Expression of BCL2L12, a new member of apoptosis-related genes, in breast tumors

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Summary

Apoptosis, a normal physiological form of cell death, is critically involved in the regulation of cellular homeostasis. If the delicate balance between cell death and cell proliferation is altered by a defect in the normal regulation of apoptosis signaling, a cell population is able to survive and accumulate, thereby favoring the acquisition of further genetic alterations and promoting tumorigenesis. Dysregulation of programmed cell death mechanisms plays an important role in the pathogenesis and progression of breast cancer, as well as in the responses of tumors to therapeutic intervention. Overexpression of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL has been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as Bax promote apoptosis and sensitize tumor cells to various anticancer therapies. Recently, a new member of the Bcl-2 family, BCL2L12, was cloned. The BCL2L12 gene is constitutively expressed in many tissues, suggesting that the encoded protein serves an important function in different cell types. In the present study, the expression of BCL2L12 gene was analyzed by reverse transcription-PCR (PT-PCR) in 70 breast cancer tissues. Our results indicate that BCL2L12 positive breast tumors are mainly of lower stage (I/II) or grade (I/II) ($p=0.02$ or $p=0.04$ respectively). Cox regression analysis revealed that BCL2L12 expression is positively related to disease-free (DFS) and overall survival (OS) at both univariate and multivariate analysis ($p=0.002$, $p=0.021$, $p=0.004$, $p=0.029$ respectively). Kaplan-Meier survival curves also demonstrated that patients with BCL2L12-positive tumors have significantly longer DFS and OS ($p=0.002$ and $p<0.001$ respectively). BCL2L12 expression may be regarded as a new independent favorable prognostic marker for breast cancer.

Keywords

Apoptosis, BCL2L12, Bcl2 gene family, breast cancer, prognostic markers

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Introduction

Apoptosis or programmed cell death is an essential physiological process that plays a critical role in a wide variety of physiological processes during fetal development and in adult tissues (1, 2). In most cases, physiological cell death occurs by apoptosis as opposed to necrosis (3). In recent years, the molecular machinery responsible for apoptosis has been elucidated, revealing a family of intracellular proteases, the caspases, which are responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis (4). Caspases segregate into two major phylogenetic subfamilies (ICE, CED-3). Based on their proteolytic specificities, caspases further divide into three groups: inflammatory caspases that mediate cytokine maturation, whereas the apoptotic caspases are either effectors of cell death or upstream activators.

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The pathogenesis of apoptosis involves the cleavage of nuclear chromatin between the nucleosomes by specific endonucleases, producing chromatin fragments. (2, 5). According to recent hypotheses, clonal expansion might occur as a premalignant event resulting from suppressed apoptosis, secondary to mutations in regulatory oncogenes. Programmed cell death and apoptosis are highly regulated physiological processes (5,6). Deregulation of apoptosis results in several diseases, including disorders where cell accumulation occurs (cancer, restenosis) or where cell loss ensues (stroke, heart failure, neurodegeneration, AIDS) (6).

Normal breast development is controlled by a balance between cell proliferation and apoptosis. There is strong evidence suggesting that tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis. The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumor in response to chemotherapy and hormonal therapy (8-11). Thus, it is possible to delineate the biology of individual tumors at the molecular and biochemical level by examining apoptosis and its control and regulation and to exploit these to clinical applications.

Apoptotic events are regulated by a number of proteins that exert either a positive (pro-apoptotic) or a negative (anti-apoptotic) effect on programmed cell death. Proteins participating in these events include members of the Bcl-2 family (12). Members of the Bcl-2 family are characterized by the presence of at least one of the BH1, BH2, BH3, BH4 domains (13). The BH1 and BH2 domains are present in all anti-apoptotic proteins, while the BH3 domain is present in the pro-apoptotic members of the family. However, BH3 domains have been identified in some anti-apoptotic proteins such as Bcl-2 and Bcl-XL (14). The anti-apoptotic Bcl-2 members promote cell survival, whereas pro-apoptotic and BH3-only members facilitate apoptosis (6).The levels of the various members of the Bcl-2 family have been shown to determine response to chemotherapy in breast and other tumors.

Proline-rich domains have been identified in a number of diverse proteins such as EGF and members of the RRAS superfamily such as the TC21 protein (15, 16). Proline-rich motifs are characterized by the presence of the consensus PXXP tetrapeptide, found in all proline-rich proteins identified to date (17). The hallmark of the proline-rich domain function is its interaction with SH3 domains. It has been shown that the proline-rich SH3-binding site is required for integrin activating function and the ability of RRAS to control cell adhesion (16)

Recently, we reported the molecular cloning, physical mapping and expression analysis of a novel gene, BCL2L12, encoding for a proline-rich protein with a highly conserved BH2 domain of the Bcl-2 family (18). The new gene maps to chromosome 19q13.3 and is located between the IRF3 and PRMT1/HRMT1L2 genes, close to RRAS gene (18-22). PCR screening for BCL2L12 transcripts using gene specific primers, revealed the presence of two bands in most of the tissue cDNAs examined, one corresponding to the classical form of the gene and the other to a splice variant. The BCL2L12 protein is composed of 334 amino acids, with a molecular mass of 36.8 kDa and it is highly expressed in mammary gland. In this paper, we describe the expression of the BCL2L12 gene, by RT-PCR, in breast tumors.

Materials and methods

Study group

Tumor specimens from 70 patients who underwent surgery for primary breast cancer at the Oncologic Hospital of Athens "Saint Savas", as well as 4 nonmalignant breast tissues (negative controls) were evaluated in this study. Informed consent was obtained from all patients. A computerized database containing information concerning each patient, together with receptor status, nodal status, size of primary tumor, tumor grade, number of positive nodes, age and menopausal status of the patients, was available for statistical analysis. Patient ages ranged from 25 to 88 years, with a median of 53 years. Follow up information (median follow-up period, 100 months) was available for 69 patients, among whom 20 (28.9%)had relapsed and 16 (23.1%) had died. All patients had a histologically confirmed diagnosis of primary breast cancer and received no treatment before surgery. Clinical staging was performed according to the Postsurgical International Union Against Cancer Tumor-Node-Metastasis (TNM) classification system (23). Histological grade of the tumors was determined according to crite-

Table 1: Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis of the BCL2L12 and actin genes.

Gene		Primer Sequence	Product size(bp)
BCL2L12	Forward	GGAGACCGCAAGTTGAGTGG	556 and 413
	Reverse	GTCATCCCGGCTACAGAACA	
Actin	Forward	ATCTCGCACCACACCTTCTA	838
	Reverse	CGTCATACTCCTGCTTGCTG	

Table 2: Distribution of numerical variables of 70 breast cancer patients examined

Variable	*No. of patients*	*Mean ± SE*	*Median*	*Range*
Age (years)	70	53.1±1.47	53.0	25-88
Tumor size (cm)	70	2.49±0.14	2.15	0.10-7.00
Lymph nodes[a]	66	3.75±0.79	1	0-29
DFS time (months)	69	66.43±3.49	84.5	8-100
OS time (months)	69	73.09±3.03	85.5	11.5-100

[a] Number of lymph nodes positive for malignancy

ria reported by Bloom and Richardson (24). The post-operative treatment modality was known for all patients; 5% received no further treatment after tumor resection, 28% were given adjuvant chemotherapy, 59% were treated with endocrine therapy and 8% were given both chemotherapy and endocrine therapy. Postoperative locoregional radiotherapy was administered to 49 patients

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the breast tissue using Trizol reagent (Gibco, BRL) following the manufacturer`s instructions. RNA concentration was determined spectrophotometrically. Two micrograms of total RNA were reverse-transcribed into first-strand cDNA using the Superscript™ pre-amplification system (Gibco BRL), following the manufacturer`s instructions. The final volume was 20 µl. Based on the information obtained from our previous report (18), two gene-specific primers were designed (see Table 1), and PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs (deoxynucleoside triphosphates), 150ng of primers, and 2.5 units of HotStar™ DNA polymerase (Qiagen Inc., Valencia, CA ,USA) on a thermal cycler (MJ Research, USA). The cycling conditions were a denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 30s, 62°C for 30 s, 72°C for 1min and a final extension step at 72°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Actin was used as internal control for the integrity of the mRNA.

Cloning and sequencing of the PCR products

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

Steroid hormone receptor analysis

Steroid hormone receptors were quantified as described elsewhere (25, 26). The results of the dual ligand binding assay, in which dextran-coated charcoal was used to separate bound ligand from free ligand, were interpreted by Scatchard analysis (27). Tumors with ER (estrogen receptors) and PR (progesterone receptors) concentrations of ≤10 fmol/mg protein were

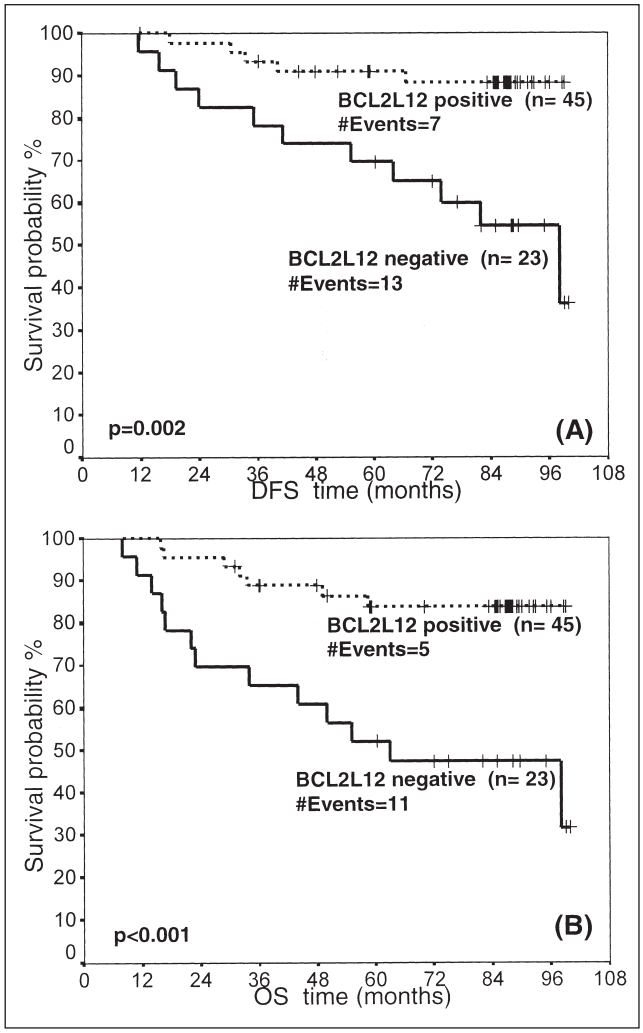

Figure 1: Expression of the BCL2L12 gene in breast cancer. The upper band represents the classical form of the gene, and the lower band is a splice variant.
M, DNA molecular weight marker; 1-11, Randomly selected breast tumors; 5,10, Simples negative for BCL2L12; 12: Normal breast tissue; Ct: negative control.

considered receptor negative, whereas, those with ER and PR concentrations of 10-300 fmol/mg were characterized as positive (28, 29).

Statistical analysis

For analysis of the data, patients were subdivided into groups on the basis of different clinical or pathological parameters. Associations between clinicopathological parameters such as stage, grade, histotype, nodal status tumor size, menopausal status, age, ER, PR and BCL2L12 expression status were analyzed by the χ^2 test or Fisher`s exact test, where appropriate. Cox proportional hazard regression model (30) was developed to evaluate the association (i.e. the hazard ratio and its confidence interval) between the prognostic marker and progression-free or overall survival. For survival analysis, two different end

Table 3: Associations between BCL2L12 expression status and clinicopathological variables of patients examined.

		No. of patients (%)		
Variable	*Total*	*BCL2L12-negative*	*BCL2L12-positive*	*p value*
Age (years)				
<45	21	6 (28.6)	15 (71.4)	
45-55	22	8 (36.4)	14 (63.6)	0.86[a]
>55	27	9 (33.3)	18 (66.7)	
Menopausal status				
Pre/peri	33	10 (30.3)	23 (69.7)	0.80[b]
Post	37	13 (35.1)	24 (64.9)	
Tumor size (cm)				
<2	28	9 (32.1)	19 (67.9)	0.97[b]
≥2	42	14 (33.3)	28 (66.7)	
Nodal status				
Negative	29	7 (24.1)	22 (75.9)	0.29[b]
Positive	37	14 (37.8)	23 (62.2)	
x	4			
Stage[c]				
I/II	58	16 (27.6)	42 (72.4)	
III	8	6 (75.0)	2 (25.0)	0.02[b]
x	4			
Grade[d]				
I/II	50	13 (26.0)	37 (74.0)	
III	18	10 (55.6)	8 (44.4)	0.04[b]
x	2			
Histology				
Ductal	43	14 (32.6)	29 (67.4)	
Lobular	7	4 (57.1)	3 (42.9)	0.24[a]
Other	18	4 (22.2)	14 (77.8)	
x	2			
ER status				
Negative	26	11 (42.3)	15 (57.7)	0.29[b]
Positive	42	12 (28.6)	30 (71.4)	
x	2			
PR status				
Negative	34	14 (41.2)	20 (58.8)	0.31[b]
Positive	34	9 (26.5)	25 (73.5)	
x	2			
Adjuvant treatment				
None	13	4 (30.8)	9 (69.2)	
Tamoxifen	25	8 (32.0)	17 (68.0)	0.94[a]
Chemotherapy ± tamoxifen	31	11 (35.5)	20 (64.5)	
x	1			

[a] χ^2 test
[b] Fisher's Exact Test
[c] TNM system
[d] Bloom-Scarff-Richardson grading system
x. Status unknown.

points, cancer relapse (either local recurrence or distant metastasis) and death were used to calculate DFS (disease free survival) and OS (overall survival) for BCL2L12-positive or negative patients, by constructing Kaplan-Meier curves (31). DFS was defined as the time interval between the date of surgery and the date of identification of recurrent or metastatic disease. OS was defined as the time interval between the date of surgery and the date of death.

Results

BCL2L12 gene expression and relation to other variables

From the 70 breast cancer patients examined (Table 2), 47 patients (67.1%) were classified as positive and 23 patients (32.9%) as negative, for expression of the BCL2L12 gene. Table 3 presents the associations between BCL2L12 gene expression and other clinical or pathological variables, including age, menopausal status, tumor size, nodal status, clinical stage, histological grade, histotype, ER and PR. Of the 58 early stage (I / II)

Table 4: Cox proportional hazard regression analysis demonstrating the association between BCL2L12 gene expression and DFS and OS of patients.

Variable	Disease-free survival			Overall survival		
	HR[a]	95% CI[b]	p value	HR[a]	95% CI[b]	p value
Univariate analysis						
BCL2L12						
Negative	1.00			1.00		
Positive	0.23	0.093-0.59	0.002	0.21	0.073-0.61	0.004
Nodal status	8.55	1.97-37.11	0.004	6.48	1.47-28.57	0.013
Grading (ordinal)	1.73	1.11-2.69	0.015	2.42	1.25-4.65	0.008
Tumor size	1.65	1.22-2.24	0.001	1.63	1.16-2.31	0.005
ER status	0.59	0.24-1.45	0.25	0.36	0.13-0.98	0.047
PR status	0.80	0.33-1.93	0.62	0.45	0.21-0.94	0.034
Histologic type[c]	0.82	0.51-1.32	0.42	0.57	0.21-1.58	0.28
Adjuvant treatment[d]	0.17	0.051-0.61	0.006	0.075	0.01-0.57	0.012
Adjuvant treatment[e]	0.21	0.073-0.56	0.002	0.16	0.045-0.55	0.004
Multivariate analysis[f]						
BCL2L12						
negative	1.00			1.00		
positive	0.25	0.66-0.89	0.032	0.24	0.06-0.96	0.044
Nodal status	3.61	0.51-25.6	0.19	2.31	0.30-17.53	0.41
Grading (ordinal)	1.97	0.68-5.71	0.21	1.64	0.54-4.96	0.37
Tumor size	2.07	1.34-3.19	0.001	1.72	1.081-2.72	0.021
ER status	0.27	0.06-1.29	0.11	0.37	0.072-1.96	0.25
PR status	0.15	0.03-0.73	0.019	0.33	0.062-1.79	0.21
Histologic type[c]	0.47	0.06-3.57	0.93	0.93	0.093-9.41	0.95
Adjuvant treatment[d]	0.26	0.06-1.11	0.069	0.33	0.061-1.82	0.21

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model

[b] Confidence interval of the estimated HR

[c] Lobular and others vs. ductal

[d] Hormonal alone vs chemo±hormonal treatment

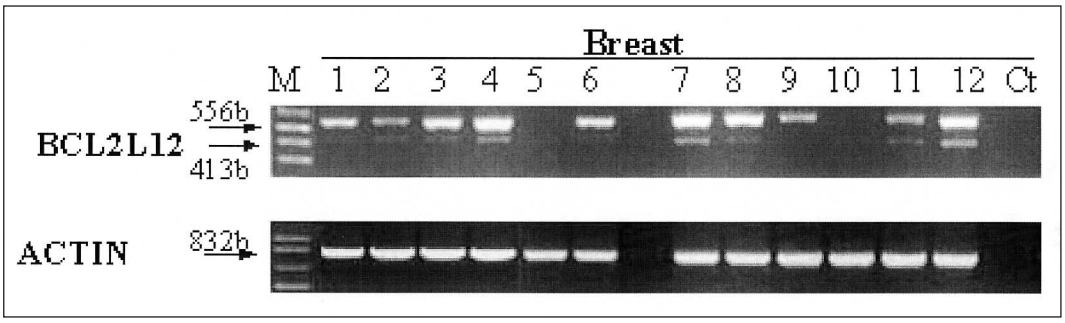

Figure 2: Kaplan-Meier survival curves presenting the associations between BCL2L12 gene expression and DFS (A) or OS (B), n=number of patients examined.

patients examined, 42 patients (72%) were BCL2L12-positive and only 16 patients (28%) BCL2L12-negative. On the other hand, of the 8 patients at advanced stage (III) examined, 6 patients (75%) were BCL2L12-negative and only 2 patients (25%) were BCL2L12-positive. BCL2L12 gene expression was significantly higher in patients with early stages (I / II) compared to patients at advanced stage III (p=0.02). Also, a higher percentage of patients with positive BCL2L12 gene expression were found to have histological grade I / II (74%) compared to patients with histological grade III disease (44%); however, this difference had lower statistical power (p=0.04). On the other hand, no significant associations were found between BCL2L12 gene expression and different histological types, nodal status, ER or PR status, age, adjuvant treatment or menopausal status.

Survival analysis

The strength of the associations between BCL2L12 gene expression and DFS or OS in univariate and multivariate analysis, are shown in Table 4. BCL2L12 gene expression showed a fairly strong association with relapse free survival and death in univariate analysis (p=0.002 and p=0.004 respectively). Breast cancer patients with positive BCL2L12 gene expression were almost 5 times less likely to relapse or die than patients with negative BCL2L12 gene expression (HR of 0.23 and 0.21 respectively). Kaplan-Meier survival curves (Fig. 2) also demonstrate that patients with BCL2L12-positive tumors have substantially longer DFS and OS (p=0.001 and p<0.001, respectively) compared to BCL2L12-negative patients. Cox multivariate analysis showed that BCL2L12 is an independent favorable prognostic marker for DFS and OS of breast cancer patients (p=0.032 and p=0.044 respectively).

Discussion

Programmed cell death plays a critical role in a wide variety of physiological processes during fetal development and in adult tissues. In most cases, physiological cell death occurs by apoptosis as opposed to necrosis. Defects in apoptotic cell death regulation contribute to many diseases, including disorders where cell accumulation occurs (cancer) or where cell loss ensues (stroke, heart failure, neurodegeneration). In recent years, the molecular machinery responsible for apoptosis has been elucidated, revealing a family of intracellular proteases, the caspases, which are responsible, directly or indirectly, for the morphological and biochemical changes that characterize the phenomenon of apoptosis. The apoptotic process is controlled by inducers and repressors; the balance between these stimuli determine whether the cell cycle enters mitosis or apoptosis (2, 5, 8, 28).

Mitochondrial and cell-surface death receptor-mediated apoptosis are the two principal pathways leading to programmed cell death (32). The mitochondrial pathway is thought to play a major role in response to cancer treatments and is mediated by the bcl-2 family proteins. More than 20 members of this family have thus so far been described in humans (5). Many examples exist of alterations in the expression of either apoptosis-suppressing or apoptosis-inducing members of the Bcl-2 family in human cancers (8). In most cases, however, the mechanisms responsible for aberrant levels of Bcl-2 family proteins probably reflect changes in the transcriptional and post-transcriptional regulatory networks that control the ultimate output of their genes (32). A positive ratio between pro- and antiapoptotic bcl-2 family members leads to cytochrome *c* release from mitochondria, which triggers the final execution of cell death by the caspase cascade (3).

In the current study, the expression of the novel gene BCL2L12 in 70 breast cancer tissues was analyzed and results indicate that increased BCL2L12 expression is associated with less aggressive forms of breast cancer. The BCL2L12 gene was highly expressed in normal mammary gland in agreement with our previous report (18) and in breast tumors of low grade or differentiation stage. However, the expression was reduced or disappeared in more advanced stages of the disease. Thus, the BCL2L12 gene seems to exert a positive (pro-apoptotic) effect on programmed cell death. Similarly Bcl-2-positive breast cancer patients had a better prognosis and an overall better survival rate, compared to Bcl-2-negative patients (33-34). These studies included groups of uniformly treated women with node-negative, node-positive, or metastatic disease, confirming an association of Bcl-2 with favorable prognosis even in multivariate analyses. Bukholm et al.. 2002 (35) also reported that overexpression of Bcl-2 was associated with better prognosis in univariate survival analysis but showed borderline association

when including other clinicopathological parameters. Krajewski et al 1999 (36) gave several possible explanations for these seemingly paradoxical results. Among these possible explanations are (i) inhibitory effects of Bcl-2 on cell proliferation; (ii) regulation of Bcl-2 expression by estrogen; and (iii) the presence of Bcl-2 antagonists, which negate its cytoprotective function. On the contrary reduced Bax expression correlated with shorter overall survival (OS) of breast cancer patients (36). Bak and Mcl-1, showed no significant correlation between tumor progression and the clinical outcome in studied cases (36).

However, it can be seen from Table 4 that patients who simply receive hormonal treatment seem to have a longer survival time than patients who receive a combination of chemotherapy and hormonal treatment. Nevertheless, this may be due to the selection of patients for treatment according to the grade and stage of their tumors.

BCL2L12 protein was predicted to contain BH2 domain (18) known to be present in most anti-apoptotic proteins. In the present study, we found that BCL2L12 gene expression fits better with a pro-apoptotic pattern, which is probably due to the simultaneous presence in the BCL2L12 protein not only of a BH2 domain, but also of proline-rich motifs. The BH2 domain is present in the anti-apoptotic proteins Bcl-2 and Bcl-XL and is important in the homo or heterodimerization of the family members (5,37-39).

The importance of Bcl-2 family members as key regulators of apoptosis is apparent from their evolutionary conservation from worm to man (40). Gene knockout data suggest that Bcl-2 family members may have both overlapping and spatially restricted functions in development and tissue homeostasis. This knowledge is clearly important when considering Bcl-2 family members, or other upsteam proteins of the apoptotic pathways that regulate the function of Bcl-2 like proteins, as targets for therapeutic intervention. The rapid progress in the molecular understanding of apoptotic pathways promises novel apoptosis-modulating therapeutics, for eliminating otherwise difficult to treat cancers.

This study suggests that breast cancer patients with positive BCL2L12 expression are almost 5 times less likely to relapse or die, in comparison to patients with negative BCL2L12 expression, confirming an association of BCL2L12 with favorable prognosis. To the best of our knowledge, this is the first study examining the expression and prognostic value of BCL2L12 gene in breast cancer.

References

1. Searle J, Kerr JFR, Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annu 1982; 17: 229-59.
2. Buja LM, Eigenbrodt ML, Eigenbrodt EH. Apoptosis and necrosis. Basic types of cell death. Arch Pathol Lab Med 1993; 117: 1208-14.
3. Reed JC. Mechanisms of apoptosis. Warner-Lambert/Park-Davis Award Lecture. Am J Pathol 2000;157: 1415-30.
4. Zimmermann KC, Bonzon Ch, Green DR. The machinery of programmed cell death. Pharmacol Ther 2001; 92: 57-70.
5. Wyllie AH. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metastasis Rev 1992; 11: 95-103.
6. Choi Ws, Yoon SY, Chang II, et al.. Correlation between structure of Bcl-2 and its inhibitory function of JNK and caspase activity in dopaminergic neuronal apoptosis. J Neurochem 2000; 74: 1621-6.
7. Zha H, Aime-Sempe C, Sato T, Reed JC. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J Biol Chem 1996; 271: 7440-4.
8. Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol 1999; 17: 2941-53.
9. Tamm I, Schriever F, Dorken B. Apoptosis implications of basic research for clinical oncology. Lancet Oncol 2001; 2: 33-42.
10. Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev 1992; 11: 121-39.
11. Verheij M, Batrelink H. Radiation-induced apoptosis. Cell Tissue Res 2000; 301: 133-42.
12. Adams JM, Cory S. The Bcl-2 protein family: Arbiters of cell survival. Science 1998; 281: 1322-6.
13. Reed JC, Zha H, Aime-Sempe C, et al.. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 1996; 406: 99-112.
14. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res 1999; 59: 1693s-1700s.
15. Bar-Sagi D, Rotin D, Batzer A, et al.. SH3 domain direct cellular localization of signaling molecules. Cell 1993; 74: 83-91.
16. Wang B, Zou JX, Ek-Rylander B et al.. R-Ras contains a proline-rich site that binds to SH3 domains and is required for integrin activation by R-Ras. J Biol Chem 2000; 275: 5222-7.
17. Alexandropoulos K, Cheng G, Baltimore D. Proline-rich sequences that bind to Src homology 3 domains with individual specificities. Proc Natl Acad Sci U S A 1995; 92: 3110-14.
18. Scorilas A, Kyriakopoulou L, Yousef G, et al.. Molecular cloning, physical mapping and expression analysis of a novel gene, BCL2L12, encoding a proline-rich protein with a highly conserved BH3 domain of the Bcl-2 family. Genomics 2001; 72: 217-21.
19. Scorilas A, Yousef G, Diamandis EP. Identification and characterization of a novel human testis specific kinase substrate gene which is down-regulated in testicular carcinomas. Biochem Biophys Res Commun 2001; 285: 400-8.
20. Scorilas A, Kyriakopoulou L, Katsaros D, et al.. Cloning of a gene (SR-A1), encoding for a new member of the human Ser/Arg-rich family of Pre-mRNA splicing factors: Over-expression in aggressive ovarian cancer. Br J Cancer 2001; 85: 190-8.
21. Scorilas A, Levesque AL, Ashworth KL, et al.. Cloning, structural characterization, physical mapping and expression pattern of the human alpha-adaptin A gene. Gene 2001; 289, 191-9.
22. Scorilas A, Black MH, Talieri M, et al.. Genomic organization of the human protein arginine methyltransferase 1 gene (PRMT1): Differential expression of splice variants in breast cancer Biochem Biophys Res Commun 2000 278, 349-259.
23. Scott-Conor CE, Christie DW. Cancer staging using the American Joint Committee on Cancer TNM System. J Am Coll Surg 1995; 181: 182-8.
24. Doussal VL, Tubiana-Hulin M, Friedman S, et al.. Prognostic value of histilogigic grade nuclear components of Scarff-Bloom-Richardson (SBR): an improved score modification based on a multivariate analysis of 1262 invasive ductal breast carcinomas. Cancer 1989; 64: 1914-22.

25. Dressler LG, Seamer LC, Owens MA, et al.. DNA flow cytometry and prognostic factors in 1131 frozen breast cancer specimens. Cancer 1988; 61: 420-7.
26. Scorilas A, Talieri M, Ardavanis A et al.. Polyadenylate polymerase enzymatic activity in mammary tumor cytosols: A new independent prognostic marker in primary breast cancer. Cancer Res 2000; 60: 5427-33.
27. Scatchard G. The attraction of proteins for small molecules and ions. Ann NY Acad Sci 1949; 51: 660-72.
28. Alexiera-Figusch J, VAN Putten WLS, Blankestein MA et al.. The prognostic value and relationships of patient characteristics, estrogen and progestin receptors and site of relapse in primary breast cancer. Cancer 1988; 61: 758-68.
29. Reiher A, Kolb R, Reiner G, et al.. Prognostic significance of steroid hormone receptor and histopathological characterization of human breast cancer. J Cancer Res Clin Oncol 1987; 113: 285-90.
30. Cox DR. Regression models and life tables R. Stat Soc. B, 1972; 34: 187-202.
31. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958; 53: 457-81.
32. Sjostrom J, Blomqvist C, von Boguslawski K, et al.. The predictive value of bcl-2, bax , bcl-Xl, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res 2002; 8: 811-6.
33. Silvestrini R, Benini E, Verneroni S, et al.. p53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. J Clin Oncol 1996; 14: 1604-10.
34. Zhang G, Kimijina I, Abe R, et al.. Apoptotic index correlated to Bcl-2 and p53 protein expression, histological grade and prognosis in invasive breast cancers. Anticancer Res 1998; 18: 1989-98.
35. Bukholm I, Bukhlm G, Nesland J. Reduced expression of both Bax and Bcl-2 is independently associated with lymph node metastasis in human breast carcinomas. APMIS 2002; 110: 214-20.
36. Krajewski S, Krajewska M, Turner BC, et al.. Prognostic significance of apoptosis regulators in breast cancer. Endocrine-Related Cancer 1999; 6: 29-40.
37. Simonian PL, Grillot DA, Andrews DW, et al.. Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death. J Biol Chem 1996; 271: 32073-77.
38. Ottilie S, Diaz JL, Chang J, et al.. Structural and functional complementation of an inactive Bcl-2 mutant by Bax truncation J Biol Chem 1997; 272: 16955-61.
39. Akahani S, Nangia-Makker P, Inohara H, et al.. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res 1997; 57: 5272-6.
40. Baliga BC, Kumar S. Role of BCL-2 family of proteins in malignancy. Hematol Oncol 2002; 20: 63-74.