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Frequent Loss of the *BLID* Gene in Early-Onset Breast Cancer

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Key Words

BLID gene · Early-onset breast cancer · Loss of heterozygosity

Abstract

The BH3-like motif-containing inducer of cell death *(BLID)* is an intronless gene localized on 11q24.1. Loss of that region has frequently been reported in early-onset breast cancer and is significantly associated with poor prognosis and reduced survival. Downregulation of *BLID* is associated with younger age, triple-negative phenotype, and reduced disease-free and overall survival of breast cancer patients. In this study, we investigated allelic loss of *BLID* in breast tumor specimens from 78 women with invasive breast cancer using 2 dinucleotide polymorphic markers closely linked to the *BLID* gene (no intragenic marker for *BLID* is available). Seventy-three cases were informative. Overall, loss of heterozygosity (LOH) at the *BLID* locus was detected in 32% of the informative cases (23/73). However, in patients 40 years old and younger, LOH was detected in 50% of the cases (9/18). Patients aged 40 years and younger were significantly more likely to experience LOH than those aged 41–55 years ($p = 0.04$). Specifically, the odds of *BLID* loss for patients aged 40 years and younger were 3.7 times the odds of loss for patients aged 41–55 years (95% CI, 1.1–13). Our findings suggest a tumor suppressor role of the *BLID* gene in early-onset breast cancer.

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Breast cancer is the most common cancer in women and the second leading cause of cancer death. Lymph node involvement, tumor size, histology, and hormone receptor status are the main prognostic factors used in breast cancer, even though the heterogeneous nature of the disease makes the prediction of recurrence difficult. Although breast cancer risk increases with age, the disease is more aggressive in women 40 years old or younger (early onset). Younger women are faced with a poorer prognosis, worse disease-free survival, higher mortality, and increased risk of recurrence even when controlling for stage, treatment and race [Chung et al., 1996; Sinha et al., 2008; Fredholm et al., 2009; Gnerlich et al., 2009; Sariego, 2010].

The more aggressive clinical picture in early-onset breast cancer as compared to later-onset disease includes larger tumor size, presence of positive lymph nodes, absence of steroid receptors, and high S-phase fraction, suggesting that young age may by itself be an independent factor for a poor outcome. Therefore, it stands to reason that identification of genes involved in the initiation and progression of early-onset breast cancer, correlation between alterations in these genes (e.g. loss of function, amplification) and outcome (e.g. response to therapy, survival) will improve our understanding of the disease and possibly its management in this age group. Loss of heterozygosity (LOH) studies are often used to investigate chro-

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mosomal deletions and identify possible regions harboring tumor suppressor genes [Wang et al., 2004; Rothenberg and Settleman, 2010]. LOH studies have shown extensive loss at 11q24–q25 in early-onset breast cancer and a significant association with poor prognosis and reduced survival [Gentile et al., 1999, 2001a, b; Chunder et al., 2004; Climent et al., 2007]. The high frequency of LOH at chromosome 11q24.1–11q25 in early-onset breast cancer and the association of this loss with poor prognosis of breast and ovarian neoplasias suggest that this chromosomal region may harbor putative tumor suppressor gene(s) [Ferti-Passantonopoulou et al., 1991; Gabra et al., 1996; di Iasio et al., 1999; Gentile et al., 1999, 2001a, b; Agrup et al., 2000; Choi et al., 2003; Weber-Mangal et al., 2003; Chunder et al., 2004; Jong et al., 2004; Climent et al., 2007].

BH3-like motif-containing inducer of cell death *(BLID*; alias *BRCC2)* is an intronless gene localized at 11q24.1 [Cavalli et al., 2008], an area of extensive LOH in cancer. This gene was first discovered in MDA-MB 231 human breast carcinoma cells as an approximately 1.2-kb transcript (GenBank accession numbers AF220061 and AF303179) [Broustas et al., 2004]. The longest predictive open reading frame (ORF) of *BLID* (327 bp) codes for an approximately 12-kDa protein (108 aa) localized predominantly in the cytosol and to a lesser extent in the mitochondria. Ectopic expression of *BLID* cDNA was also found in both the cytosol and mitochondria, and *BLID* was found to have a negative impact on cell survival. Functional studies have identified BLID as a novel BH3-like domain containing a pro-apoptotic molecule [Broustas et al., 2010]. Exogenous expression of *BLID* caused apoptotic cell death in 3 different cell lines (monkey kidney cells, COS-1; human prostate carcinoma cells, PC-3; and human cervical carcinoma cells, ME-180), as evidenced by enhanced chromatin condensation, DNA fragmentation or enhanced number of cells in the sub-G1 phase [Broustas et al., 2004]. Translocation of endogenous *BLID* to the mitochondria is increased in response to doxorubicin and hydrogen peroxide, known cytotoxic agents. Recently, *BLID* has been identified as a new binding partner of BCL-X_L, and BLID-induced apoptosis has been associated with activation of BAX and an increase in cytosolic cytochrome c [Broustas et al., 2010]. Unlike most other pro-apoptotic members of the *BCL2* family, *BLID* is unique, as it appears to be an emerging prognostic and therapeutic target in breast cancer. Downregulation of *BLID* expression was correlated with poor prognostic factors including younger age (median 40 years), African American ethnicity, tumor size, disease-free and overall survival, and triple-negative disease [Broustas et al., 2010].

Since somatic loss is one of the key features of tumor suppressor genes, we designed this study to determine the incidence of *BLID* loss in a series of 78 invasive sporadic breast tumors and the association of this loss with early-onset disease. The high frequency of LOH at chromosome 11q24.1–11q25 in early-onset breast cancer and the association of this loss with poor prognosis strongly suggest a role for the pro-apoptotic molecule, *BLID*, located on 11q24.1, as a tumor suppressor gene in breast cancer; loss of this gene may contribute to the development of early-onset breast cancer in women 40 years old and younger. A better understanding of the mechanisms of tumorigenesis in early-onset breast cancer may lead to better treatment strategies and improved survival in younger women.

Material and Methods

We determined allelic loss of *BLID* by LOH analysis of breast tissue specimens from 78 patients with sporadic breast cancer obtained from the tumor bank of the Histopathology and Tissue Shared Resources (HTSR) of Georgetown University Lombardi Comprehensive Cancer Center (LCCC). In each case, a paraffin block with breast tumor tissues and a second block with normal tissues (skin, negative lymph node or a normal breast tissue non-adjacent to the tumor to avoid potential field effect of genetic abnormalities) were identified. An H&E (hematoxylin/eosin-stained) slide from each block was evaluated by a pathologist to confirm the diagnosis and mark the areas with malignant tissue or normal tissue. A 100-μm consecutive section was obtained from each block, and the tissues of interest were grossly microdissected with a razor blade to insure that over 90% of the sample consisted of malignant cells. Corresponding normal cells from a different block from the same patient were obtained for each case. DNA was extracted from the tissue using the DNeasy kit (Qiagen Inc., Valencia, Calif., USA) according to the manufacturer's instructions. For the purpose of this study, we defined early-onset breast cancer as cancer occurring at the age of 40 years and younger.

To study LOH at the *BLID* locus, we selected 2 dinucleotide polymorphic markers closely linked to, and flanking, the *BLID* gene because there are no intragenic markers for *BLID*: D11S4107 (120,554,216–120,554,534) and D11S4167 (121,655,531–121,655,846) (*BLID*: 121,491,272–121,492,133). These 2 markers have a high degree of heterozygosity. The sequences of the oligonucleotide primers were obtained from the UCSC genome database (http://www.genome.ucsc.edu). Primers were fluorescently labeled, and PCR amplification was performed using a standard protocol. Allele sizes were determined by electrophoresis of PCR products in 6% denaturing polyacrylamide gels and compared to ROX 500 size standards (Applied Biosystems, Foster City, Calif., USA), using an automated sequencer (ABI 377), according to the manufacturer's instructions (Applied Biosystems). Fluorescent

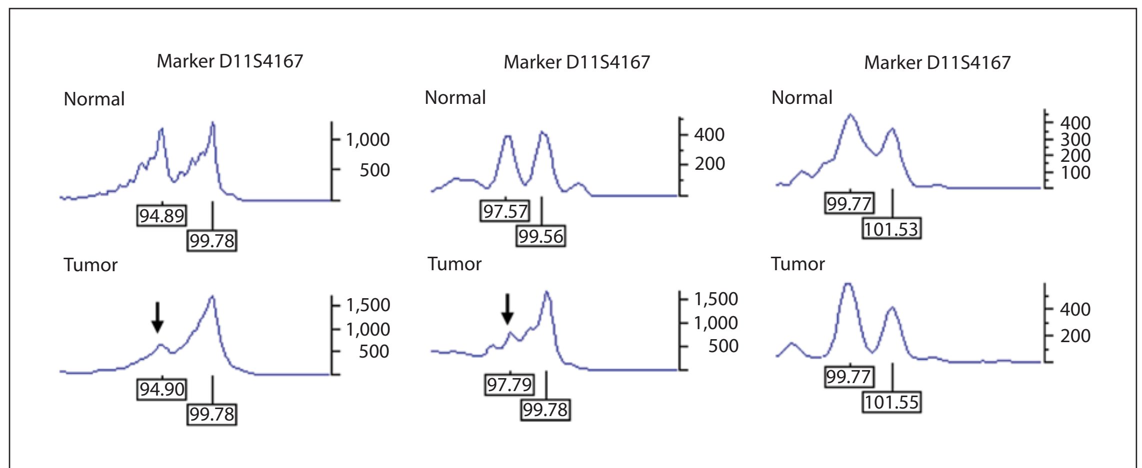

Fig. 1. Two representative cases with LOH (left and middle panels) and another representative case with no LOH (right panel) for marker D11S4167. In each case, the arrow indicates the lost allele.

Table 1. Patient characteristics by LOH status and unadjusted and adjusted odds ratios modeling the probability of loss

	Total (n = 73)		LOH (n = 23)		No LOH (n = 50)		Unadjusted			Adjusted		
							OR*	95% CI	p value	OR**	95% CI	p value
Age (years)												
≤40	18	25%	9	50%	9	50%	3.7	1.1–13	0.04	3.5	1.0–12	0.05
41–55	33	45%	7	21%	26	79%	ref			ref		
≥56	22	30%	7	32%	15	68%	1.7	0.5–5.9	0.38	1.9	0.5–6.7	0.31
Stage												
I	27	37%	6	22%	21	78%	ref			ref		
II–IV	46	63%	17	37%	29	63%	2.1	0.7–6.1	0.20	1.9	0.6–6.0	0.26

* Odds ratios describe univariable associations, modeling probability of loss. ** Odds ratios describe adjusted multivariable associations, modeling probability of loss. Referent category is denoted (ref).

signals from the different-sized alleles were recorded and analyzed using GENOTYPER version 2.1 and GENESCAN version 3.1 software (Applied Biosystems), respectively. Following visual examination of computer printouts by 2 independent observers, LOH was determined mathematically according to the Genotyper User's Manual (Applied Biosystems).

Patient characteristics were age (≤40, 41–55, ≥56) and disease stage (I, II–IV) (table 1). Logistic regression was used to evaluate the association between age and stage with LOH and to provide unadjusted and adjusted odds ratios (OR) and 95% confidence intervals for these predictors of interest. Statistical analyses were performed using SAS statistical software, version 9.2 (SAS Institute, Inc., Cary, N.C., USA).

Results

In this study, 73/78 cases (94%) were informative for the *BLID* dinucleotide markers used for LOH analysis. LOH was detected in 23 of these informative cases (23/73; 32%). Figure 1 shows LOH analysis at marker D11S4167. Two representative cases with LOH (left and middle panels) and another representative case with no LOH (right panel) are shown. In each case with LOH, the arrow indicates the lost allele. Patient characteristics and LOH results are summarized in table 1. Fifty percent of the patients with early-onset disease (age ≤40) had LOH, as opposed to 21% and 32% in the 41–55 and ≥56 age

groups, respectively. In addition, early-onset patients were significantly more likely to experience LOH at the *BLID* locus than those aged 41–55 (p = 0.04; table 1). Specifically, the odds of loss for early-onset patients were 3.7 times the odds of loss for patients aged 41–55 (95% CI, 1.1–13). Odds of loss for patients 56 years and older were not significantly different from either the early-onset or the 41–55 age group.

LOH in tumors from patients with stage I disease was observed in 22% of the cases versus 37% of the tumors from patients with later-stage disease (stages II–IV). A test for the association between LOH and age yielded nonsignificant results (χ^2 test, p = 0.88 vs. p = 0.06 for early and late stage, respectively). Therefore, stage seems to play a nonsignificant role in this relationship. In the early-onset group, 78% of the cases were of late stage compared to 58% of the cases in the late-onset group (χ^2 test, p = 0.14 for the association between stage and age). After adjusting for stage, the odds of loss for early-onset patients compared to patients aged 41–55 was slightly attenuated but remained statistically significant (OR = 3.5, 95% CI, 1.0–12, p = 0.05).

Discussion

Chromosomal deletions involving 11q is frequent in a number of different tumor types [Spitz et al., 2006; Watanabe et al., 2006; Parikh et al., 2007; Buckley et al., 2010; Fischer et al., 2010] and are associated with poor prognostic features. Recent studies on neuroblastoma, where allelic losses on 11q occur in approximately 30% of the tumors, reported that tumors with 11q loss are a distinct subgroup with different clinical and genetic behavior, as determined by array-CGH and gene expression (miRNA) analysis [Buckley et al., 2010; Fischer et al., 2010]. LOH is a powerful tool to identify areas that might harbor tumor suppressor genes. A high frequency of LOH has been reported at the 11q23–q24 region [Gudmundsson et al., 1995; Martin et al., 2003; Wang et al., 2004; Climent et al., 2007]. In this study, we observed LOH at 2 microsatellite markers closely linked and flanking the *BLID* gene on 11q24.1 in 50% of breast tumors from patients with early-onset disease (age ≤40), compared to 21 and 32% of tumors from patients in the 41–55-year-old and ≥56-year-old groups, respectively. In addition, the odds of *BLID* loss for patients 40 years and younger were 3.7 times the odds of loss for patients aged 41–55 (95% CI, 1.1–13). Despite the fact that one of the limitations of our study is the small sample size, our findings are consistent with other studies from our group showing that downregulation of the BLID protein was correlated with younger age (median 40 years), large tumor size, and decreased disease-free and overall survival, all factors of poor prognosis [Broustas et al., 2010]. In spite of the heterogeneous nature of breast cancer and the variation in incidence among age groups in different countries, studies have shown that breast cancer diagnosed at a younger age is associated with a worse prognosis and that this subset of tumors shares similar patterns of gene expression that are different than tumors from older patients [Chunder et al., 2004; Elkum et al., 2007; Anders et al., 2008a, b; Fredholm et al., 2009; Gnerlich et al., 2009].

Other studies in early-onset breast tumors support our present data, reporting frequent allelic losses on chromosome 11q. Gudmundsson et al. [1995] found 3 distinct regions of LOH on chromosome 11 in breast cancer patients of young age, with the highest frequency of LOH (37–43%) at 11q22–qter. Loss of the whole arm of this chromosome was found in 20% of early-onset cases (<35 years old, n = 44) compared to 7.4% of late-onset cases (>63 years old, n = 54) by Jong et al. [2004]. Frequent allelic deletions were reported at chromosome 11q23–q24 in approximately 50% of breast cancer tissues from mixed populations [Ferti-Passantonopoulou et al., 1991; di Iasio et al., 1999; Wang et al., 2004] and at 11q23–q25 in 11% of breast cancer tissues from women younger than 35 years [Weber-Mangal et al., 2003]. A compelling evidence for involvement of 11q (11q24.1–q25) in breast cancer in younger women (22–36 years old at diagnosis, n = 102) was provided by Gentile et al. [2001a]. In this study, LOH frequencies for the 3 markers in the 11q24.1–q25 region ranged from 51–54%. Loss at the most centromeric marker (D11S969) was associated with a high histological grade (p = 0.02). Two markers in the 11q25 region (D11S387 and D11S4125) exhibited LOH frequencies of 42 and 53%, respectively. Additional studies of LOH on 11q in early- and late-onset breast cancer were reported by Chunder et al. [2004]. This group described 6 highly deleted regions on chromosome 11 in younger and older breast cancer patients, namely 11p15.5, 11p11.2, 11q13.2, 11q22.3–23.1, 11q23.1–24.1, and 11q25. They also observed that the pattern of associations between these deletions and the amplification of the *CCND1* gene, mapped at 11q13, differed between age groups.

Genomic loss at the 11q23 region in early-onset breast cancer patients was associated with increased sensitivity to anthracycline treatment [Climent et al., 2007]. The presence of genes mapped to this region, *CHK1, H2A, ATM*, and *ZW10,* which are involved in DNA repair pathways,

suggests that deletions on 11q can lead to a functional impairment of these genes in early-onset breast cancer cases.

MicroRNA (miRNA) analysis demonstrated that deletions on 11q can also target noncoding miRNAs [Iorio et al., 2005]. The miR125b-1, which maps very closely to the *BLID* gene, has been found to be downregulated in breast tumors with 11q deletions. This miRNA may potentially regulate oncogenes such as *ETS1*, which plays a role in cell growth and is overexpressed in breast cancer. Other miRNAs closely mapped to the *BLID* gene are the mirLet7-a-2 and miR100. Overexpression of miR100 was linked to low expression of the *ATM* gene in a glioma cell line [Ng et al., 2010]. Other studies have identified alterations in miRNAs, such as miR146a, miR-453 and miR-502, to be associated with early-onset breast tumors [Song et al., 2009; Tchatchou et al., 2009; Pastrello et al., 2010].

All of these studies point to a hot spot of activity on 11q region and suggest that chromosomal loss in this region is implicated in the development of early-onset breast cancer. The idea that a chromosomal region may be the site of a potential 'tumor suppressor gene cluster' has been described for the 3p21.3 region [Ji et al., 2005] and may also apply to the telomeric region of chromosome 11q. In this respect and based on the findings of this study as well as other studies from our group [Broustas et al., 2010], we propose that the *BLID* gene located in this hot spot plays a relevant role in this group of patients. However, additional studies are needed to determine the incidence and type of *BLID* mutations. There is a definite need to identify markers for earlier detection and more targeted therapies in early-onset breast cancer patients who have poorer outcomes and higher mortality rates than older women. The *BLID* gene can potentially be one of these markers.

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