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Short Communication

Expression analysis of BCL2L12, a new member of apoptosis-related genes, in colon cancer

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Abstract

Apoptosis is an active process regulated by a variety of genes. Recently, the molecular cloning, physical mapping and expression analysis of a novel gene of the Bcl-2 family, BCL2L12, was reported. Expression analysis of the BCL2L12 gene in breast cancer confirmed an association of BCL2L12 with favorable prognosis of patients. In the present study, the expression of the BCL2L12 gene was analyzed in colon cancer by RT-PCR. Two transcripts, BCL2L12 and BCL2L12-A, were overexpressed in the cancer tissues as compared to their paired normal mucosa. An association was found between BCL2L12-A transcript expression and nodal status, as well as Dukes' stage. The BCL2L12-A transcript appears to be of importance for colon cancer since its expression is associated with disease progression.

Keywords: apoptosis; BCL2L12; colon cancer.

Apoptosis is a tightly regulated process essential to maintaining tissue homeostasis. Acquisition of resistance to apoptosis plays a pivotal role in tumorigenesis by disrupting the balance between cell proliferation and cell destruction and by allowing cancer cells to resist radiation and chemotherapy. Identification of mechanisms that can antagonize apoptosis is essential to devise therapeutic strategies aimed at enhancing the efficiency of cancer treatment (Reed, 1999, 2002, 2003). Furthermore, because many anti-cancer drugs rely on inducing apoptosis in target cells, a cell population with defects in normal apoptosis signaling will often be refractory to such treatment (Tamm et al., 2001; Floros et al., 2003; Floros et al., submitted). In recent years substantial efforts have concentrated on understanding which molecules are important in the regulation of apoptosis, with the hope of discovering therapeutic targets that can overcome the cancer cell's inherent resistance to apoptosis.

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 gene family (Adams et al., 1998). Its gene products are either anti-apoptotic molecules, e.g. Bcl-2, Bcl-xL, Bcl-w and Al, or pro-apoptotic molecules, e.g. Bax, Bak, Bcl-xS, Bad, Bik, Bid and Bim. Members of the Bcl-2 family are characterized by the presence of at least one of the BH1, BH2, BH3, BH4 domains (Reed et al., 1996). 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, Bcl-xL, and Bcl-w (Korsmeyer et al., 1999). The anti-apoptotic Bcl-2 family members promote cell survival, whereas pro-apoptotic and BH3-only members facilitate apoptosis (Choi et al., 2000). The expression levels of the various members of the Bcl-2 family have been shown to determine response to chemotherapy in breast, colon and other tumors (Reed, 2002, 2003; Mariadason et al., 2003; Nahta et al., 2003).

The molecular cloning, physical mapping and expression analysis of a novel gene, BCL2L12, encoding a proline-rich protein with a highly conserved BH2 domain of the Bcl-2 family, was recently reported by members of our group (Scorilas et al., 2001). This new gene maps to chromosome 19q13.3 and is located between the IRF3 and PRMT1/HRMT1L2 genes (Scorilas et al., 2000, 2001). PCR screening for BCL2L12 transcripts revealed the presence of two bands in most of the tissue cDNAs examined, one corresponding to the classical form of the gene (BCL2L12) and the other to a splice variant (BCL2L12-A). We also examined the expression of the BCL2L12 gene in breast cancer tissues (Talieri et al., 2003) and our results indicated that BCL2L12 gene expression may be regarded as a new independent favorable prognostic marker for breast cancer.

Colorectal cancer is the second most common malignancy of both sexes in developed countries. The prognosis of a colorectal carcinoma patient is generally predicted by the well-established Dukes' classification based on the depth of tumor invasion and metastasis. It is known, however, that in the different Dukes' stages, particularly stages B and C, the prognosis of patients can vary considerably. The reason for this variation could be

Table 1 Distribution of the different variables of the 95 colon cancer patients examined.

Variable	No. of patients	Mean±SEM	Median	Range
Age (years)	95	67.17±1.25	70.0	31–92
Tumor size (cm)	91	4.49±0.17	4.00	2.00–11.50

Dichotomous variable	No. of patients	(%)
BCL2L12		
Positive	47	49.5
Negative	48	50.5
BCL2L12-A		
Positive	18	18.9
Negative	77	81.1
Nodal status		
Negative	35	38.9
Positive	55	61.1
x	5	
Dukes' stage		
A	8	8.8
B	30	33.0
C	44	48.4
D	9	9.9
x	4	
Grade		
I	7	7.7
II	34	37.4
III	42	46.2
IV	8	8.8
x	4	

the fact that the Dukes' classification reflects a stage in the course of cancer growth rather than its biological behavior. Although in recent years several major studies have been performed to evaluate adjuvant chemotherapy for colorectal cancer, it is still of great relevance to probe other biological factors for their prognostic impact and their effect on patient selection. In this regard, new biological factors can help to identify patients for adjuvant systemic treatment. In accordance with our previous results (Talieri et al., 2003; Floros et al., submitted), we decided to examine the expression of the novel apoptotic BCL2L12 gene in colon cancer cells.

The study group consisted of 20 pairs of colorectal carcinomas and their distal normal colonic mucosa in proximal surgical margin and 75 samples of colon cancer tissue, as well as 8 adenomas and 18 samples of inflamed colon tissues collected at the Oncologic Hospital of Athens 'Saint Savas'. Informed consent was obtained from all patients for the scientific analysis of tumor tissues. The patients' mean age±SEM was 67.17±1.25 years with a range of 31–92 years. Clinical and pathological information documented at the time of surgery included stage and grade of the disease, histological type, size and nodal status. Of the 95 tumors included in the study, 8 were of Dukes' stage A, 30 of stage B, 44 of stage C, 9 of stage D and 4 were unclassified (Table 1). Of the 8 adenomas, 4 were highly dysplastic tubuluvilous, having *in situ* adenocarcinoma, 2 tubulous moderately dysplastic and 2 of low dysplasia. Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

Total RNA extracted by the Triazol method (Invitrogen, Carlsbad, USA) from all tissues was reverse-transcribed

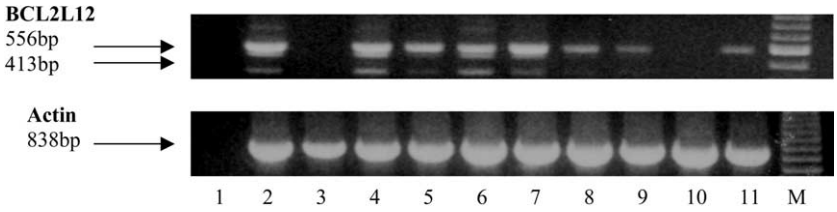

Figure 1 Expression of the BCL2L12 gene in colon tumors.
Lane M: DNA size markers; lane 1: blank (H_2O); lanes 2–10: randomly selected colon cancer tissues.
PCR was performed in a 20 μl reaction mixture containing 0.8 μl of cDNA, 2 μl 10× PCR buffer (Invitrogen), 0.8 μl 50 mM $MgCl_2$, 0.4 μl 10 mM dNTPs mix, 0.4 μl of each primer (0.1 μg/μl) and 2.5 units of *Taq* DNA polymerase (Invitrogen) on an MJ Research thermal cycler. Incubation protocol consisted of: initial incubation at 95°C for 15 min, followed by 40 cycles consisting of 94°C for 30 s (denaturing step), 62°C for 45 s (annealing step) and 72°C for 1 min (extension step) and a final extension step of 72°C for 10 min. Sequencing verified the identity of the products. Colon tissues were then classified as BCL2L12 (classical form) or BCL2L12-A (splice variant) positive or negative, based on ethidium bromide visualization of the PCR product on 2% agarose gels. All experiments were repeated twice and in rare cases, where inconsistencies were found, were repeated for a third time. Actin was used as a control gene.

Table 2 Primers used for reverse-transcription polymerase chain reaction (RT-PCR) analysis of the BCL2L12 and actin genes.

Gene	Sequence	Length of PCR product (bp)
BCL2L12	5′-GGAGACCGCAAGTTGAGTGG-3′ 5′-GTCATCCCGGCTACAGAACA-3′	556 and 413
Actin	5′-ATCTCGCACCACACCTTCTA-3′ 5′-CGTCATACTCCTGCTTGCTG-3′	838

by RT-PCR (Figure 1). The primers used for the amplification of the BCL2L12 gene, as well as for the actin gene (control), are shown in Table 2.

The results obtained were used for statistical analysis. BCL2L12 transcripts displayed 2 bands. The upper band of 556 bp represented the classical form of the BCL2L12 gene, and the lower band BCL2L12-A of 413 bp represented the splice variant. Either both transcripts were expressed in the samples examined or only the BCL2L12 transcript was found. In only one case (cancer sample), was BCL2L12-A expressed alone. A similar picture was seen in the expression of the BCL2L12 gene in breast cancer (Talieri et al., 2003), but in this case only the expression of the classical form BCL2L12 was associated with clinicopathological parameters. Both transcripts, BCL2L12 and BCL2L12-A, were differentially expressed in the colon tissues examined (Table 3). BCL2L12 and BCL2L12-A were overexpressed in the cancer tissues as compared to their paired normal mucosa (non-cancer) and this overexpression was statistically significant ($p=0.014$ and $p=0.025$, respectively; Table 4). When compared to clinicopathological variables, no association was observed between BCL2L12 transcript expression and lymph node involvement, Dukes' stage or grade of the tumors. Nevertheless, a statistically significant association was found between the BCL2L12-A transcript and nodal status as well as Dukes' stage ($p=0.025$ and $p=0.032$ respectively; Table 5). Positive BCL2L12 expression was associated with tumors of larger size and this association was statistically significant ($p=0.009$; Figure 2).

Follow-up information (median follow-up period 29 months, range 10–96 months) was available for 82 patients and included survival status (alive or deceased) and disease status (disease-free or recurrence/metastasis) along with the dates of the events and cause of death, if applicable. During their respective follow-up periods, 28 patients (34.1%) developed cancer relapse and 13 (15.8%) died. Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that BCL2L12 positivity was associated with a short time of relapse ($p=0.043$; Figure 3). However, when entered into a Cox multivariate model, adjusted for other clinicopathological features studied, it was not found to be of independent prognostic significance. When the relationship between BCL2L12-A and survival was examined, no difference between the two parameters was statistically significant, due to the small number of events in BCL2L12-A-positive cases.

Table 4 BCL2L12 and BCL2L12-A transcript expression in 20 pairs of cancerous and non-cancerous colon tissues.

Variable	Number of patients (%)	*p* value*
BCL2L12		
Higher in cancer vs. normal	6 (30.0)	0.014
Lower in cancer vs. normal	0 (0.0)	
Approx. equal in both tissues	14 (70.0)	
BCL2L12-A		
Higher in cancer vs. normal	5 (25.0)	0.025
Lower in cancer vs. normal	0 (0.0)	
Approx. equal in both tissues	15 (75.0)	

*Calculated by the Wilcoxon Signed Ranks Test.

In the current study, the classical form of the gene BCL2L12 was positively associated with the disease-free survival (DFS) of colon cancer patients, in contrast with our previous finding for breast cancer, where it was found to be negatively associated with the DFS (Talieri et al., 2003), while the splice variant, BCL2L12-A, seems to be associated with the progress of colon cancer since it is associated with clinicopathological parameters.

To the best of our knowledge this is the first study examining the expression of the BCL2L12 gene in colon cancer. Many other studies have intensively investigated the expression of other apoptosis-related proteins in colorectal cancer mainly by immunohistochemistry, however the results were controversial. Ogura et al. (1999) reported that Bcl-2, Bcl-xL/S and Bax expression was not related to any clinicopathological factors examined, while Leahy et al. (1999) showed that Bcl-2 protein expression is associated with better prognosis in colorectal cancer. Meterissian et al. (2001) reported that Bcl-2 is a useful prognostic marker in Dukes' B colon cancer, whereas Kanavaros et al. (1999) reported that low levels of Bax expression play a role in late stage colorectal cancer. Recently, Sun et al. (2003) concluded that Bcl-2 protein may play variable prognostic roles in the subgroups of patients with colorectal cancer and the same may apply for the other members of the family. From our results obtained so far, we also conclude the same for BCL2L12 and work is in progress, including large sample numbers and quantitative determination of the gene's expression. The presence of the BH2 domain in the

Table 3 Expression of BCL2L12 and BCL2L12-A transcripts in the different colon tissues analyzed.

Type of tissue	BCL2L12 expression		BCL2L12-A expression	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Carcinomas	47 (49.5)	48 (50.5)	18 (19)	77 (81)
Adenomas	2 (25)	6 (75)	1 (12.5)	7 (87.5)
Normal tissue	3 (15)	17 (85)	0 (0)	20 (100)
Inflamed colon tissues	9 (50)	9 (50)	2 (11.11)	16 (88.89)

Table 5 Association between BCL2L12 and BCL2L12-A transcript expression and other clinicopathological variables.

Variable	Total	Number of patients (%)		
		BCL2L12-negative	BCL2L12-positive	*p* value
Nodal status				
Negative	35	16 (45.7)	19 (54.3)	0.67[a]
Positive	55	29 (52.7)	26 (47.3)	
x	5			
Dukes' stage				
A/B	38	17 (44.7)	21 (55.3)	
C/D	53	28 (52.8)	25 (47.2)	0.53[a]
x	4			
Grade				
I/II	41	20 (48.8)	21 (51.2)	
III/IV	50	25 (50.0)	25 (50.0)	0.54[a]
x	4			
		BCL2L12-A negative	BCL2L12-A positive	*p* value
Nodal status				
Negative	35	24 (68.6)	11 (31.4)	0.025[a]
Positive	55	49 (89.1)	6 (10.9)	
x	5			
Dukes' stage				
A/B	38	27 (71.1)	11 (28.9)	
C/D	53	47 (88.7)	6 (11.3)	0.032[a]
x	4			
Grade				
I/II	41	30 (73.2)	11 (26.8)	
III/IV	50	44 (88.0)	6 (12.0)	0.11[a]
x	4			

[a]Fisher's Exact Test.
x: status unknown.

BCL2L12 protein advocates for its anti-apoptotic role, so does the association of its positive expression with lower disease-free survival of colon cancer patients. However, our previous finding that positive expression of BCL2L12 is associated with longer survival of breast cancer patients (Talieri et al., 2003) suggests a pro-apoptotic role for the gene. Thus, it is still not clear from our work whether the BCL2L12 gene serves a pro- or anti-apoptotic process.

Even though extensive research is underway, the results trying to relate apoptosis markers and chemotherapy are not yet clear. The recent data by Mariadason et al. (2003) comparing the expression of apoptosis-relat-

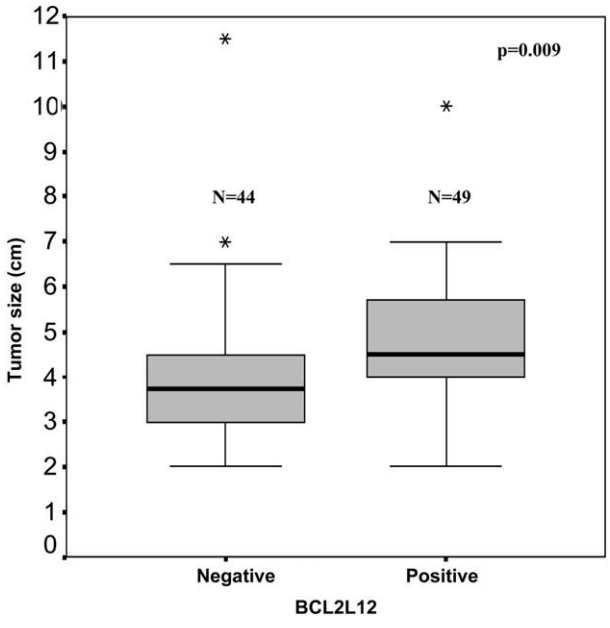

Figure 2 Association of BCL2L12 gene expression and tumor size.
The *p* value was calculated by the Mann-Whitney test. The Figure shows that tumors of larger size are statistically positive for BCL2L12 gene expression in contrast to smaller tumors, which are BCL2L12-negative (p=0.009).

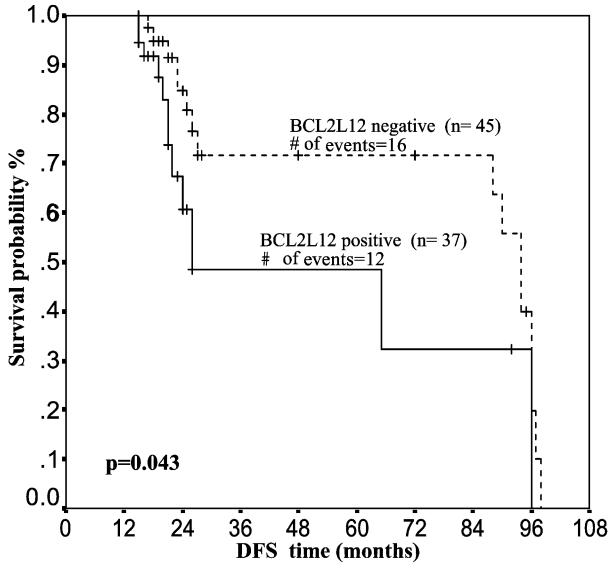

Figure 3 Disease-free survival curves of patients with BCL2L12-positive and BCL2L12-negative colon tumors.

ed markers and clinical outcome of patients with advanced colorectal cancer did not show clinically relevant associations between markers favoring, delaying or controlling apoptosis such as bax, Bcl-2 and p53, and response to a polychemotherapy regimen including MTX and 5-FU/LV.

In conclusion, the classical transcript form of BCL2L12 is significantly associated with the DFS of colon cancer patients, while the BCL2L12-A variant appears to be a promoting determinant for colon cancer since its appearance seems to be associated with disease progression.

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