Differential roles of Bcl2L12 and its short variant in breast cancer lymph node metastasis

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Abstract. Bcl2L12 plays a role in post-mitochondrial apoptosis through multiple mechanisms involving p53, αB-crystallin, caspase-3 and -7 in glioblastoma. Bcl2L12 is reported to be a good prognostic marker in breast cancer and correlated with ER and Bcl2 expression status. However, the mechanisms by which Bcl2L12 regulates apoptosis in breast cancer (BCa) remain unknown. Recent studies have shown that Bcl2L12 expression is a useful biomarker in other types of cancer. Thus, we examined whether Bcl2L12 and Bcl2L12A mRNA were associated with breast cancer progression or a specific subtype. In total, 106 paraffin-embedded, different stage breast cancer specimens were prepared and quantified for Bcl2L12 and Bcl2L12A expression by PCR. The correlation between Bcl2L12 and Bcl2L12A mRNA levels and clinico-pathological characteristics was statistically analyzed. The results showed that Bcl2L12 and Bcl2L12A mRNA expression was not significantly different across the different stage, grade and TNM classification groups (P>0.005). Using linear regression, Bcl2L12 mRNA was associated with Bcl2L12A mRNA, grade 3 tumor and the triple-negative breast cancer (TNBC) subtype. In non-TNBC specimens, Bcl2L12 mRNA was only correlated with Bcl2L12A mRNA. Bcl2L12A mRNA was positively associated with Bcl2L12 mRNA and the number of lymph node metastases, but negatively correlated with staging in the non-TNBC group. Specifically, Bcl2L12, but not Bcl2L12A, mRNA was significantly higher in TNBC and grade 3 tumors, respectively. In non-TNBC, Bcl2L12A mRNA was significantly highly expressed in tumors with ≥12 metastatic lymph nodes. Bcl2L12 and its variant mRNA were highly expressed in carcinoma *in situ* (CIS) samples. In addition, they were estimated to be correlated with the total sample and non-TNBC, but not the TNBC group. In summary, a high Bcl2L12 mRNA expression was associated with the high-grade BCa and TNBC subtype. In addition, the interplay between Bcl2L12 and its variant may be associated with high lymph node metastasis in non-TNBC tumors.

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Introduction

Breast cancer (BCa) is the leading cause of cancer-related mortality in women worldwide (1). The incidence of BCa in Taiwan has increased from 6.23/100,000 in 1970 to 23.76 in 2000. In a 2005 report, breast cancer was the second most frequent cancer in women with an incident rate of 42.3/100,000 (2). Cases in men are extremely rare and the ratio of males to females is 0.3:100. The Bureau of Health Promotion Data from 1998 to 2002 indicate that 5-year survival for all stages was 78.37%. The 5- and 10-year survival rates were 98 and 95% for stage 0; 96 and 89% for stage I; 90 and 82% for stage II; 65 and 53% for stage III; and 22 and 10% for stage IV (3). Triple-negative breast cancer (TNBC) accounts for 15-25% of the breast cancer cases. This subtype of BCa refers to any type of breast cancer that does not express the genes for estrogen (ER) and progesterone (PR) receptors, and Her2/neu (4). It is thought to be more aggressive and to respond poorly to hormone therapy, and is more difficult to treat since there is no receptor target to be antagonized. The risk of relapse in TNBC is also much higher for the first 3-5 years.

The Bcl2-like 12 (*Bcl2L12*) gene was identified and cloned by Scorilas *et al* (5) in 2001, and is a proline-rich (PxxP) protein and a newly identified member of the Bcl2 family, containing a highly conserved BH2 domain, a BH3-like motif and a proline-rich region. Currently, two splicing variants of the *Bcl2L12* gene are known: one consisting of seven coding exons and producing a 334-amino acid protein with a molecular mass of 36.8 kDa, and another resulting from alternative splicing, leading to a protein of 176 amino acids, a splice variant known as Bcl2L12A which lacks exon 3 (143 bp) (5).

Expression of the full-length mRNA transcript has been observed in many tissues, including breast, thymus, prostate, fetal liver, colon, placenta, pancreas, small intestine, spinal cord, kidney and bone marrow, whereas Bcl2L12A is mainly expressed in fetal liver, spinal cord and skeletal muscle (5). Bcl2L12 and Bcl2L12A are localized within the nucleus (6). The biological role of Bcl2L12 is not yet completely understood and remains paradoxical. In previous studies, Bcl2L12 and Bcl2L12A exhibited pro-apoptotic activity in BCa and gastric cancer (7-9). A 3-fold increase of Bcl2L12 levels was demonstrated in non-cancerous compared to cancerous stomach tissues (9). In BCa, the two proteins are highly expressed in normal breast tissue, and Bcl2L12 has been identified as a favorable prognostic marker. Knockdown of its expression leads to cisplatin-resistance in the MDA-MB-231 BCa cell line (7). In nasopharyngeal cancer, th eBcl2L12 expression status was also found to be positively associated with distant metastases and to be an unfavorable and independent prognostic indicator of short-term relapse. Bcl2L12 mRNA expression may thus constitute a novel biomarker for the prediction of short-term relapse in nasopharyngeal carcinoma. By contrast, Bcl2L12 and Bcl2L12A are ubiquitously overexpressed in primary human GBMs and may be associated with resistance to chemotherapeutic agent-induced apoptosis, which is an important hallmark of this disease (10). Furthermore, Bcl2L12 plays an anti-apoptotic role in GBM and blocks post-mitochondrial apoptotic signaling by inhibiting effectors caspase-3 and -7 (11-13). Besides that, Bcl2L12 attenuates endogenous p53-directed transcriptomic changes after genotoxic stress and inhibits p53-dependent DNA damage-induced apoptosis (10). The anti-apoptotic role of Bcl2L12 and Bcl2L12A was found to be regulated by GSK3β in glioblastoma and was inhibited by LiCl (14). ERβ5 was observed to interact with Bcl2L12 in a novel estrogen-independent molecular pathway that promotes cisplatin and/or doxorubicin-induced *in vitro* apoptosis of the MCF-7 and MDA-MD-231 BCa cell lines (15). Taken together, the roles of Bcl2L12 and its short variant in BCa remain largely unknown and contradictory. Moreover, it is also unclear whether Bcl2L12 and Bcl2L12A mRNA can be used as biomarkers for Bca progression and/or a subtype of BCa. Therefore, in this study we screened and analyzed the expression of Bcl212 and Bcl2L12A mRNA in clinical specimens to address these issues.

Table I. Clinicopathological characteristics of BCa specimens.

Clinical diagnostic BCa type			Total
Invasive tumors	102		106[a]
Invasive ductal carcinoma	89		
Invasive lobular carcinoma	4		
Invasive papillary carcinoma	1		
Medullary carcinoma, atypical	1		
Mucinous adenocarcinoma	7		
Non-invasive tumors	4		
Ductal CIS	2		
Intraductal papillary carcinoma	2		
Histological grade			105
Low (I and II)	97		
High (III)	8		
HER2/neu IHC			105
Negative (0 to +1)	56		
Positive (+2 to +3)	49		
Lymph node metastasis			104[b]
Negative	60		
Positive	34		
TNM staging			100[c]
T1 : 31	n0 : 64	m0 : 95	
T2 : 58	n1a : 17	m1 : 5	
T3 : 5	n2a : 9		
T4 : 3	n3a : 10		
Tis : 2			
Tx : 1			

[a]One case of medullary carcinoma, atypical was not analyzed, [b]One case was not examined for lymph node metastasis, [c]Five cases without TNM staging data. BCa, breast cancer; CIS, carcinoma *in situ*; IHC, immunohistochemistry.

Materials and methods

Tissue collection. A total of 106 paraffin-embedded BCa tissues and 1 flesh tumor tissue were collected with the permission of the Institutional Review Board of Kaohsiung Armed Forces General Hospital in 2013. The expression profile of Bcl2L12 and Bcl2L12A was assessed and analyzed to determine whether they were correlated. The clinicopathological characteristics of these tumors are shown in Table I. There were 102 (96.23%) invasive and 4 (3.77%) non-invasive tumors. The invasive tumor group included ductal (n=89, 87.25%), lobular (n=4, 3.92%), papillary (n=1, 0.98%) carcinomas, medullary carcinomas, atypical (n=1, 0.98%) and mucinous adenocarcinoma (n=7, 6.86%). The non-invasive tumors included ductal carcinoma *in situ* (CIS) (n=2, 50.0%) and intraductal papillary carcinoma (n=2, 50.0%). Histological grades were classified into the low (grade I and II, n=97, 91.5%) and high (grade III, n=8, 8.5%) grade groups. HER2/neu protein expression was negative (0 to +1) in 56 specimens and positive in 49 (+2 to +3). In TNM staging for tumor size or direct extent of the primary tumor, T2 stage (n=58) predominated followed by the T1 stage (n=31), T3 stage (n=3), T4 stage (n=3) and CIS (Tis, n=2). Regarding the spread to regional lymph nodes, tumor cells were absent from regional lymph nodes in 64 (N0) and regional lymph node metastasis was present in 36 (N1). For distant metastasis, samples were grouped into no distant metastasis (n=95) and metastasis to distant organs (n=5).

RNA extraction. Total RNA was prepared from paraffin-embedded breast cancerous or normal tissue using a PureLink FFPE Total RNA Isolation kit (Invitrogen-Life Technologies, Carlsbad, CA, USA). Deparaffination, purification and washing were conducted according to the manufacturer's instructions. RNA quality was determined by the ratio of OD260 vs OD280 nm. The RNA concentration was deter-

mined by detecting absorbance at 280 nm on a Tecan Infinite M200 multiscan spectrophotometer (Tecan Group, Bayonne, NJ, USA).

cDNA synthesis. First-strand cDNA synthesis was carried out using the ImProm-II Reverse Transcription system (Promega, Madison, WI, USA). Briefly, up to 1 μg of total RNA was premixed with Oligo (dT) and random hexamers in a vial, then heated at 70˚C for 5 min and chilled at 4˚C for 5 min for pre-denaturation. Subsequently, 4 μl ImProm-II 5X reaction buffer, 25 mM $MgCl_2$, 1 μl 10 mM dNTP Mix, 20 units ribonuclease inhibitor and nuclease-free water were added to a 1.5 ml eppendorf tube. Then, 1 μl ImProm-II Reverse Transcriptase was added to a final volume of 15 μl and incubated at 25˚C for 5 min for primer annealing, 42˚C for 60 min for synthesis, and 70˚C for 15 min to inactivate the enzyme. The generated cDNA were stored at -20˚C for quantitative PCR (qPCR).

Quantitative PCR. A total of 106 BCa tissues underwent qPCR. Detection of mRNA expression levels with respect to endogenous Bcl2L12 and Bcl2L12A was performed by EZtime 2X SYBR-Green Premix real-time PCR (Yeastern Biotech, Taipei, Taiwan). The thermal cycling program was carried out on an IQ5 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The increase in fluorescence emission (Rn) was measured during PCR amplification, and the difference (ΔRn) between the fluorescence emission of the product and the baseline was calculated with IQ5 Optical System software (Bio-Rad) and plotted against the cycle number. Threshold cycle values were then calculated by determining the point at which the emitted fluorescence exceeded the threshold, determined as 10-fold the standard deviation of the baseline of cycles 3-15 (16). The reaction mixture contained 10 ng cDNA diluted in 2.5 μl diethylpyrocarbonate-treated water (Applied Biosystems, Foster City, CA, USA), 12.5 μl EZtime 2X SYBR-Green Premix PCR and 2 μl gene-specific primers (final concentration: 50 nmol/l each), in a final reaction volume of 25 μl. The reaction conditions were set up as follows: For Bcl2L12 and Bcl2L2A: denaturation of the template and activation of DNA polymerase at 95˚C for 10 min, followed by 45 cycles of 95˚C for 20 sec; for denaturation of the PCR products, 58˚C for 1 min for primer annealing and 72˚C for 15 sec for extension. The conditions for β-actin were: denaturation of the template and activation of DNA polymerase at 95˚C for 10 min followed by 45 cycles of 95˚C for 10 sec for denaturation of the PCR products, 58˚C for 1 min for primer annealing and 55˚C for 15 sec for extension. Each RT-PCR experiment was performed in duplicate to evaluate data reproducibility. To distinguish the main PCR products from primer-dimers or other non-specific products, a melting curve analysis of the PCR products was generated after amplification by heating the reaction mixtures at 60-95˚C with a heat ramping rate of 0.1˚C/sec while continuously acquiring fluorescence emission data (18). The melting temperatures (Tm) of the target genes and β-actin amplicons were expected to be 80 and 85˚C respectively, whereas primer-dimers or other non-specific products were characterized by a much lower Tm (up to 75.0˚C). The calculations and validation of the comparative CT ($2^{-\Delta\Delta CT}$) method were used for target gene mRNA quantification. The application of this method was based on the hypothesis that the PCR amplification efficiencies of the target and the reference genes were similar to each other and close to 100% (17). The prerequisites for the application of the $2^{-\Delta\Delta CT}$ method were checked in a validation experiment, in which CT values of target genes and β-actin were measured in a dilution series of control cDNA over a 10^6-fold range and then plotted against log cDNA dilution according to a previous study (18). RT-PCR efficiency (E%) for amplification of each gene was calculated using the formula: $E\% = [-1 + 10^{(-1/\alpha)}] \times 100$, where α is the slope of the corresponding amplification plot (18). β-actin was used as a reference gene to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. Normalized results were expressed in the ratio of target gene mRNA copies to β-actin mRNA copies (c/c). The results were the average of data from at least triplicate experiments and were shown as fold increase ± SEM. All primer pairs with respect to Bcl2L12, Bcl2L12A and β-actin were designed using a web-based program provided by GeneScript.com. The primer pairs were qualified and demonstrated: i) High amplification efficiency (≥96%) across a wide range of cDNA dilutions (see Fig. 3); ii) specific single products in dissociation curve analysis; and iii) Tm similar to those predicted by oligonucleotide software. The sequences of each primer pair were as follows: Bcl2L12 forward, 5'-GGTCCAA GAGCAGCTGAAAT-3' and Bcl2L12 and reverse, 5'-AGGCC AGCTTCTGGTTAATG-3'; Bcl2L12A forward, 5'-CCACCT AGGCCCAGCTACT-3' and Bcl2L12A and reverse, 5'-CGGA GATTTCAGCTGCTCTT-3'; β-actin forward, 5'-GACATC CGCAAAGACCTGTA-3', and β-actin reverse, 5'-GGAGCA ATGATCTTGATCTTCA-3'. The amplification strategy for Bcl2L12 and Bcl2L12A mRNA is shown in Fig. 1.

Statistical analysis. Statistical procedures were conducted using SPSS 22.0 statistical software (IBM). A Kruskal-Wallis test was applied to discriminate whether expression levels with respect to Bcl2L12 and Bcl2L12A mRNA were significantly different across different stage, grade and TNM staging breast cancer groups. The Stepwise and Enter methods, respectively, were used in linear regression to determine associated factors for Bcl2L12 and Bcl2L12A mRNA expression in total, TNBC and non-TNBC samples. The analyzed variables included diagnostic age, clinical diagnosis type, tumor size, number of metastastic lymph nodes, TNM staging, staging, Her2 expression, ER status, PR status, histological grade, lymph node metastasis, invasive status, grade 3, TNBC, Bcl2L12 mRNA and Bcl2L12A mRNA. The different expression levels of Bcl2L12 and Bcl2L12A in dichotomous groups were assessed using the independent sample Student's t-test. The linear regression formula and plot of Bcl2L12 against Bcl2L12A were estimated using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA). $P<0.05$, $\beta>0.14$ and $r^2>0.14$ were considered statistically significant.

Results

Since the amplification efficiencies of the target (Bcl2L12 and Bcl2L12A) and reference gene (β-actin) were approximately

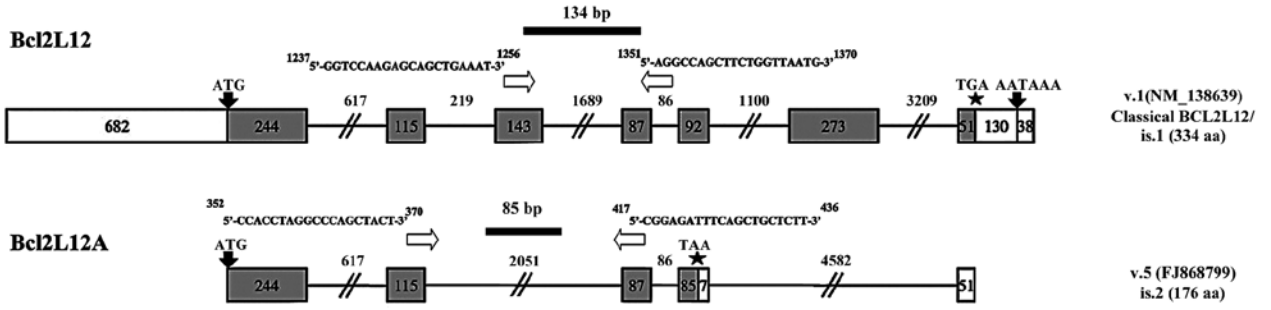

Figure 1. Bcl2L12 and Bcl2L12A amplification strategy in RT-PCR. The black lines are the amplicon of each gene. Empty arrows refer to the orientation of primer annealing.

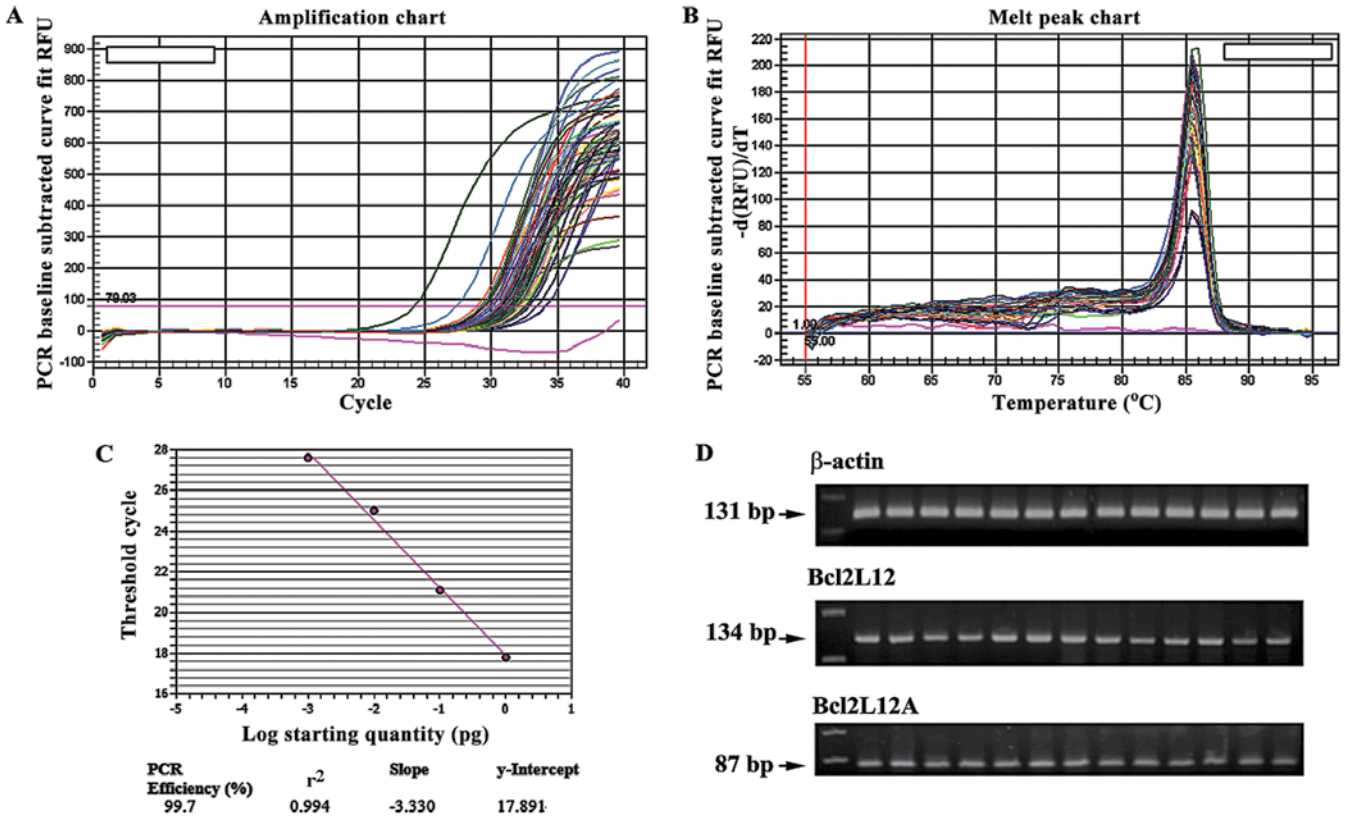

Figure 2. Amplification plot, melting curve analysis and standard curve of Bcl2L12 quantitative PCR. (A) Amplification plot shows Bcl2L12 mRNA expression after baseline Rn signaling subtraction. (B) A single peak was detected in the melting curve analysis. (C) The standard curve of Bcl2L12. (D) Gel electrophoretic patterns for β-actin, Bcl2L12 and Bcl2L12A.

equal, the ΔΔCt calculations of our RT-PCR data were valid. The slopes of the Bcl2L12, Bcl2L12A and β-actin standard curves were similar (-3.361, -3.341 and -3.440, respectively), and showed similar efficiencies to the corresponding amplicons (98.4, 99.0 and 98.3%, respectively). To confirm the efficient and specific amplification, the standard curve and melting curve analysis was generated for each target gene and the reference gene. As shown in Fig. 2, Bcl2L12 amplification showed a good amplification efficiency of 99.7 and r^2 number of 0.994 of the standard curve. Checking the products by gel electrophoresis, the three target gene amplifications had the expected size bands as shown in Fig. 2D.

The expression levels with respect to Bcl2L12 and Bcl2L12A mRNA in our samples were estimated for the skewness value of 3.16-6.58 and kurtosis index of 9.38-44.828 (data not shown), suggesting that they were unlikely to be normally distributed. Therefore, a non-parametric statistical method, the Kruskal-Wallis test, was applied to discriminate whether expression levels with respect to Bcl2L12 and Bcl2L12A mRNA were significantly different across categories of stage, grade and TNM staging. As shown in Table II, there was no significant difference in the Bcl2L12 and Bcl2L12A mRNA expression for the categories of stage, grade and TNM staging ($P>0.05$). This result suggested that Bcl2L2 and Bcl2L12A mRNA expression were unlikely to be associated with BCa progression.

The correlation between Bcl2L12 and Bcl2L12A mRNA levels and clinicopathological characteristics was analyzed

Table II. mRNA expression of Bcl2L12 and its variant in different BCa groups.

Variables	Stage	No.	Mean rank	Kruskal-Wallis chi-square	df	Asymp. Sig.[a]
Bcl2L12	0 (CIS)	3	89.33	12.959	7	0.073
	IA	24	45.42			
	IIA	37	47.00			
	IIB	13	46.69			
	IIIA	7	53.29			
	IIIC	9	67.67			
	IV	4	27.75			
	V	1	54.00			
	Total	98				
Bcl2L12A	0 (CIS)	3	72.33	5.072	7	0.651
	IA	24	48.79			
	IIA	37	45.57			
	IIB	13	47.62			
	IIIA	7	51.43			
	IIIC	9	50.44			
	IV	4	69.50			
	V	1	66.00			
	Total	98				
	Grade	N	Mean rank	Kruskal-Wallis chi-square	df	Asymp. Sig.[a]
Bcl2L12	I	5	44.80	0.561	2	0.756
	II	90	52.82			
	III	8	47.25			
	Total	103				
Bcl2L12A	I	5	53.80	0.028	2	0.986
	II	90	51.82			
	III	8	52.88			
	Total	103				
	TNM	N	Mean rank	Kruskal-Wallis chi-square	df	Asymp. Sig.[a]
Bcl2L12	T0N0M0	3	87.67	16.616	15	0.342
	T1N0M0	24	44.79			
	T1N1M0	3	38.33			
	T1N2M0	1	11.00			
	T1N2M1	1	53.00			
	T1N3M0	1	67.00			
	T2N0M0	33	47.15			
	T2N1M0	10	48.10			
	T2N1M1	1	27.00			
	T2N2M0	6	59.33			
	T2N3M0	6	69.50			
	T2N3M1	1	34.00			
	T3N0M0	3	39.33			
	T3N1M0	1	38.00			
	T3N1M1	1	16.00			
	T3N3M0	1	29.00			
	Total	96				
Bcl2L12A	T0N0M0	3	72.00	12.588	15	0.634
	T1N0M0	24	48.63			
	T1N1M0	3	19.00			

Table II. Continued. mRNA expression of Bcl2L12 and its variant in different BCa groups.

Variables	Stage	No.	Mean rank	Kruskal-Wallis chi-square	df	Asymp. Sig.[a]
	T1N2M0	1	18.00			
	T1N2M1	1	66.00			
	T2N0M0	33	48.88			
	T2N1M0	10	44.90			
	T2N1M1	1	75.00			
	T2N2M0	6	56.50			
	T2N3M0	6	46.67			
	T2N3M1	1	53.00			
	T3N0M0	3	56.67			
	T3N1M0	1	11.00			
	T3N1M1	1	65.00			
	T3N3M0	1	60.00			
	Total	96				

[a]Kruskal Wallis test.

Table III. Parsimonious results of linear regression by stepwise method to identify the associated factors with respect to mRNA expression of Bcl2L12 and its variant.

		Unstandardized coefficients		Standardized coefficients			95.0% CI for B	
Model	factors	B	Std. Error	β	t	Sig.	Lower bound	Upper bound
1	(Constant)	-98.068	155.768		-0.630	0.531	-407.623	211.487
	Bcl2L12A mRNA	0.103	0.027	0.356	3.782	<0.001	0.049	0.157
	Grade 3	1060.372	485.531	0.205	2.184	0.032	95.481	2025.263
	TNBCa	617.757	309.898	0.189	1.993	0.049	1.900	1233.614
2	(Constant)	80.170	35.256		2.274	0.026	9.836	150.503
	Bcl2L12A mRNA	0.037	0.008	0.473	4.465	<0.001	0.021	0.054
3	(Constant)	1707.252	474.109		3.601	0.001	765.351	2649.153
	Bcl2L12 mRNA	1.327	0.336	0.385	3.953	<0.001	0.660	1.995
4	(Constant)	3231.182	1084.556		2.979	0.004	1066.399	5395.964
	Bcl2L12 mRNA	4.865	1.236	0.384	3.936	<0.001	2.398	7.332
	Number of metastatic lymph nodes	381.132	88.158	0.505	4.323	<0.001	205.168	557.095
	Staging	-142.309	51.131	-0.324	-2.783	0.007	-244.367	-40.250

Models 1-2: Dependent variable is Bcl2L12 mRNA; models 3-4: Dependent variable is Bcl2L12A mRNA; models 1 and 3 denote total samples; models 2 and 4 denote non-TNBC group; Sig.: P-value. CI, confidence interval.

using linear regression. As shown in Table III, when the stepwise method was applied, the Bcl2L12 mRNA level was highly correlated with the Bcl2L12A mRNA level (β=0.356, P<0.001), grade 3 tumors (β=0.205, P=0.032) and TNBC status (β=0.189, P=0.049) in the total sample. However, grade 3 was no longer associated with a high Bcl2L12 mRNA expression in the total sample (β=0.196, P=0.229) when the Enter method was used (Table IV). Alternatively, Bcl2L12 was relevant to the number of metastatic lymph nodes (β=0.837, P=0.030) and grade 3 tumors (β=0.904, P=0.016) in the TNBC group (Table IV). Bcl2L12 mRNA was only correlated with Bcl2L12A mRNA expression in the non-TNBC group (β=0.473, P<0.001). Conversely, Bcl2L12A was solely associated with Bcl2L12 mRNA expression in the total sample (Table III, β=0.385, P<0.001) and was correlated with Bcl2L12 mRNA (Table III, β=0.384, P<0.001), number of metastatic lymph nodes (Table III, β=0.505, P<0.001) and staging (Table III, β=-0.324, P=0.007) in the non-TNBC

Table IV. Results of linear regression using the Enter method to identify the associated factors with respect to mRNA expression of Bcl2L12 and its variant.

Model	factors	Unstandardized Coefficients		Standardized Coefficients			95.0% CI for B	
		B	Std. Error	β	t	Sig.	Lower bound	Upper bound
1	(Constant)	1201.310	2207.699		0.544	0.588	-3193.005	5595.625
	Bcl2L12A mRNA	0.096	0.031	0.332	3.122	0.003	0.035	0.158
	TNBCa	741.683	335.668	0.227	2.210	0.030	73.553	1409.813
	TNM staging	-.309	4.092	-0.017	-0.075	0.940	-8.453	7.836
	Grade 3	1009.777	832.876	0.196	1.212	0.229	-648.022	2667.575
	Staging	8.852	26.723	0.057	0.331	0.741	-44.338	62.042
	Histological grade	455.355	627.208	0.120	0.726	0.470	-793.071	1703.782
	No. of metastatic lymph nodes	8.389	41.075	0.029	0.204	0.839	-73.368	90.147
	Invasive	-1460.935	1134.381	-0.346	-1.288	0.202	-3718.863	796.993
	Lymph node metastasis	40.941	415.754	0.014	0.098	0.922	-786.596	868.479
	Tumor size	-108.108	128.346	-0.153	-0.842	0.402	-363.574	147.359
	Clinical diagnosis type	-193.893	313.043	-0.178	-0.619	0.537	-816.990	429.203
	Diagnostic age	-8.884	11.252	-0.083	-0.789	0.432	-31.281	13.514
2	(Constant)	6960.557	9841.433		0.707	0.499	-15733.829	29654.942
	Bcl2L12A mRNA	0.077	0.090	0.194	0.850	0.420	-0.131	0.285
	Diagnostic age	36.151	44.194	0.203	0.818	0.437	-65.761	138.063
	Clinical diagnosis type	-1822.850	1963.595	-0.738	-0.928	0.380	-6350.909	2705.209
	Tumor size	-1478.243	645.415	-1.366	-2.290	0.051	-2966.572	10.087
	No. of metastatic lymph nodes	679.731	258.063	0.837	2.634	0.030	84.636	1274.825
	TNM staging	38.209	19.514	1.120	1.958	0.086	-6.789	83.208
	Staging	-123.668	96.783	-0.436	-1.278	0.237	-346.851	99.514
	Her2 expression	2298.805	1374.020	0.362	1.673	0.133	-869.692	5467.301
	Histological grade	1078.154	1614.687	0.213	0.668	0.523	-2645.320	4801.629
	Lymph node metastasis	-4828.536	2355.310	-0.842	-2.050	0.075	-10259.890	602.818
	Invasive	-9889.988	6180.396	-1.280	-1.600	0.148	-24142.006	4362.030
	Grade 3	6985.884	2306.121	0.904	3.029	0.016	1667.960	12303.808
3	(Constant)	-415.603	601.281		-0.691	0.492	-1620.114	788.909
	Bcl2L12A mRNA	0.035	0.010	0.450	3.409	0.001	0.015	0.056
	Diagnostic age	4.948	3.159	0.194	1.566	0.123	-1.380	11.276
	Clinical diagnosis type	110.386	77.675	0.476	1.421	0.161	-45.215	265.987
	Tumor size	68.108	34.841	0.382	1.955	0.056	-1.687	137.903
	No. of metastatic lymph nodes	3.603	10.436	0.061	0.345	0.731	-17.304	24.509
	TNM staging	-1.482	1.025	-0.352	-1.447	0.154	-3.535	0.570
	Staging	5.746	7.202	0.166	0.798	0.428	-8.681	20.172
	Her2 expression	-85.187	73.381	-0.141	-1.161	0.251	-232.186	61.812
	Histological grade	53.824	94.210	0.090	0.571	0.570	-134.902	242.550
	Lymph node metastasis	21.775	33.721	0.088	0.646	0.521	-45.777	89.326
	Invasive	-168.374	217.986	-0.162	-0.772	0.443	-605.053	268.305
	Grade 3	-57.712	99.344	-0.092	-0.581	0.564	-256.722	141.298
4	(Constant)	-13851.468	7453.258		-1.858	0.067	-28686.807	983.872
	TNBCa	1055.147	1184.807	0.094	0.891	0.376	-1303.152	3413.446
	TNM staging	17.390	13.950	0.271	1.247	0.216	-10.377	45.158
	Grade 3	-3106.166	2872.626	-0.174	-1.081	0.283	-8823.986	2611.653
	Staging	-45.721	91.915	-0.086	-0.497	0.620	-228.674	137.232

Table IV. Continued. Results of linear regression using the Enter method to identify the associated factors with respect to mRNA expression of Bcl2L12 and its variant.

Model	factors	Unstandardized Coefficients		Standardized Coefficients			95.0% CI for B	
		B	Std. Error	β	t	Sig.	Lower bound	Upper bound
	Histological grade	2444.395	2148.886	0.187	1.138	0.259	-1832.856	6721.646
	No. of metastatic lymph nodes	328.809	136.518	0.328	2.409	0.018	57.077	600.542
	Invasive	5633.146	3894.765	0.387	1.446	0.152	-2119.189	13385.480
	Tumor size	-370.230	441.864	-0.151	-0.838	0.405	-1249.738	509.278
	Clinical diagnosis type	1825.681	1060.586	0.484	1.721	0.089	-285.363	3936.725
	Diagnostic age	41.296	38.611	0.112	1.070	0.288	-35.558	118.150
	Bcl2L12 mRNA	1.141	0.365	0.331	3.122	0.003	0.414	1.868
5	(Constant)	-16618.637	37593.474		-0.442	0.670	-103309.343	70072.069
	Diagnostic age	137.144	165.604	0.305	0.828	0.432	-244.740	519.029
	Clinical diagnosis type	-473.151	7749.804	-0.076	-0.061	0.953	-18344.230	17397.928
	Tumor size	-2196.546	3016.639	-0.803	-0.728	0.487	-9152.928	4759.836
	No. of metastatic lymph nodes	-473.285	1312.034	-0.231	-0.361	0.728	-3498.840	2552.270
	TNM staging	47.881	87.395	0.555	0.548	0.599	-153.653	249.416
	Staging	143.306	395.105	0.200	0.363	0.726	-767.806	1054.419
	Her2 expression	-1857.754	5951.652	-0.116	-0.312	0.763	-15582.287	11866.780
	Histological grade	6187.159	5825.695	0.484	1.062	0.319	-7246.917	19621.235
	Lymph node metastasis	-5737.446	10720.572	-0.396	-0.535	0.607	-30459.130	18984.238
	Invasive	-1871.498	26626.121	-0.096	-0.070	0.946	-63271.444	59528.448
	Grade 3	-7020.365	12429.037	-0.360	-0.565	0.588	-35681.777	21641.046
	Bcl2L12 mRNA	1.079	1.270	0.427	0.850	0.420	-1.849	4.008
6	(Constant)	-1754.655	7050.335		-0.249	0.804	-15878.173	12368.863
	Diagnostic age	-29.233	37.502	-0.090	-0.780	0.439	-104.358	45.892
	Clinical diagnosis type	654.643	919.482	0.223	0.712	0.479	-1187.302	2496.587
	Tumor size	-179.240	419.998	-0.079	-0.427	0.671	-1020.597	662.117
	No. of metastatic lymph nodes	358.237	112.268	0.475	3.191	0.002	133.336	583.137
	TNM staging	15.254	12.021	0.286	1.269	0.210	-8.826	39.335
	Staging	-178.224	81.186	-0.406	-2.195	0.032	-340.860	-15.589
	Her2 expression	585.023	863.977	0.077	0.677	0.501	-1145.730	2315.776
	ER status	-634.557	1100.531	-0.083	-0.577	0.567	-2839.186	1570.071
	PR status	116.982	395.094	0.037	0.296	0.768	-674.485	908.450
	Histological grade	1196.637	2555.108	0.091	0.468	0.641	-3921.859	6315.133
	Lymph metastasis	-920.613	1157.537	-0.116	-0.795	0.430	-3239.437	1398.211
	Invasive	2211.404	3558.910	0.184	0.621	0.537	-4917.949	9340.756
	Grade 3	828.127	3043.055	0.050	0.272	0.787	-5267.844	6924.098
	Bcl2L12 mRNA	4.843	1.421	0.382	3.409	0.001	1.997	7.689

Models 1-3: Dependent variable is Bcl2L12 mRNA; models 4-6: Dependent variable is Bcl2L12A mRNA; clinical diagnostic type indicates diagnostic BCa type; models 1 and 4 denote total samples; models 2 and 5 denote TNBC group; models 3 and 6 denote non-TNBC group. CI, confidence interval.

group when using the stepwise method. Nevertheless, when the Enter method was used, Bcl2L12A mRNA was additionally associated with the number of metastatic lymph nodes (Table IV, β=0.328, P=0.018) in the total sample and correlated with Bcl2L12 mRNA (Table IV, β=0.382, P=0.001), number of metastatic lymph nodes (Table IV, β=0.475,

Table V. The Student's t-test was used to discriminate the difference between dichotomous groups of BCa.

Variables	Groups	N	Mean	Std. Error Mean	t-value	P-value
Bcl2L12	**Non-TNBC**	**79**	**157.3000**	**39.02000**	**-2.487**	**0.014**
	TNBC	**24**	**899.7200**	**531.58000**		
Bcl2L12A	**Non-TNBC**	**79**	**1665.8200**	**412.22000**	**-1.371**	**0.174**
	TNBC	**24**	**3104.3300**	**1346.37000**		
Bcl2L12	Non-grade 3	97	245.6278	92.84499	-2.203	0.030
	Grade 3	8	1280.0088	1250.11850		
Bcl2L12A	Non-grade 3	97	1976.7996	468.62588	-0.013	0.989
	Grade 3	8	1998.6525	882.57020		
Bcl2L12	≥12 metastatic lymph nodes	6	264.4200	124.34000	0.989	0.326
	<12 metastatic lymph nodes	65	342.9000	148.20000		
Bcl2L12A	≥12 metastatic lymph nodes	6	5230.9800	3212.87000	2.363	0.021
	<12 metastatic lymph nodes	65	1484.1300	393.26000		
Bcl2L12	≥ Stage IA	73	147.7100	38.71000	-2.654	0.010
	< Stage IA	2	795.8700	546.37000		
Bcl2L12A	≥ Stage IA	73	1559.1700	397.70000	-2.710	0.008
	< Stage IA	2	8538.6600	7461.89000		

Bold area denotes the results from analysis of the non-TNBC group.

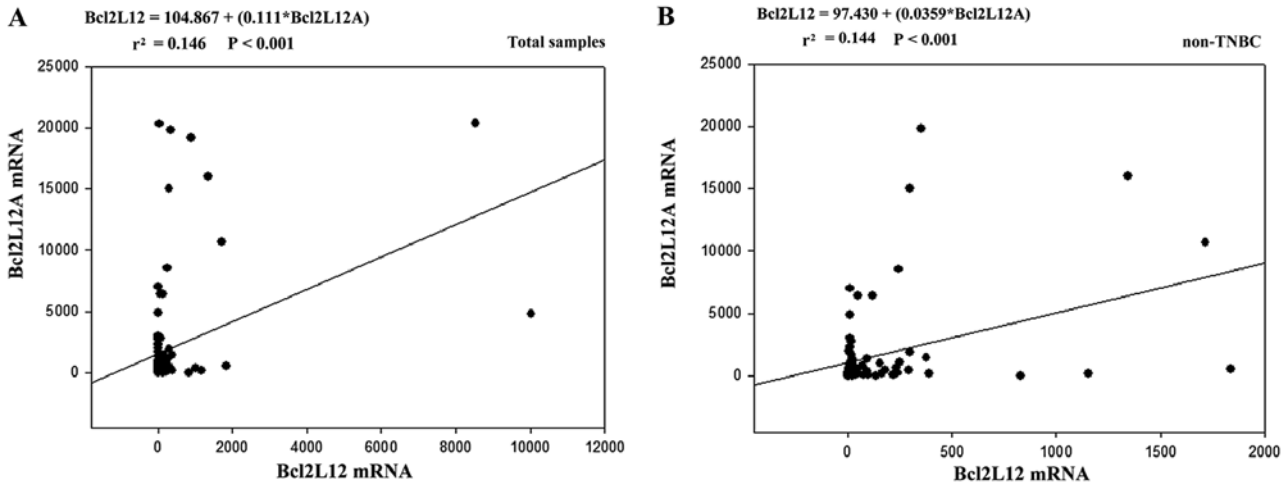

Figure 3. Regression plots of Bcl2L12 mRNA expression versus Bcl2L12A mRNA in total sample (A) or the non-TNBC group (B).

P=0.002) and staging (Table IV, β=-0.406, P=0.032) in the non-TNBC group, but not correlated with any analyzed variable in TNBC tumors (Table IV, P>0.05). These results suggested that Bcl2L12 and Bcl2L12A have an unequal impact on TNBC and non-TNBC. Bcl2L12 has a unique role in TNBC, and a high expression correlated with high-grade tumor and the number of metastatic lymph nodes. Bcl2L12A was correlated with Bcl2L12 in non-TNBC and their association was possibly linked to the severity of lymph node metastasis. We assessed whether Bcl2L12 and/or Bcl2L12A were unequally expressed in the dichotomous group of BCa. As a result, we observed that Bcl2L12, but not Bcl2L12A, mRNA had a significantly higher expression in the TNBC than that in the non-TNBC group (Table V, P=0.014) and was also more highly expressed in grade 3 than non-grade 3 tumors (Table V, P=0.030). Conversely, in the non-TNBC group, tumors with metastatic lymph nodes ≥12 were found to have a higher Bcl2L12A expression (Table V, P=0.021) than their counterparts. The CIS samples also showed a higher expression of Bcl2L12 and Bcl2L12A than other samples (Table V, Bcl2L12: P=0.010; Bcl2L12A: P=0.008). Since, as mentioned above, Bcl2L12 was correlated with Bcl2L12A expression in the total sample and non-TNBC group, we plotted the regression line and estimated the regression formula as shown in Fig. 3 (total sample: Bcl2L12=104.867 +(0.111*Bcl2L12A); non-TNBC: Bcl2L12=97.430+(0.0359* Bcl2L12A). According to the result and regression formula, Bcl2L12A mRNA expression had a 3-fold enhancement in the non-TNBC group compared to the total sample. In addition, Bcl2L12 was correlated with Bcl2L12A expression

with a low level index (r^2=0.144-0.146, P<0.001). These data indicated that the interplay between Bcl2L12 and its variant may be linked to non-TNBC tumors.

Discussion

In BCa, Bcl2L12 expression is initially recognized as a good prognostic marker and is associated with long-term survival (19). Using a conventional RT-PCR approach, Bcl2L12 mRNA shows a relatively higher expression in low stage (I/II) and grade (I/II) tumors. The chemotherapeutic agent taxol, used to treat the MCF-7 BCa line, downregulates Bcl2L12A and caspase-9 expression, but causes elevation of Bax expression. Thomadaki *et al* (20) suggested that Bcl2L12 and Bc12L12A were potential biomarkers for predicting patient outcome after chemotherapy. Antineoplastic agents, such as cisplatin, carboplatin and doxorubicin have also been tested in different BCa lines. Bcl2L12 has been found to be associated with BCa chemoresistance associated with Bcl2 and can be modulated by chemotherapeutic drugs. The hypothesis of Bcl2L12 as a marker of favorable outcome in BCa is based on the finding that Bcl2L12 is highly expressed in low-stage clinical samples (8) and on acquired resistance to cisplatin consequent to RNAi-based knockdown of Bcl2L12 in the BCa MDA-MB-231 cancer cell line (7). Apparently, ectopically expressed GFP-tagged Bcl2L12 and Bcl2L12A inhibit CHO cell growth. However, Bcl2L12 is more likely to trigger apoptosis, whereas Bcl2L12A as a nuclear protein affects phosphorylation of cyclin B1 and interferes with the G2/M transition in the cell cycle to cause growth arrest (6). Investigation of the interaction of Bcl2L12 and its variant revealed that HSP70 protected Bcl2L12 and Bcl2L12A from N-terminal ubiquitin-mediated proteosomal degradation (21). More recently, in contrast to previous findings on the role of Bcl2L12 in BCa, a putative tumor suppressor of BCa, ERβ5, was found to interact with Bcl2L12, which may compete with the interaction of Bcl2L12-caspase-7 and result in sensitization to chemotherapeutic agent-induced apoptosis (15). This finding supports the anti-apoptotic role of Bcl2L12in GBM, by interacting with caspase-7 to antagonize apoptotic activity. Of note, the use of chemotherapeutic agents to treat cancer cell lines generally results in the downregulation of Bcl2L12 and activation of pro-apoptotic members in the Bcl2 protein family, such as Bax. Thus, the reason for chemotherapeutic agent-associated signaling downregulating a favorable prognosis marker such as Bcl2L12, should be investigated. However, Bcl2L12 and/or its variant may have different impacts on drug response, tumorigenesis and patient outcome. Bcl2L12 and Bcl2L12A need to be evaluated synchronously to elucidate their interplay in BCa.

Regarding the correlation between ER and Bcl2L12, a previous study demonstrated that Bcl2 is more highly expressed in ER-positive and Bcl2L12-positive BCa (8). A high Bcl2L12 expression is suggested to be associated with ER and Bcl2 expression. However, in the present study, we observed that Bcl2L12 mRNA was more abundantly expressed in TNBC tumors. Furthermore, Bcl2L12A mRNA was elevated in TNBC compared to non-TNBC tumors although the result was not statistically significant. In non-TNBC, Bcl2L12A expression was markedly enhanced, correlated with Bcl2L12 mRNA level and was associated with the severity of lymph node metastasis. In another study, Bcl2L12 was found to interact with ERs through ERβ5, but not ERα, ERβ1 and β2 (15). The expression profiles of ERβ5, caspase-7 and Bcl2L12 in TNBC and non-TNBC subtypes should be further investigated.

Despite the lack of knowledge concerning the role of Bcl2L12 in BCa, the molecular mechanism of Bcl2L12 involvement in GBM tumorigenesis is better known. Its caspase-inhibiting role is dependent on negatively regulating p53 transcription and the subsequent triggering of the αB-crystallin/caspase-3 interaction (22,23). Recent findings have demonstrated that αB-crystallin overexpression promoted brain metastasis, while silencing αB-crystallin inhibited brain metastasis in orthotopic TNBC (ER/PR/Her2 negative BCa) (24). αB-crystallin promoted the adhesion of TNBC cells to HBMECs at least in part through an α3β1 integrin-dependent mechanism. These findings suggest a role for Bcl2L12 in TNBC brain metastasis. In the present study, we also found that TNBC highly expressed Bcl2L12 and Bcl2L12A. In GBM, αB-crystallin is known to be a downstream gene of Bcl212 and may be important for inactivating caspase-3 during tumorigenesis. More studies are needed to determine whether Bcl2L12 is an upstream activator of αB-crystallin in promoting distal metastasis of TNBC. Thus, our results have shown that, a high Bcl2L12 and Bcl2L12A mRNA expression was not associated with BCa progression, but that Bcl2L12 mRNA was correlated with high-grade BCa and the TNBC subtype. In addition, the interplay between Bcl2L12 and its variant may be associated with high lymph node metastasis in non-TNBC tumors.

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