The Proapoptotic Molecule BLID Interacts with Bcl-X<sub>L</sub> and Its Downregulation in Breast Cancer Correlates with Poor Disease-Free and Overall Survival

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Abstract

Purpose: BLID is a BH3-like motif containing apoptotic member of the Bcl-2 family of proteins. This study was designed to investigate the mechanism of BLID-induced apoptosis and to assess the significance of BLID expression in breast cancer.

Experimental Design: The interaction between BLID and Bcl-X<sub>L</sub> was examined using in vitro transcription/translation, coimmunoprecipitation, and immunofluorescence assays. The relationship between BLID mRNA expression and pathologic measures in breast cancer specimens (n = 55) was examined using the publicly available ONCOMINE microarray database. Immunohistochemistry was done using formalin-fixed, paraffin-embedded sections of 148 cases of invasive ductal breast carcinomas (IDC) and 58 cases of invasive lobular breast carcinomas, and breast tissue microarrays representing additional 437 cases (>85% IDC) with associated clinicopathologic database and long-term clinical follow-up (median 7 years).

Results: BLID was found to interact with Bcl-X<sub>L</sub> and the binding was enhanced in cancer cells exposed to doxorubicin or cisplatin. Exogenous expression of BLID correlated with activation of Bax and an increase in cytosolic cytochrome c. BLID mRNA expression was significantly reduced in grade 3 relative to grade 1 and 2 breast cancer (P = 0.023). Cytoplasmic BLID immunoreactivity was absent in IDC compared with invasive lobular breast carcinoma (P < 0.001). Lack of BLID expression was associated with younger age (median 40 years), African American ethnicity, tumor size, and triple-negative breast cancer (estrogen receptor negative, progesterone receptor negative, and human epidermal growth factor receptor 2 negative; all P < 0.005). Significant correlations were observed between BLID negativity and declines in overall, cause-specific, and local relapse-free survival (all P < 0.03). Multivariate analysis indicated that BLID is an independent prognostic factor of distant metastasis-free survival (hazard ratio, 0.302; 95% confidence interval, 0.160-0.570, P = 0.0002).

Conclusion: BLID is a new binding partner of Bcl-X<sub>L</sub> and a significant prognostic factor in breast cancer.

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**Translational Relevance**

BLID is a BH3-like motif containing apoptotic member of the Bcl-2 family. This study is aimed at the mechanism of BLID-induced cell death and its significance in breast cancer. BLID was found to interact with Bcl-XL, and the expression of BLID was associated with activation of Bax and an increase in cytosolic cytochrome c. BLID mRNA expression was reduced in grade 3 relative to grade 1 and grade 2 breast cancer. BLID protein was frequently lost or decreased in invasive ductal breast carcinomas but not in invasive lobular breast carcinomas. Lack of BLID protein was associated with poor prognostic factors such as African American ethnicity, younger age, and triple-negative breast cancer. Significant correlations were also observed between BLID negativity and declines in overall survival, local relapse-free survival, and distant metastasis-free survival. These findings show that BLID is a promising new clinical target, and further evaluation of this molecule may lead to improvements in breast cancer prognosis and therapy.

However, one of the significant drawbacks of doxorubicin, in both the metastatic and locally advanced breast cancer settings, is the significant risk for developing cardiac toxicities (11–13). Better understanding of the molecules and signaling pathways pertinent to breast cancer should lead to development of more effective and safer treatment strategies, and improvements in the quality of life and life span of breast cancer patients. Here, we have investigated the mechanism of BLID-induced apoptosis, compared BLID mRNA expression in high-grade versus low-grade breast tumor specimens (n = 55), and assessed the significance of BLID protein expression in breast cancer (n = 643). We show that (a) BLID is a novel binding partner of Bcl-XL and this interaction is enhanced by chemotherapeutic drugs, (b) BLID mRNA expression is reduced in high-grade relative to low-grade breast cancer, (c) BLID protein expression is frequently downregulated in invasive ductal breast carcinoma (IDC) compared with invasive lobular breast carcinoma (ILC), and (d) loss of BLID protein correlates with poor disease-free and overall survival of breast cancer patients.

**Materials and Methods**

**Cell lines, culture conditions, cDNA constructs, and transient transfections**

Normal human mammary epithelial cells (HMEC) were purchased from Cambrex and grown in mammary epithelial growth medium according to the supplier's recommendations. Human breast (MCF-10A, MCF-7, MDA-MB-231), ovarian (PA-1), and prostate cancer cell lines (PC-3) were obtained from the Tissue Culture Shared Resource of the Lombardi Comprehensive Cancer Center. Human epithelial kidney cells (HEK293T) were obtained from American Type Culture Collection. MCF-10A cells were grown in mammary epithelial growth medium (Cambrex), and all other cell lines were cultured in Dulbecco's minimum essential medium/GlutaMAX supplemented with 10% heat-inactivated fetal bovine serum, and penicillin (100 units/mL)/streptomycin (100 μg/mL; all from Invitrogen). All cultures were maintained in a humidified atmosphere at 37°C in the presence of 5% CO₂ and 95% air.

Constructions of the wild-type and mutant BLID plasmids using pCR3.1 expression vector [Invitrogen; MCR-Blind, Myc-Blind (ΔN-16), and Myc-Blind (L5E)] as well as pCR3.1 HA-Bcl-XL and pCR3.1 HA-p53 plasmids have been described previously (7). Myc-tagged Bad and HA-tagged Bax cDNAs were generated by PCR using human BAD (Genbank accession no. BC001901, IMAGE clone 3537914) and human Bax cDNAs (Genbank accession no. BC014175, IMAGE clone 4576562) as templates, respectively (Supplementary Materials and Methods). A full-length cDNA encoding wild-type BLID was cloned into prokaryotic PET-15b vector carrying the NH₂-terminal hexa-histidine (His₆) tag (Novagen; Supplementary Materials and Methods).

Intronless gene localized to human chromosome 11q24.1 (8). The UCSC Genome Browser v161 Web site shows human expressed sequence tags and conserved sequence information on BLID (MGC: 163235; IMAGE: 40146394; http://mgc.nci.nih.gov/). The longest predicticive open reading frame of BLID (327 bp) codes for ~12 kDa predominately cytosolic protein. We have shown that BLID functions as a proapoptotic molecule and that the amino-terminal region of BLID, including a BH3-like motif, is important for BLID-induced apoptosis. Consistently, a NH₂-terminal deletion mutant of BLID (10.2 kDa; Δ 1aa-16aa, ΔN16) lacking the BH3-like motif (1aa-16aa, LPIEGEQEI) or BLID containing a mutant BH3-like motif (leucine 5→glutamate; L5E) failed to induce apoptosis, whereas a COOH-terminal deletion mutant (6.8 kDa; Δ 62aa-108aa, ΔC47) retained apoptotic activity comparable with the full-length BLID (7). In human cancer cells, BLID-induced DNA fragmentation could be efficiently blocked by coexpression of the antiapoptotic molecule Bcl-XL, raising the possibility that BLID-induced cell death may involve binding to and antagonizing Bcl-XL.

In 2009, 192,370 estimated new cases of breast cancer and ~40,170 breast cancer–related deaths have been reported in the United States (9). Of note, African American women show a higher incidence rate before age 40 years, and higher overall breast cancer–related mortality compared with white women (9, 10). Resistance to chemotherapy and disease progression is a major challenge in the clinical management of breast cancer across all ethnic and age groups. Adjuvant chemotherapy that includes an anthracycline such as doxorubicin has been at the forefront of breast cancer clinical practice for many years.
The transient transfections of various plasmid cDNA constructs were done using either LipofectAMINE 2000 (Invitrogen) or FuGene HD (Roche Applied Sciences) according to the manufacturers' protocols.

**Antibodies, reagents, and chemicals**

Rabbit polyclonal anti-peptide BLID antibody was raised against the amino acid residues 42 to 61 (KARPLEALLGSNKEPMLPK) of human BLID (Zymed Laboratories), as described earlier (7). All other antibodies, reagents, and chemicals were commercially obtained as detailed in Supplementary Materials and Methods.

**Doxorubicin, cisplatin, and hydrogen peroxide treatments**

Cells (∼5 × 10^5) were seeded in serum-containing medium overnight. The next day, the medium was replaced with fresh serum-containing medium with DMSO (vehicle) or 100 ng/mL doxorubicin and incubations continued for 24 or 48 hours, followed by trypsinization. The adherent and floating cells were collected and lysed in 0.2% NP40 lysis buffer. For cisplatin treatment, cells were seeded overnight as above and treated for 24 hours. For hydrogen peroxide treatment, cells were seeded as above and then the medium was replaced with fresh serum-free medium containing hydrogen peroxide at a final concentration of 100 μmol/L for 2 hours at 37°C.

**In vitro transcription/translation and coimmunoprecipitation**

[35S]methionine-labeled Myc-BLID and HA-Bcl-X_L proteins were generated using pCRII-3 HA-Bcl-X_L constructs, respectively, by using the TNT Coupled Reticulocyte Lysate transcription/translation system (Promega) and [35S]methionine (specific activity 1,175.0 Ci/mmol; 2 μL in final 50 μL of reaction volume) according to the manufacturer’s instructions, followed by in vitro coimmunoprecipitation assay (Supplementary Materials and Methods).

**Subcellular fractionation**

The cytosolic and heavy membrane fractions were isolated as described earlier (7). Soluble and membrane fractions were isolated by the digitonin permeabilization procedure (Supplementary Materials and Methods).

**Immunoprecipitation and immunoblotting assays**

For immunoprecipitation, adherent cells were lysed in 0.3 mL of NP40 lysis buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L KCl, 0.2% NP40, 2.5 mmol/L MgCl2, 1 mmol/L EGTA, one tablet of Complete Protease Inhibitor (Roche)] for 30 minutes on ice. Whole cell lysates (2-6 mg protein) were precleared with protein A/ G-PLUS agarose (25 μL) for 2 hours in a cold room, followed by immunoprecipitation and immunoblotting (Supplementary Materials and Methods).

**Immunofluorescence assay**

HEK293T cells grown on coverslips were cotransfected with 1 μg of Myc-BLID or Myc-Bad and 1 μg HA-Bcl-X_L plasmids using Lipofectamine 2000. Twenty-four hours later, cells were fixed in 3.7% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. The nonspecific binding was blocked in 5% nonfat dry milk (blocking buffer) for 1 hour, and cells were sequentially labeled first with polyclonal anti-HA antibody (1:500, 2 hours, room temperature), followed by monoclonal anti-Myc antibody (1:100; overnight at 4°C). The primary antibodies were detected by sequential treatments first with anti-rabbit secondary antibody conjugated to Alexa Fluor 596 (1:2,000 dilution, 45 minutes) to detect the HA signal (red) and later with anti-mouse secondary antibody conjugated to Alexa Fluor 488 (1:2,000 dilution, 45 minutes) to detect the Myc signal (green). All antibodies were diluted in blocking buffer. The coverslips were mounted in glass slides with ProLong Antifade solution (Molecular Probes), and the cells were examined under an Olympus confocal microscope (laser scanning microscope FV300, Olympus America).

**Clinical specimens, tissue microarrays, and pathology evaluation**

A total of 643 cases of breast cancer were evaluable in this study. Formalin-fixed, paraffin-embedded sections of IDC or ILC and matched or adjacent benign breast tissues from 206 cases were obtained from two sites. The archival breast tissues from 80 cases (68 IDC and 12 ILC) were obtained from Georgetown University Hospital (GUH) Human Tumor Bank, and the remaining 126 cases (80 IDC and 46 ILC) were obtained from the Albany Medical College (AMC). The hematoxylin and eosin–stained sections (4 μm) of each tumor specimen and the matched or adjacent benign tissue were reviewed to confirm the presence of tumor and other histologic features, including tumor grade. Tumor pathologic staging was determined in each case according to the American Joint Committee on Cancer criteria using the tumor-node-metastasis classification scheme. The invasive carcinomas were graded using the modified Bloom and Richardson method, described by Elston and Ellis (14), and tumors were classified as either low or intermediate grade (grade 1 or 2, with scores of <8) and high grade (grade 3, with scores of 8 or 9).

Two breast tissue microarrays (TMA) used in this study represented an additional 437 cases (86% IDC, 7% ILC, and 7% other histologic subtypes) with associated clinicopathologic database and long-term clinical follow-up (median 7 years). The TMAs were constructed as follows. A pathologist examined hematoxylin and eosin–stained slides of the archived paraffin blocks and circled representative tumor sections. Areas of tumor, distinct from benign elements, were identified on the stained slides by a pathologist and marked for subsequent analysis. From these tumor sections, two 0.6-mm cores were extracted using a Tissue Microarrayer (Beecher Instruments), resulting in a total of 874 cores for the 437 patients in the study.
As previously described, this 2-fold redundancy has been validated and correlates highly with whole-section staining (15–17). Five-micrometer sections were cut with a tape-based tissue transfer system (Intrumedics) and processed as described previously (15–17).

**Immunohistochemistry**

The paraffin-embedded breast tissue sections were immunostained by either an automated or manual method using the avidin-biotin-peroxidase and dianinobenzidine kits (Vector Laboratories) according to the manufacturer's instructions with minor modifications (Supplementary Materials and Methods). The BLID immunoreactivity was localized in the cytoplasm and semiquantitatively scored based on both distribution (percentage of positive cells) and intensity of staining. The distribution of staining in the tumor cells was graded as focal (≤10%), regional (11–50%), or diffuse (>50%). The intensity of staining was graded as negative, weak, moderate, or intense. The BLID immunoreactivity was assigned one of the two final scores: 0, negative (focal distribution, or regional distribution and negative/weak staining); 1, positive (diffuse distribution, or regional distribution and moderate/intense staining). The TMAs were immunostained using the manual procedure. The presence of cytoplasmic BLID in ≤10% of tumor cells was scored as negative (0), and the presence of cytoplasmic BLID in >10% of tumor cells was scored as positive (1). The sections or cores with discrepant scores were reexamined and restained, and rare repeat conflicts considered nonevaluable were excluded from analysis.

**Statistical analysis**

Fisher's exact test and the Mantel-Haenszel procedure were used to compare BLID protein expression in IDC versus ILC tumor subtypes from the GUH and AMC sites. Analysis based on the Mantel-Haenszel test controls for sample provenance.

Statistical analysis of BLID expression in the TMAs was done using SAS version 9.1 (SAS Institute) as we described previously (18–20). All tests of statistical significance were two-sided. P values <0.05 were considered statistically significant. Local recurrence was defined as clinically and biopsy-proven relapse in the ipsilateral breast. Distant metastasis was defined as clinically and biopsy-proven metastasis in the contralateral breast or other sites. Distant metastasis-free survival was calculated as the interval between the date of diagnosis and the date of first recurrence of disease. Cause-specific survival was calculated as the interval between the date of diagnosis and the date of patient death from breast cancer (Fig. 1A). The survival analyses for the associations between predictor variables and BLID status included the date of diagnosis and the date of first recurrence of disease. Disease-free survival was calculated as the interval between the date of diagnosis and the date of first recurrence of disease. Bivariate analysis for the association between covariables and BLID status included the χ² test. Bivariate analyses for the associations between predictor variables and local, regional, and distant recurrence and overall survival were conducted by using the Kaplan-Meier log-rank test and the χ² test for linear trend. Univariate survival analysis was done with Cox proportional hazards model, which was used to calculate unadjusted relative risks for 15-year local relapse, 15-year distant metastasis-free survival, 15-year cause-specific survival, and 15-year poor overall survival. In multivariate analysis, Cox proportional hazards regression evaluated the effects of BLID on local relapse, distant metastasis-free survival, cause-specific survival, and overall survival after adjusting for age, race, tumor size, and nodal status.

**Results**

Interaction of BLID with Bcl-X<sub>L</sub> and correlations of BLID expression with activation of Bax and an increase in cytosolic cytochrome c

A variety of in vitro and in vivo binding assays were done (Fig. 1; Supplementary Fig. S1). [³⁵S]methionine-labeled in vitro translated Myc-BLID was found to bind directly to [³⁵S]methionine-labeled in vitro translated HA-Bcl-X<sub>L</sub> protein (Fig. 1A). The binding of purified His-BLID protein to HA-Bcl-X<sub>L</sub> transiently expressed in HEK293T cells was shown by a combination of immunoprecipitation and in vitro binding and pull-down assays (Fig. 1B; Supplementary Fig. S1A and S1B). Purified His-BLID protein did not seem to bind to HA-Bax expressed in HEK293T cells (Fig. 1B). In in vitro studies, wild-type Myc-BLID but not an amino-terminal deletion mutant of BLID (Myc-BLID ΔN-16) lacking the BH3-like domain was found to bind to HA-Bcl-X<sub>L</sub> and interaction of Myc-BLID carrying a point mutation in the BH3-like domain with HA-Bcl-X<sub>L</sub> was significantly reduced (Fig. 1C and D; Supplementary Fig. S1C). Wild-type HA-p53 was used as a negative control (Fig. 1D). Myc-BLID expressed in HEK293T cells was found to coimmunoprecipitate with Bcl-X<sub>L</sub> and Bcl-2 but not Bak or Bax (Supplementary Fig. S1D, S1F, and S1G). The colocalization of ectopic Myc-BLID and HA-Bcl-X<sub>L</sub> proteins was shown by immunofluorescence, and known colocalization of Myc-Bad and HA-Bcl-X<sub>L</sub> was used as a positive control (Supplementary Fig. S1H). Ectopic expression of Myc-BLID in HEK293T cells resulted in activation of Bax as detected by the conformation-specific anti-Bax antibody 6A7 (Supplementary Fig. S1I). In addition, increased expression of cytosolic cytochrome c was found in Myc-BLID transfectants compared with control vector transfectants (Supplementary Fig. S1E and S1J). These data show that the BLID-induced apoptotic pathway involves its binding to Bcl-X<sub>L</sub>, activation of Bax, and an increase in cytosolic cytochrome c.

Enhanced interaction between endogenous BLID and Bcl-X<sub>L</sub> proteins in cancer cells treated with chemotherapeutic drugs or oxidative stress

Next, we tested the hypothesis that exposure of cancer cells to chemotherapeutic drugs and potent oxidants may stimulate the binding of BLID to Bcl-X<sub>L</sub>. As shown in Fig. 2A, BLID was found to coimmunoprecipitate with Bcl-X<sub>L</sub> in doxorubicin-treated MDA-MB-231 breast cancer cells but not in untreated cells. As anticipated, doxorubicin-treated cells also showed activation of caspase-9 and caspase-3 (Fig. 2B). Similar results were observed in PC-3 prostate cancer cells treated with doxorubicin, PA-1 ovarian cancer cells treated with...
cisplatin, and MDA-MB-231 cells treated with hydrogen peroxide (Fig. 2C and D; Supplementary Fig. S2A-D).

BLID mRNA expression in human breast tumors

Using the publicly available ONCOMINE cancer gene expression microarray database (ref. 21; http://www.oncomine.org), we searched for any relationship between BLID mRNA expression and clinical outcome or pathologic measures in breast cancer clinical studies. Normalized Affymetrix U133 Plus 2.0 gene expression data, originally published by Ginestier and colleagues (22), were downloaded from ONCOMINE. The Ginestier et al. data set includes 55 tumor samples from women with invasive breast cancer. These patients represent a wide range of clinical and pathologic characteristics (including age, grade, estrogen receptor status, and lymph node status). In this study, median BLID mRNA expression was significantly reduced in grade 3 relative to grade 1 and grade 2 breast cancers (Fig. 3A). We also analyzed the Ginestier et al. data set for a potential correlation between Bcl-X<sub>L</sub> mRNA expression and high-grade versus low-grade tumors. Median Bcl-X<sub>L</sub> mRNA expression was significantly increased in grade 3 versus grade 1 breast cancer (P = 0.05).

Optimization and validation of BLID immunostaining in breast tissues

The anti-BLID antibody clearly detected the anticipated -12 kDa band in whole cell lysates from HMEC, MCF-10A, and MDA-MB-231 cells, and this signal was significantly reduced in the presence of the BLID peptide-blocked antibody (Supplementary Materials and Methods and Fig. S3), confirming the expression of BLID protein in several widely used models of normal mammary epithelium and breast cancer. No signal was seen with the preimmune serum (data not shown). In the immunohistochemical assay, the anti-BLID antibody showed remarkable sensitivity (at dilution 1:10,000) and specificity. The negative controls in the immunohistochemical assay included the preimmune serum used at the same dilution as the primary antibody and absence of the primary antibody (data not shown). A primarily cytoplasmic pattern of BLID protein expression was observed to varying degrees in both benign breast epithelium and certain invasive tumors. In a unique specimen with

**Fig. 1.** BLID interacts with Bcl-X<sub>L</sub> in vitro and in vivo, and expression of BLID is associated with activation of Bax and an increase in cytosolic cytochrome c. A, interaction between in vitro transcribed/translated and radiolabeled Myc-BLID (∼12 kDa) and HA-Bcl-X<sub>L</sub> proteins (∼30 kDa). Data shown are representative of two independent experiments. B, interaction of purified His-BLID with HA-Bcl-X<sub>L</sub> expressed in HEK293T cells. HA-Bax plasmid was used as a negative control. Top, top band is nonspecific. C and D, reciprocal coimmunoprecipitation of Myc-BLID and HA-Bcl-X<sub>L</sub>. HEK293T cells were cotransfected with HA-Bcl-X<sub>L</sub> and indicated wild-type Myc-BLID, mutant Myc-BLID, or vector pCR3.1. Whole cell lysate was analyzed by immunoprecipitation followed by sequential immunoblotting as indicated. In D (right lane only), cotransfection was done using a control HA-epitope-tagged plasmid HA-p53 and wild-type Myc-BLID. IP, immunoprecipitation, IB, immunoblotting, WCE, whole cell extract, UT, untransfected.
benign tissue, IDC and ILC areas were located on the same slide, positive cytoplasmic BLID immunostaining was seen in the benign and ILC areas, whereas negative BLID staining was seen in the adjacent IDC field (Fig. 3B). In independent sets of benign and matched malignant specimens from two other patients (A and B), the benign and ILC tissues from patient A, and the benign tissue from patient B, were found to be BLID positive, whereas the IDC tissue from patient B showed negative staining for BLID (Supplementary Fig. S4). Next, we tested the validity of BLID immunostaining in a breast TMA generated from an independent cohort of breast tumor tissues. Representative BLID-positive and BLID-negative immunostaining patterns identified in two independent cores spotted on the same TMA slide are shown in Fig. 3C. These results show that the custom-made BLID antibody is suitable for detection of BLID in human normal and malignant mammary cells, and the immunohistochemical staining procedure established in this study is sensitive and reliable for localization of cytoplasmic BLID in human benign and malignant breast tissues.

**Differential expression of BLID in IDC versus invasive lobular breast carcinoma**

Of a total of 206 cases of breast cancer obtained from two sites, GUH and AMC, 161 cases had available matched benign specimens. In this cohort, 23 of 116 IDC (19.8%) and 1 of 45 ILC (2.2%) were found to be BLID negative compared with matched benign samples (Table 1). We consistently observed that cytoplasmic BLID immunoreactivity was significantly low or absent in most IDC compared with ILC. As shown in Table 1, IDC specimens are more likely to show loss of BLID expression compared with ILC specimens. BLID expression was also compared between all available IDC versus ILC specimens from the two sites using the Fisher’s exact test. IDC were
found to be BLID negative compared with ILC [BLID negativity: IDC 43.2% (64 of 148); ILC, 5.2% (3 of 58), \(P < 0.001\)]. A statistically significant difference in BLID expression in IDC versus ILC was also observed after controlling for site using the Mantel-Haenszel test (\(P < 0.001\)). By site, the odds of positive BLID expression in ILC are 7.8 times higher than the odds in IDC (95% confidence interval, 2.5-24).

**Correlations of the negative status of BLID with age, race, tumor size, estrogen receptor/progesterone receptor–negative/triple-negative breast cancer, and poor survival**

Of a total of 437 independent cases spotted on two TMAs, 73 cases (17%) were found to be BLID negative, and of these 65 cases (89%) represented IDC. Lack of BLID expression was found to be associated with several known prognostic factors such as younger age (range 25-45 years, median 40 years), African American ethnicity, and increased tumor size (>2 cm; all \(P < 0.005\); Table 2). Lack of BLID expression was also found to be associated with estrogen receptor (ER)–negative, progesterone receptor (PR)–negative, and triple-negative breast cancer [ER-negative, PR-negative, and human epidermal growth factor receptor 2 (HER-2) negative; all \(P < 0.0001\); Table 2], although BLID status did not correlate with HER-2–negative breast cancer. To determine the prognostic power of BLID, we evaluated BLID expression status with respect to the four survival outcomes: local relapse-free survival, cause-specific survival, distant metastasis-free survival, and overall survival. As shown in Fig. 4, significant correlations were observed between BLID negativity and declines in all four survival categories (all \(P < 0.03\)). Multivariate analysis indicated that BLID is an independent prognostic factor of distant metastasis-free survival after adjusting for risk factors (hazard ratio, 0.302; 95% confidence interval, 0.160-0.570, \(P = 0.0002\)).

**Discussion**

This is the first report to show that BLID is a novel binding partner of Bcl-X\(_L\). Very limited information is
Despite the complex nature of breast cancer biology and therapy response, a limited number of markers and multiparameter tests are available for routine clinical practice (29). Our findings that cytoplasmic BLID immunoreactivity is significantly low or absent in most IDC compared with ILC may have important clinical implications. IDC and ILC are the two most common malignancies of the breast, accounting for ~80% and ~15% of all invasive breast tumors, respectively (30). Molecular differences between the two tumor subtypes exist, the most notable one being a moderate to strong membranous pattern of E-cadherin immunoreactivity in a majority of IDC versus the near-complete absence of E-cadherin expression in a

Table 1. BLID protein expression in IDC versus ILC

<table>
<thead>
<tr>
<th>Loss of BLID expression*</th>
<th>IDC (Positive, %)</th>
<th>ILC (Positive, %)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>93/116 (80.2%)</td>
<td>44/45 (97.8%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Yes</td>
<td>23/116 (19.8%)</td>
<td>1/45 (2.2%)</td>
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</table>

*Immunohistochemistry was done using paraffin-embedded tumor sections of a total of 161 breast cancer cases with available matched benign samples from two sites—GUH and AMC. To evaluate the relationship of BLID expression with IDC versus ILC, a new binary classification was created in which patients with BLID expression in the tumor lower than BLID expression in the benign specimen (i.e., loss of expression associated with the tumor) were included in one group, and all other patients comprised the other group. Fisher’s exact test was used to compare tumor subtypes.

available on the role of BH3-only proteins in cellular signaling response to cytotoxic drugs (23). Using in vitro and in vivo binding assays and 35S-labeled BLID as well as recombinant purified His-BLID protein, we have presented evidence that BLID directly binds Bcl-XL. In contrast, mutant BLID that compromised the integrity of the BH3-like motif failed to bind Bcl-XL, or the binding was greatly diminished. BLID also binds to Bcl-2, but not Bax or Bak, and therefore BLID should be considered as an enabler of apoptosis. Bcl-2 and Bcl-XL proteins have been associated with resistance of breast cancer to anti-neoplastic therapies (24, 25). In MDA-MB-231 breast cancer cells exposed to doxorubicin, BLID was found to coimmunoprecipitate with Bcl-XL. Furthermore, expression of BLID in breast cancer cells also results in enhanced cytotoxic effects of doxorubicin and docetaxel.4 This study suggests that interaction of BLID with Bcl-XL and Bcl-2 offers an important mechanism for sequestering these molecules in vivo and cell death induced by cytotoxic agents.

The mechanism of regulation of BLID expression in breast cancer is unknown. Several members of the forkhead box (Fox) family of transcriptional regulators play an important role in breast pathogenesis (26–28). A sequence homology search for the DNA binding motifs within the putative BLID promoter region revealed potential binding sites for several members of the FoxO subfamily and FoxA1. Further studies in our laboratory are aimed at the investigation of transcriptional regulation of BLID and role of Fox→BLID→Bcl-XL→→→caspase pathway in chemosensitization of breast cancer.

Table 2. BLID expression and known prognostic factors in breast cancer

<table>
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<tr>
<th>Prognostic factor</th>
<th>BLID expression status*</th>
<th>P†</th>
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<tbody>
<tr>
<td>Age (y)‡</td>
<td>Negative (%), Positive (%)</td>
<td>&lt;0.0001</td>
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<tr>
<td>≤45</td>
<td>45 (32.4), 94 (67.6)</td>
<td></td>
</tr>
<tr>
<td>&gt;45</td>
<td>28 (9.5), 267 (90.5)</td>
<td></td>
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<tr>
<td>Strong family history</td>
<td>No</td>
<td>54 (16.0), 283 (84.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>13 (22.8), 44 (77.2)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>≤2</td>
<td>42 (13.8), 263 (86.2)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>25 (28.0), 64 (72.0)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td>Caucasian</td>
<td>53 (14.4), 315 (85.6)</td>
</tr>
<tr>
<td>African American</td>
<td>17 (32.0), 36 (68.0)</td>
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<tr>
<td>Nodal status</td>
<td>Negative</td>
<td>38 (18.8), 164 (81.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>18 (25.3), 53 (74.7)</td>
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<td>TN status§</td>
<td>Yes</td>
<td>42 (34.7), 79 (65.3)</td>
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<td>No</td>
<td>30 (9.6), 282 (90.4)</td>
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<tr>
<td>ER status</td>
<td>Negative</td>
<td>52 (27.8), 135 (72.2)</td>
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<td>Positive</td>
<td>20 (8.2), 225 (91.8)</td>
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<td>PR status</td>
<td>Negative</td>
<td>56 (27.2), 150 (72.8)</td>
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<td>Positive</td>
<td>16 (7.0), 212 (93.0)</td>
<td></td>
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<tr>
<td>HER-2/Neu status</td>
<td>Negative</td>
<td>61 (17.0), 298 (83.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>12 (15.4), 66 (84.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Immunohistochemistry was done on two breast tissue microarrays representing a total of 437 cases.

†Two-sided P value (χ² test); values shown in bold are statistically significant (P < 0.05).

‡Age, ≤45 years, range 25 to 45 years, median age 40 years; >45 years, range 46 to 88 years, median age 52 years.

§TN, triple-negative breast cancer (ER negative, PR negative, and HER-2/Neu negative).

4 E.H. Chang and U. Kasid, unpublished data.
A side-by-side comparison of the E-cadherin and BLID immunostaining patterns in a limited number of ILC specimens from our tumor tissues indicated that whereas E-cadherin immunoreactivity was lost in 26 of 31 (84%) ILC, diffuse BLID cytoplasmic positivity was noted in 37 of 37 (100%) ILC. Most noteworthy, however, was that 91% of ILC showed a profile of BLID positive/E-cadherin negative. It therefore seems that in conjunction with the loss of E-cadherin immunoreactivity, BLID may have an improved diagnostic utility in distinguishing the lobular from ductal phenotype in invasive mammary carcinomas.

In this study, ~20% of IDC showed loss of BLID expression compared with matched benign tissue specimens (n = 116; Table 1). Further investigations using a larger sample size of tumor and matched benign specimens will be necessary to establish the loss of BLID in IDC versus matched benign tissue. Nonetheless, these data are consistent with the independent data set used in our breast tumor TMAs, in which ~17% of breast tumors (n = 437, 86% IDC) were found to be BLID negative. Based on TMA data, this unique cohort lacking BLID seems to have a worse prognosis and is at high risk for poor disease-free and overall survival (Fig. 4).

Remarkable associations were found between BLID negativity and younger women, African American population, tumor size, and hormone receptor-negative and triple-negative subgroups of breast cancer (Table 2). We did not find a correlation between BLID and either HER-2 status or lymph node status, two other poor prognostic factors. It has been reported that African American women show a higher breast cancer incidence rate before age 40 years and a higher overall breast cancer–related death rate compared with white women (9, 10). In addition, premenopausal African American patients have a significantly higher prevalence of triple-negative or basal-like breast tumors (negative for ER, PR, and HER-2, and positive for cytokeratin 5/6 and/or HER-1) compared with postmenopausal African American and non–African American populations (32, 33). Interestingly, the triple-negative subgroup has been suggested as an independent predictor of distant breast cancer metastasis in African American women (34). Based on this study, BLID may be a unique prognostic marker for African American breast cancer patients. The negative status of BLID was found to correlate with poor relapse-free, distant metastasis-free, cause-specific, and overall survivals in all breast cancer patients in our study, regardless of ethnicity or

Fig. 4. Fifteen-year outcomes as a function of BLID expression in breast cancer patients (age range 25-88 years, median age 51 years). Total number of specimens: BLID positive, 361; BLID negative, 73. Statistical significance was determined using the log-rank test.

5 B.V.S. Kallakury, C.E. Sheehan, and J.S. Ross, unpublished data.
age. Together with the function of BLID in the chemotherapeutic drug–stimulated apoptotic pathway, these data suggest that loss of BLID may significantly affect the clinical course of breast cancer.

Disclosure of Potential Conflicts of Interest

C.G. Broustas and U.N. Kasid are among the inventors of Georgetown University’s patented BLID technology titled Gene BRCC2 and Diagnostic and Therapeutic Uses Thereof (U.S. Patent 7,253,272).

References


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