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The Pro-apoptotic Molecule BLID Interacts with Bcl-XL and Its Downregulation in Breast Cancer Correlates with Poor Disease Free and Overall Survival

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Note: C. G. Broustas, J. S. Ross, and Q. Yang contributed equally to this work.

Running title: BLID in Apoptosis and Breast Cancer
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-X\textsubscript{L} and 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 the 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 demonstrate that BLID is a promising new clinical target and further evaluation of this molecule may lead to improvements in breast cancer prognosis and therapy.
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 assess the significance of BLID expression in breast cancer.

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

Results: BLID was found to interact with Bcl-X\textsubscript{L} 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 as compared with ILC ($P < 0.001$). Lack of BLID expression was associated with younger age (median 40 years), African American population, tumor size, and triple-negative breast cancer (estrogen receptor negative, progesterone receptor negative and HER2 negative) (all $P$ values < 0.005). Significant correlations were observed between BLID negativity and declines in overall, cause-specific, and local relapse-free survival.
(all $P$ values < 0.03). Multivariate analysis indicated that BLID is an independent prognostic factor of distant metastasis-free survival ($HR = 0.302$, 95% CI: 0.160-0.570, $P = 0.0002$).

**Conclusion:** BLID is a new binding partner of Bcl-X$_L$ and a significant prognostic factor in breast cancer.
Introduction

The Bcl-2 family of proteins plays a pivotal role in the control of cell death and response of cancer cells to antineoplastic drugs and γ-radiation (1, 2). The anti-apoptotic members (e.g. Bcl-2, Bcl-XL, Mcl-1) share homology within three or four BH (Bcl-2 homology) domains (BH 1-4). The pro-apoptotic proteins contain either two or three BH domains (e.g. Bax, Bak) or only the short (9-16 amino acids) BH3 domain (BH3-only proteins, e.g. Bad, Puma, Noxa) (3). Under unstimulated conditions, Bax and Bak are sequestered by one or more prosurvival members. Numerous landmark studies have demonstrated that binding of a BH3-only apoptotic molecule to an anti-apoptotic member is a critical step in activation of Bax/Bak, release of cytochrome c, and activation of caspase-9 and caspase-3 (4, 5). More recently, a new class of pro-apoptotic molecules has emerged that contains a BH3-like motif with highly conserved leucine but not aspartate in the BH3 domain (6). We have identified BLID, earlier reported as BRCC2, a BH3-like motif containing inducer of cell death (7). We first discovered BLID as an approximately 1.0 kb transcript in MDA-MB-231 human breast cancer cells (GenBank accession numbers AF220061 and AF303179). BLID is an intronless gene localized to human chromosome 11q24.1 (8). The UCSC Genome Browser v161 website shows human ESTs and conserved sequence information on BLID (MGC: 163235; IMAGE: 40146394). The longest predictive open reading frame of BLID (327 bp) codes for ~ 12 kDa predominately cytosolic protein. We have demonstrated that BLID functions as a pro-apoptotic molecule, and that the amino-terminal region of BLID including a BH3-like motif is important for BLID-induced apoptosis. Consistently, a N-terminal deletion mutant of BLID (10.2 kDa; Δ 1aa-16aa, ΔN16) lacking the BH3-like motif (5aa-12aa, LPIEGQE1) or BLID containing a mutant BH3-like motif

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(Leucine5→Glutamate) (L5E) failed to induce apoptosis, whereas a C-terminal deletion mutant (6.8 kDa; Δ 62aa-108aa, ΔC47) retained apoptotic activity comparable to the full-length BLID (7). In human cancer cells, BLID-induced DNA fragmentation could be efficiently blocked by coexpression of the anti-apoptotic molecule Bcl-XL, raising a 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 approximately 40,170 breast cancer-related deaths have been reported in the United States (9). Of note, African American women show higher incidence rate before age 40, and higher overall breast cancer-related mortality as compared to 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. 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 demonstrate that 1) BLID is a novel binding partner of Bcl-XL and this interaction is enhanced by chemotherapeutic drugs, 2) BLID mRNA expression is reduced in high grade relative to low grade breast cancer, 3) BLID protein expression is frequently downregulated in IDC as compared
to ILC, and 4) 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 (MEGM) 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 ATCC. MCF-10A cells were grown in MEGM (Cambrex), and all other cell lines were cultured in Dulbecco's minimum essential medium DMEM/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) (Myc-BLID, Myc-BLID (ΔN-16), and Myc-BLID (L5E)) as well as pCR3.1 HA-Bcl-Xₐ 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# BC001901, IMAGE clone 3537914) and human Bax cDNAs (GenBank accession# BC014175, IMAGE clone 4578562) as templates, respectively (Supplemental Materials and Methods). A full-length cDNA encoding wild type BLID was cloned into prokaryotic pET-15b vector.
carrying the N-terminal hexa-histidine (His₆) tag (Novagen) (Supplemental Materials and Methods).

The transient transfections of various plasmid cDNA constructs were performed 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 42-61 (KARLPLEALLGNSKEPMLPK) of human BLID (Zymed Laboratories, South San Francisco, CA) as described earlier (7). All other antibodies, reagents, and chemicals were commercially obtained as detailed in the Supplemental Materials and Methods.

**Doxorubicin, cisplatin, and hydrogen peroxide treatments.** Cells (approximately 5 x 10⁶) were seeded in serum-containing medium overnight. The next day medium was replaced with fresh serum-containing medium with DMSO (vehicle) or 100 ng/mL DXR and incubations continued for 24 hr or 48 hr, followed by trypsinization. The adherent and floating cells were collected and lysed in 0.2% NP-40 lysis buffer. For cisplatin treatment, cells were seeded overnight as above and treated for 24 hr. For hydrogen peroxide treatment, cells were seeded as above and then medium was replaced with fresh serum-free medium containing hydrogen peroxide at a final concentration of 100 µM for 2 hr at 37°C.

**In vitro transcription/translation and co-immunoprecipitation.** [³⁵S]methionine-labeled Myc-BLID and HA-Bcl-XL proteins were generated using pCR3.1 Myc-BLID and pCR3.1 HA-Bcl-XL constructs, respectively, the TNT Coupled Reticulocyte Lysate transcription/translation system (Promega), and [³⁵S]methionine (specific activity 1175.0 Ci/mmol; 2 µL in final 50 µL of
reaction volume) according to the manufacturer's instructions, followed by in vitro co-immunoprecipitation assay (Supplemental Materials and Methods).

**Subcellular fractionation.** The cytosolic and heavy membrane fractions (HM) were isolated as described earlier (7). Soluble (S) and membrane (M) fractions were isolated by the digitonin permeabilization procedure (Supplemental Materials and Methods).

**Immunoprecipitation and immunoblotting assays.** For immunoprecipitation, adherent cells were lysed in 0.3 mL of NP-40 lysis buffer (20mM HEPES, pH 7.5, 150mM KCl, 0.2% Nonidet P-40, 2.5mM MgCl₂, 1mM EGTA, one tablet of complete™ protease inhibitors [Roche]) for 30 min on ice. Whole cell lysates (2-6 mg protein) were precleared with protein A/G-PLUS agarose (25 μL) for 2 hr in cold room, followed by immunoprecipitation and immunoblotting (Supplemental Materials and Methods).

**Immunofluorescence assay.** HEK293T cells grown on coverslips were co-transfected with 1μg of Myc-BLID or Myc-Bad and 1 μg HA-Bcl-X₇ plasmids using LipofectAMINE 2000. Twenty-four hr later, the cells were fixed in 3.7% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 5 min. The non-specific binding was blocked in 5% non-fat dry milk (blocking buffer) for 1 hr, and cells were sequentially labeled first with polyclonal anti-HA antibody (1:500, 2 hr, RT), 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 (1:2000 dilution, 45 min) to detect the HA signal (red) and later with anti-mouse secondary antibody conjugated to Alexa Fluor 488 (1:2000 dilution, 45 min) to detect the Myc signal (green). All antibodies were diluted in blocking buffer. The coverslips were mounted in glass slides with ProLong Antifade solution.
and the cells were examined under an Olympus Confocal microscope (Laser scanning microscope FV300, Olympus America, Center Valley, PA).

**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 (H & E)-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 histological features including tumor grade. Tumor pathologic staging was determined in each case according to American Joint Committee on Cancer criteria using the TNM 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 (TMAs) used in this study represented additional 437 cases (86% IDC, 7% ILC, and 7% other histological subtypes) with associated clinicopathologic database and long-term clinical follow-up (median 7 years). The TMAs were constructed as follows. A pathologist examined H & E–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, Silver Spring, MD, U.S.A.), resulting in a total of 874 cores for the 437 patients in the study. As
previously described, this two-fold redundancy has been validated and correlates highly with whole section staining (15-17). Five micron sections were cut with a tape-based tissue transfer system (Intrumedics, Hackensack, NJ, U.S.A.) 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 diaminobenzidine kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions with minor modifications (Supplemental Materials and Methods). The BLID immunoreactivity was localized to 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: negative (0) (focal distribution, or regional distribution and negative/weak staining) or positive (1) (diffuse distribution, or regional distribution and moderate/intense staining). The TMAs were immunostained using the manual procedure. Presence of cytoplasmic BLID in ≤10% of tumor cells was scored as negative (0), and presence of cytoplasmic BLID in >10% of tumor cells was scored as positive (1). The sections or cores with discrepant scores were reexamined, restained, and rare repeat conflicts considered non-evaluable 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. The analysis based on the Mantel-Haenszel test controls for sample provenance.

Statistical analysis of BLID expression in the TMAs was performed using SAS Version 9.1 (SAS Institute, Cary, NC) as we described earlier (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 metastases were defined as clinical evidence of distant disease based on clinical and/or radiographic evidence. 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 Chi-square 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 Chi-square 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 performed (Fig. 1 and supplemental Fig. S1). [<sup>35</sup>S] methionine-labeled in vitro-translated Myc-BLID was found to bind directly to [<sup>35</sup>S] 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 the immunoprecipitation and in vitro binding and pull-down assays (Fig. 1B and supplemental Fig. S1A and S1B). Purified His-BLID protein did not seem to bind to HA-Bax expressed in HEK293T cells (Fig. 1B). In in vivo 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 1D, and supplemental Fig. S1C). Wild type HA-p53 was used as a negative control (Fig. 1D). Myc-BLID expressed in HEK293T cells was found to co-immunoprecipitate with Bcl-X<sub>L</sub> and Bcl-2 but not Bak or Bax (Supplemental Fig. S1D, S1F and S1G). The colocalization of ectopic Myc-BLID and HA-Bcl-X<sub>L</sub> proteins was demonstrated by immunofluorescence and known colocalization of Myc-Bad and HA-Bcl-X<sub>L</sub> was used as a positive control (Supplemental 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 (Supplemental Fig. S1I). In addition, increased expression of cytosolic cytochrome c was found in Myc-BLID transfectants as compared to control vector transfectants (Supplemental Fig. S1E and S1J). These data demonstrate that 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\textsubscript{L} 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\textsubscript{L}. As shown in Fig. 2A, BLID was found to co-immunoprecipitate with Bcl-X\textsubscript{L} in DXR-treated MDA-MB-231 breast cancer cells but not in untreated cells. As anticipated, DXR-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 DXR, PA-1 ovarian cancer cells treated with cisplatin, and MDA-MB-231 cells treated with hydrogen peroxide (Fig. 2C and 2D, and Supplemental Fig. S2A –S2D).

BLID mRNA expression in human breast tumors. Using the publicly-available ONCOMINE cancer gene expression microarray database (21), we searched for any relationship between BLID mRNA expression and clinical outcome or pathological 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. dataset includes 55 tumor samples from women with invasive breast cancer. These patients represent a wide range of clinical and pathological 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 interrogated the Ginestier et al. dataset for a potential correlation between Bcl-X\textsubscript{L} mRNA expression and high grade versus low grade tumors. Median Bcl-X\textsubscript{L} mRNA expression was significantly increased in grade 3 versus grade 1 breast cancer ($P=0.01$, Student’s t test). In contrast, in this same subset ($n=43$), BLID mRNA was significantly reduced 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 (Supplemental 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 pre-immune serum (data not shown). In the IHC assay, the anti-BLID antibody showed a remarkable sensitivity (at dilution 1:10,000) and specificity. The negative controls in the IHC assay included the pre-immune 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 benign tissue, invasive ductal breast carcinoma (IDC) and invasive lobular breast carcinoma (ILC) areas 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 (Supplemental Fig. S4). Next, we tested the validity of the 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 demonstrate 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 invasive ductal breast carcinoma 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, 23/116 IDC (19.8%) and 1/45 ILC (2.2%) were found to be BLID negative as compared to matched benign samples (Table 1). We consistently observed that cytoplasmic BLID immunoreactivity was significantly low or absent in most IDC as compared to ILC. As shown in Table 1, IDC specimens are more likely to show loss of BLID expression as compared to 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 as compared to ILC (BLID negativity: IDC 43.2% (64/148); ILC, 5.2% (3/58), 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% CI: 2.5-24).

**Correlations of the negative status of BLID with age, race, tumor size, ER/PR-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 values < 0.005) (Table 2). Lack of BLID expression was also found to be associated with ER-negative, PR-negative, and triple-negative breast cancer (ER-negative, PR-negative and HER-2 negative) (all P values < 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 \( P \) values < 0.03). Multivariate analysis indicated BLID is an independent prognostic factor of distant metastasis-free survival after adjusting for risk factors (Hazard Ratio (HR) = 0.302, 95%CI: 0.160-0.570, \( P = 0.0002 \)).
Discussion

This is the first report showing BLID is a novel binding partner of Bcl-X<sub>L</sub>. Very limited information is available on the role of BH3-only proteins in cellular signaling response to cytotoxic drugs (23). Using <i>in vitro</i> and <i>in vivo</i> binding assays and <sup>35</sup>S-labeled BLID as well as recombinant purified His-BLID protein, we have presented evidence that BLID directly binds Bcl-X<sub>L</sub>. In contrast, mutant BLID that compromised the integrity of the BH3-like motif failed to bind Bcl-X<sub>L</sub> 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-X<sub>L</sub> proteins have been associated with resistance of breast cancer to antineoplastic therapies (24, 25). In MDA-MB-231 breast cancer cells exposed to DXR, BLID was found to co-immunoprecipitate with Bcl-X<sub>L</sub>. Furthermore, expression of BLID in breast cancer cells also results in enhanced cytotoxic effects of DXR and docetaxel (E.H.Chang, personal communication).<sup>6</sup> This study suggests that interaction of BLID with Bcl-X<sub>L</sub> and Bcl-2 offers an important mechanism for sequestering these molecules <i>in vivo</i> and cell death-induced by cytotoxic agents.

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 <i>BLID</i> and role of Fox→BLID→Bcl-X<sub>L</sub>→caspase pathway in chemosensitization of breast cancer.

<sup>6</sup>E. H. Chang and U. Kasid, unpublished data
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 as compared to 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 do 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 majority of ILC (31). 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 while E-cadherin immunoreactivity was lost in 26/31 (84%) of ILC, diffuse BLID cytoplasmic positivity was noted in 37/37 (100%) ILC. Most noteworthy, however, was that 91% of the ILC demonstrated a profile of BLID positive/E-cadherin negative. It therefore appears 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, approximately 20% of IDC showed loss of BLID expression as compared to matched benign tissue specimens (n = 116, Table 1). Further investigations using 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 dataset used in our breast tumor TMAs, where approximately 17% of breast tumors (n = 437, 7B.V.S.K., C.S., and J.R., unpublished data
86% IDC) were found to be BLID negative. Based on the 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 TN subgroups of breast cancer (Table 2). We did not find a correlation between BLID and either HER2 status or lymph node status, two other poor prognostic factors. It has been reported that African American women show higher breast cancer incidence rate before age 40, and higher overall breast cancer-related death rate as compared to white women (9, 10). In addition, pre-menopausal African American patients have a significantly higher prevalence of TN or basal-like breast tumors (negative for ER, PR and HER2, and positive for cytokeratin 5/6 and/or HER1) compared with post-menopausal African American and non-African American populations (32, 33). Interestingly, the TN 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 age. Together with the function of BLID in the chemotherapeutic drug-stimulated apoptotic pathway, these data suggest that loss of BLID may significantly impact the clinical course of breast cancer.
Disclosure of Potential Conflicts of Interest

None declared.

Acknowledgment

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


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<th>ILC †</th>
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<td>No</td>
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*Immunohistochemistry was performed 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.

†IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.
Table 2. BLID expression and known prognostic factors in breast cancer

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

*Immunohistochemistry was performed on two breast tissue micro-arrays representing a total of 437 cases.

\(^+\)Two-sided \( p \) value (Chi-square test); values shown in bold are statistically significant (\( p < 0.05 \)).

\(^‡\)Age, \( \leq 45 \) years, range 25 – 45 years, median age 40 years; > 45 years, range 46 – 88 years, median age 52.

\(^§\)TN, triple-negative breast cancer (ER negative, PR negative and HER-2/Neu negative), ER, estrogen receptor; PR, progesterone receptor.
Figure Legends

**Fig. 1.** BLID interacts with Bcl-X\(_L\) *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\(_L\) proteins (~30 kDa). Data shown are representative of 2 independent experiments.  

*\(B\)*, interaction of purified His-BLID with HA-Bcl-X\(_L\) expressed in HEK293T cells. HA-Bax plasmid was used as a negative control. Top panel, top band is non-specific.  

*\(C\)* and *\(D\)*, reciprocal co-immunoprecipitation of Myc-BLID and HA-Bcl-X\(_L\). HEK293T cells were co-transfected with HA-Bcl-X\(_L\) and indicated wild type Myc-BLID, mutant Myc-BLID, or vector pCR3.1 and whole cell lysate was analyzed by immunoprecipitation followed by sequential immunoblotting as indicated. In panel D (right lane only), cotransfection was performed using a control HA-epitope-tagged plasmid HA-p53 and wild type Myc-BLID. IP, immunoprecipitation; IB, immunoblotting; WCE, whole cell extract; UT, untransfected.

**Fig. 2.** Association of endogenous BLID with Bcl-X\(_L\) and activation of caspases in cancer cells treated with doxorubicin or cisplatin.  

*\(A\)*, doxorubicin (DXR, 0.2 \(\mu\)M) induces association of endogenous BLID with Bcl-X\(_L\) in MDA-MB-231 cells. Whole cell lysate (5 mg protein) was used for immunoprecipitation (IP) with monoclonal anti-Bcl-X\(_L\) antibody (4 \(\mu\)g), followed by sequential immunoblotting (IB) with anti-BLID (1:3000 dilution) and polyclonal anti-Bcl-X\(_L\) antibodies (1:2000 dilution).  

*\(B\)*, doxorubicin (DXR, 0.2 \(\mu\)M) activates caspase-9 and caspase-3 in MDA-MB 231 cells. Cells were lysed in RIPA buffer, followed by 4-12% NuPAGE and sequential immunoblotting with anti-caspase-9 antibody (1:2,000 dilution) and anti-caspase-3 antibody (1:2,000 dilution).  

*\(C\)*, cisplatin induces association of endogenous BLID with Bcl-X\(_L\).
in PA-1 cells. Cells were lysed in 0.2% NP-40 buffer and whole cell lysate (3.5 mg protein) was first pre-cleared with 30 µL of protein A/G-PLUS agarose (2 hr, 4°C), followed by incubation with 4 µg of monoclonal anti-Bcl-XL antibody and 30 µL protein A/G-PLUS agarose. The same blot was probed with anti-BLID antibody (1:3,000 dilution) and polyclonal anti-Bcl-XL antibody (1:3,000 dilution). D, cisplatin activates caspase-3 in PA-1 cells. Whole cell lysate in 1% NP-40 buffer (50 µg protein) was sequentially immunoblotted with anti-BLID, anti-caspase-3, and polyclonal anti-Bcl-XL antibodies. Whole cell extract, WCE; UT, untreated.

**Fig. 3.** A, association of reduced BLID mRNA expression with high grade breast tumors. Statistical analysis was performed using SigmaStat (Systat Software Inc.) and median BLID expression values were compared by Mann-Whitney Rank-Sum test. Grades 1 and 2, n =16; Grade 3, n = 39. B and C, immunostaining of cytoplasmic BLID in breast tissues. B, differential immunostaining of BLID in benign, IDC and ILC components located on the same tissue section from a rare case of mammary carcinoma. C, BLID immunostaining patterns in two independent IDC cores on the same tissue micro-array slide. The IHC assay was performed using 1:10000 (B) and 1:8000 dilutions (C) of the anti-BLID antibody. X200 magnification.

**Fig. 4.** Fifteen-year outcomes as a function of BLID expression in breast cancer patients (age range 25 -88 years, median age 51). Total number of specimens: BLID positive, 361; BLID negative, 73. Statistical significance was determined using the log-rank test.
Fig. 3A
B  BLID immunostaining in adjacent benign, IDC, and ILC areas on the same paraffin-embedded tissue section

C  BLID expression profiles in two independent IDC cores on the same breast TMA
**Fig. 4**

**Breast Relapse-Free Survival (years)**

- **BLID+ve**
- **BLID-ve**

**Cause-Specific Survival (years)**

- **BLID+ve**
- **BLID-ve**

**Distant Metastasis-Free Survival (years)**

- **BLID+ve**
- **BLID-ve**

**Overall Survival (years)**

- **BLID+ve**
- **BLID-ve**

- $P = 0.0114$
- $P = 0.0031$
- $P < 0.0001$
- $P = 0.0251$
The Pro-apoptotic Molecule BLID Interacts with Bcl-XL and Its Downregulation in Breast Cancer Correlates with Poor Disease Free and Overall Survival

Constantinos Broustas, Jeffrey S Ross, Qifeng Yang, et al.

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