cancer. Our preliminary results indicate that Bax is not of prognostic significance in early-stage patients treated with surgery and local radiation therapy. However, Bax may be of prognostic utility for patients with metastatic disease who are treated with combination chemotherapy, presumably because of its role in promoting apoptosis in response to genotoxic injury induced by anticancer drugs. Both the agonist of apoptosis, Bak, expressed mainly in myoepithelial cells in benign breast tissue, and the antagonist, Mcl-1, showed no significant correlation between tumor progression and the clinical outcome in studied cases.

Bcl-x<sub>L</sub> expression is commonly elevated in breast cancers, suggesting a role for this protein in the pathogenesis of mammary tumors. Similar to Bax, immunohistochemical assessment of Bcl-x may not be of strong prognostic significance for patients with early-stage disease who are treated with surgery and local radiation. However, Bcl-x expression has not been evaluated in patients who received anti-hormonal or chemotherapy, where a higher level of this anti-apoptotic protein would be anticipated to be associated with poor outcome.

Though highly preliminary, BAG-1 immunostaining may provide prognostic information for patients with early-stage cancers with higher levels of BAG-1 being associated with longer survival. Of particular interest is the assessment of the nuclear expression of BAG-1 in breast cancer prior to, and during, therapies, which could be correlated with less malignant phenotypes of mammary tumors. The biological rationale for an association between BAG-1 and improved outcome is unclear. Similar to Bcl-2, it is conceivable that BAG-1 expression is reflective of the origin of breast cancers from a genetic pathway that results in less aggressive tumors.

Because the full repertoire of genetic and epigenetic alterations that lead to breast cancer is presently unknown, it would be inappropriate to infer a great deal of functional significance from evaluation of any one gene product. Indeed, since most apoptosis genes exist as large families with enormous potential for redundancy, it is somewhat surprising that immunohistochemical assessment of any single gene product within highly regulated and complex apoptosis pathways can be of prognostic significance. Regardless of the biological reasons for the associations of particular changes in the expression of apoptosis proteins and clinical outcome, laboratory assessments of specific proteins involved in cell death control hold the promise for better segregating patients into prognostic groups so that individual therapeutic strategies can be better optimized. The studies of apoptosis proteins and their prognostic

value therefore should be continued using larger groups of patients. In particular, it will be important to compare uniformly treated cohorts of patients representing the full spectrum of breast cancer, including persons with early-stage localized disease, with locally advanced disease, and with metastatic disease.

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