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Molecular analysis and prognostic impact of the novel apoptotic gene *BCL2L12* in gastric cancer

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ABSTRACT

Stomach cancer comprises a malignancy with feeble prognosis. In gastric carcinogenesis, molecular alterations in the apoptosis-related genes have been described. In this study, the expression of *BCL2-like-12* (*BCL2L12*) gene, discovered and cloned by members of our group, was investigated in a statistically significant sample size of cancerous and non-cancerous stomach tissues and gastric cancer cells with quantitative real-time PCR methodology. *BCL2L12* transcript was indicated in cancer gastric tissues to range from 29 to 53200 mRNA copies *BCL2L12*/10^6 mRNA copies *GAPDH*. Significant associations of *BCL2L12* with gastric tumors of the early stages (I/II) ($p = 0.044$) and of intestinal histotype ($p = 0.034$) was substantiated. Both univariate and multivariate analyses disclosed, respectively, *BCL2L12* relationship with disease-free ($p = 0.006$ and $p = 0.025$) and overall patients' survival ($p = 0.007$ and $p = 0.022$). Our results open new horizons for the possible application of *BCL2L12* as a novel prognostic indicator of gastric cancer.

Introduction

Gastric carcinogenesis is considered as a multistage, multifactorial and long-term process [1] associated with multiple epigenetic changes, leading to altered gene expression [2]. Universally, gastric carcinoma belongs to the most common and lethal neoplasms with its frequency to exhibit a great geographical distribution [3].

Gastric cancer presents a particularly heterogeneous morphology [4]. Nevertheless, its classification into the two main and biologically distinct types, the diffuse and intestinal [5] has been successful in microscopically characterizing these tumors as clinically dissimilar entities [1,6]. In general, 10–20% of the gastric cancer cases are referred to as mixed (unclassified) types [1].

Owing to their asymptomatic nature, diagnosis of gastric carcinomas of the early stages (I and II) often delays [6]. Consequently, by the time of their diagnosis, locally advanced or metastatic tumors will be surgically unresectable, thus rendering patients' cure not feasible [6,7].

In attempting to elucidate the molecular mechanisms of gastric carcinogenesis, scientists have eagerly focused on the discovery of a repertoire of candidate molecular markers for the early detection of the disease. Among the potential biomarkers that have gained popularity, in gastric cancer, are: E-cadherin, p53, CD-34, c-ErbB2, CEA, CA 19–9 and CA 72–4 [8–11]. Their utility, however, as prognostic factors, in stomach cancer, is still fragmentary. The inefficiency of the prognostic value of the currently used markers is ascribed to their inadequate sensitivity and specificity as well as to lack of their diagnostic accuracy in early detecting gastric cancer [12].

Notwithstanding the increased survival rates in gastric cancer patients, thanks to innovative diagnostic techniques and improved therapeutic strategies, high mortality continues to predominate [13]. After decades of intensive work, it has become perceptible that deregulation of apoptosis could play a critical role in the growth or development of human neoplasias [14]. In accordance with this notion, transformation of gastric epithelium to a malignant phenotype appears to result from aberrant programmed cell death [15].

Closely related to apoptosis, BCL-2 family members could either function as cell survival promoters (anti-apoptotic) such as BCL-2, BCL-X_L, MCL-1, BCL-W or might act as inducers of apoptosis (pro-apoptotic) among which are: BAX, BCL-X_S, BAK, BAD, BID, BIK, BIM, BOK, HRK, NOXA [16–18]. Among the members, exhibiting pro-apoptotic activity, are the BAX-like subgroup and the BH3-single domain containing only proteins [17,19,20]. The substantial role of the BCL-2 family members in the initiation and progression of cancer has been well documented. Nevertheless, the precise biochemical mechanism of how this is achieved and of how their countervailing activities are regulated merits further clarification [20,21].

A much promising as a tumor marker, but rather intricate due to its enigmatic pro-apoptotic [22–24] or anti-apoptotic behavior [23,25,26], is the apoptosis-related gene, *BCL2L12* [27]. Expression

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analysis of *BCL2L12* revealed high expression of both transcripts of this gene in colon cancer tissues compared to their normal counterparts [23]. Its prognostic importance was, also, indicated in the case of breast tumors [22]. Less probability for relapse or death was shown in *BCL2L12*-positive than *BCL2L12*-negative women with breast carcinomas [24]. Evidence on the anti-apoptotic function of the protein BCL2L12 was provided by a different team, according to which, not only BCL2L12 over-expression conferred intensive resistance to post-mitochondrial apoptotic signaling, in human primary glioblastoma tissues, but also it engendered a necrogenic response [25]. Moreover, the upregulated BCL2L12 oncoprotein functioned as a potent apoptotic inhibitor by neutralizing the activation of the effector caspases 3 and 7 [26]. On the context of the HL-60 leukemic [28–30], MCF-7 [31,32] and MDA-MB-231 [33] breast cancer cells as well as of the gastric cancer SGC7901 cell line [34], the differential *BCL2L12* mRNA expression, after exposure of these cells to various antineoplastic agents, further pointed out its involvement in apoptosis.

With the current work we aimed to investigate and evaluate the prognostic significance of *BCL2L12* in primary gastric tumors. Towards this direction we developed a remarkably sensitive quantitative real-time PCR (qRT-PCR) in order to uncover any possible differences at the mRNA expression levels of *BCL2L12* between stomach cancer and non-cancerous gastric epithelia.

Materials and methods

Gastric cancer cells. AGS, a cell line (ATCC, CRL-1739) isolated from a single biopsy of a freshly resected human stomach adenocarcinoma, was established as an *in vitro* model system for gastric cancer [35]. AGS cells were grown to subconfluence in RPMI 1640 containing 10% FBS, 0.1 g/L streptomycin, 100 KU/L penicillin, 0.3 g/L L-glutamine and 0.85 g/L $NaHCO_3$ (PAA, Austria). They were incubated in a humidified environment adjusted at 37 °C and 5% (v/v) CO_2.

Patients' tissue specimens. Eighty samples (45 cancerous and 35 non-cancerous) were obtained from 48 patients who had undergone surgery for histologically verified primary gastric carcinomas at the 4th Surgery Department, in "Attikon" University Hospital, Athens, Greece, between 2000 and 2007. For 32 patients paired normal gastric tissues were, also, available. No patient had preoperatively received treatment with cytotoxic drugs. All scientific investigations, approved by the Ethics Committee of the "Attikon" University Hospital, Athens, Greece, were conducted in accordance with the ethical standards of the revised in 2000, Helsinki Declaration of 1975 [36]. Histopathological assessment of the stomach tumors was according to Laurén's classification [5]. The mean patients' age, from 39 to 90 years, was 66.7 ± 2.04. The median follow-up period for the disease-free (DFS) and overall (OS) survivals was 6 and 10 months, respectively, with a range of 1–64 months.

cDNA synthesis. Total RNA was isolated from AGS cells and 80 stomach specimens with the guanidinium isothiocyanate method (TRI reagent) (Ambion, Europe). RNA concentration and purity were determined, with a photospectrometer, at the absorbance ratio of 260/280. cDNA (20 μL) were synthesized from 1 μg of cellular or tissue RNA with the TaKaRa AMV reverse-transcriptase RNA PCR kit (Ver. 3.0), Japan.

Polymerase chain reaction (PCR). Based on the NCBI information regarding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH* ID: 2597) and *BCL-2-like-12* (*BCL2L12* ID: 83596) sequences, two gene-specific pairs of primers were designed (Supplementary Table).

PCR amplification of the 50 μL reaction mixture, containing 100 ng of cDNA, 10× Reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM from each primer and 5 U/μL Taq DNA polymerase (HyTest, Russia), was performed in a thermal cycler (LabNet, USA). The PCR procedure included the steps: 95 °C for 2 min, 38–39 cycles at 95 °C for 30 s, 60 °C or 62 °C for 30 s, 72 °C for 1 min and finally for 5 min. PCR-products (equal amounts) were separated on 1.5% (w/v) agarose gel (Invitrogen Life Technologies, UK) and visualized under ultra-violet light after ethidium bromide staining (Research Organics, USA). They were, then, photographed with a digital camera (KODAC DC120). Using *GAPDH* as an endogenous control gene, mRNA integrity was checked.

Quantitative Real-time PCR (qRT-PCR). For the molecular analysis of *BCL2L12* in gastric tissues, a qRT-PCR methodology was developed, utilizing the SYBR Green® I chemistry. Based on the published cDNA sequences (for accession numbers see above), synthesis of two pairs of gene-specific primers followed (Supplementary Table). Real-time PCR for *GAPDH* and *BCL2L12* (classical form) genes was conducted, in triplicates, on the same 96-microwell plate in a 7500 Thermal Cycler (Applied Biosystems, USA). Ten nanograms of cDNA, 50 nM of each primer and (2×) Power SYBR® Green Master-Mix (Applied Biosystems, USA) were diluted in DEPC-treated water comprising the 10 μL reaction mixture. The PCR protocol consisted of the steps: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was, additionally, generated to distinguish the PCR-products of interest from any primer–dimers. The relative quantification, calculated using the comparative C_T (2^{-ddC_T}) method [37], was validated for *GAPDH* and *BCL2L12* with the amplification of serially diluted AGS cDNA, ranging from (100 to 0.1) ng and covering 10 masses: (100, 50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1) ng. For all stomach specimens, mRNA expression of the target gene (*BCL2L12*) was normalized to the internal control gene (*GAPDH*) using the differences in their threshold cycles and relatively to the calibrator (AGS cells). These normalized (2^{-ddC_T}) amounts were multiplied by 1000 in order to yield *BCL2L12* mRNA copies/10^3 *GAPDH* mRNA copies (c/kc).

Statistical analysis. Owning to the non Gaussian distribution of *BCL2L12* expression, in the stomach cancer patients, analysis of the differences in *BCL2L12* profiles between cancerous and non-cancerous gastric tissues was performed with the non-parametric Mann–Whitney *U* and the Wilcoxon Sign tests. Relationships between different continuous variables were assessed by Spearman's correlation coefficient (r_s). The ability of variables to predict relapse or death was studied using univariate and multivariate unconditional logistic regression models.

Results

BCL2L12 expression in stomach specimens

Investigation of *BCL2L12* expression, in the AGS cells, with regular-PCR disclosed the presence of the classical gene isoform, corresponding to a product of 578 bp, and its shorter splice variant (*BCL2L12-A*) of 435 bp (Supplementary Fig. 1B). This evidence prompted us to utilize AGS cells as positive control and calibrator for all regular-PCR and quantitative Real-time PCR experiments, respectively. Modulations in the expression profile, principally, of the classical form of *BCL2L12* were noticed in gastric cancer, as well as in the non-cancerous, paired or non-paired specimens (Supplementary Fig. 1B). Prerequisite for the study of *BCL2L12* mRNA levels was the expression of *GAPDH* in all 80 gastric tissues of the present work (Supplementary Fig. 1A).

Quantitative analysis of BCL2L12 in gastric tissues

When the number of threshold cycles (C_T values) was plotted against AGS serially diluted cDNAs, two graphs were generated (Supplementary Fig. 2). These graphs, one referring to *GAPDH* (internal control) and the other to *BCL2L12* (target gene), demon-

Table 1
Distribution of the numerical variables of the study in stomach cancer patients.

Variable	Mean ± SE	Range	Quartiles (Median)		
			25	50	75
BCL2L12 in non cancer tissues (c/Kc) $N = 35$	9.53 ± 2.53	0.029–65.8	0.31	3.08	14.8
BCL2L12 in cancer tissues (c/Kc) $N = 45$	8.48 ± 2.13	0.029–53.2	0.029	1.03	10.1
Patient age (years)	66.7 ± 2.04	39.0–90.0	55.0	68.0	77.0
Tumor size (cm)	6.11 ± 0.49	2.0–15.0	4.5	5.0	7.2
DFS (months)	10.5 ± 2.0	1.0–64.0	3.0	6.0	13.0
OS (months)	13.3 ± 2.1	1.0–64.0	4.0	10.0	18.0

c/Kc: mRNA copies *BCL2L12*/10^3 mRNA copies *GAPDH*.
p: calculated by the Mann–Whitney *U* test.

strated the almost equal amplification efficiencies of these two genes, so as for the $2^{-ddC}{}_T$ method to be applicable to any Real-time quantification studies [37]. On the basis of previous findings concerning the differential expression of the classical *BCL2L12* form, in colon [23] and breast [22] carcinomas we tried to unravel its potential predictive role in cancerous gastric epithelia. The relative mRNA patterns of *BCL2L12*, quantified with Real-time PCR, are depicted in Supplementary Fig. 3A and B.

Correlation of BCL2L12 expression with the numerical variables of the study

A threefold increase of *BCL2L12* levels was demonstrated in non-cancerous compared to cancerous stomach tissues. However, this over-expression lacked a statistical significance (Table 1). Furthermore, *BCL2L12* expression was not found to be significantly correlated with the patients' age. As far as tumor size and *BCL2L12* expression are concerned, a statistically significant ($p = 0.022$) negative correlation ($r_s = -0.366$) between these two continuous variables was illustrated (Fig. 1).

BCL2L12 status and patients' clinicopathological characteristics

Association of *BCL2L12* expression with some clinicopathological features of the patients was, then, looked at (Table 2). According to our results, *BCL2L12* status, adjusted for the grade of stomach tumors, indicated a statistically insignificant probability ($p = 0.87$). On the contrary, *BCL2L12* expression was significantly elevated in patients of the early TNM stages (I/II) ($p = 0.044$), whilst its very low mRNA levels were related with advanced disease stages (III/IV). Furthermore, *BCL2L12* expressional profiles exhibited a statistically powerful relationship with Laurén's classification system. In particular, these were significantly lower ($p = 0.034$) in the diffuse-type gastric tumors as opposed to the intestinal or mixed histotypes, denoted as others, in Table 2.

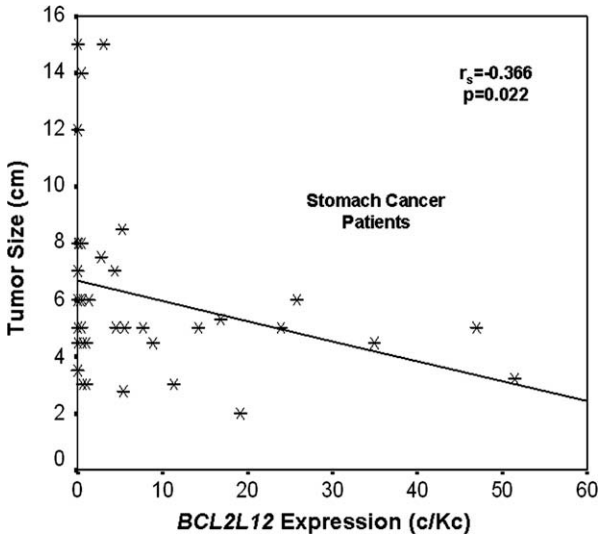

Fig. 1. Association of *BCL2L12* expression with tumor size in gastric cancer patients. c/Kc: copies *BCL2L12*/10^3 copies *GAPDH*.

Table 2
Association between *BCL2L12* status (c/kc) and clinicopathological variables.

Variable	Mean ± SE	Median	*p* Value[a]
Grade			
I/II	7.64 ± 3.63	0.56	
III	4.23 ± 1.53	0.51	0.87
x			
TNM stage			
I/II	13.0 ± 4.93	5.29	
III/IV	6.21 ± 2.15	0.50	0.044
Histotype			
Diffuse	5.63 ± 3.77	0.19	
Others	9.24 ± 2.41	4.31	0.034

[a] Calculated by the Mann–Whitney *U* test.

The predictive strength of BCL2L12 in disease-free and overall survivals

In order to determine the prognostic significance of *BCL2L12* in regard to DFS and OS for gastric cancer patients, univariate and multivariate analyses were performed exploiting the logistic regression models (Table 3). A strong association between *BCL2L12* gene and DFS or OS was disclosed ($p = 0.006$ or $p = 0.007$, respectively), in the univariate analysis. *BCL2L12* expression was shown, in the multivariate analysis, to possess the most significant association with gastric cancer prognosis with respect to DFS ($p = 0.025$) or OS ($p = 0.022$). The fact that crude odds ratio was found to be (<1) for DFS and OS in both analyses, further supported the statistically important prognostic value of *BCL2L12* gene.

Discussion

The prognosis of stomach cancer remains poor and it is unfortunate the fact that even after curative treatment more than half of patients develop local or distant recurrences and eventually pass away because of the disease [38,39]. Early and accurate detection as well as effective monitoring and pertinent therapies are expectations being raised by the discovery of gastric cancer biomarkers, whose prognostic value is still debatable.

It is considered that suppression of apoptosis is implicated in the initiation and progression of human cancers. The effect exerted by such a dysfunctional mechanism results in the survival of tumor cells and their concomitant acquired capability to resist apoptosis [14]. Over the last years, a great bulk of work has led to the iden-

Table 3
Logistic regression analyses of *BCL2L12* in regard to disease-free and overall survival.

Covariant	Disease-free Survival			Overall Survival		
	Crude Odds Ratio	95% CI	*p* value*	Crude Odds Ratio	95% CI	*p* value*
Univariate analysis						
BCL2L12	0.88	0.81–0.96	0.006	0.87	0.79–0.96	0.007
Grade	1.52	1.13–2.05	0.005	1.49	1.12–2.00	0.007
TNM stage	1.53	1.18–1.96	0.014	1.39	1.11–1.75	0.005
Multivariate analysis						
BCL2L12	0.84	0.71–0.97	0.025	0.79	0.65–0.96	0.022
Grade	0.21	0.03–1.44	0.11	0.15	0.018–1.36	0.093
TNM Stage	10.5	1.35–81.4	0.024	15.4	1.38–172.0	0.026

CI: confidence intervals.
* Test for trend.

tification of the ever-growing BCL-2 family members. Their down-regulation or elevated expression has been related to the development of human malignancies [17,20].

A complexity delineates the alterations of the BCL-2 family of proteins, which have been important during the course of gastric cancer formation. Depending on the histological type of the gastric tumors, five members of this family were shown to be frequently expressed. The intensity of BCL-2 immunostaining was more often reduced in adjacent normal gastric epithelium compared to that seen in the case of stomach neoplasms [40]. Elsewhere, particularly BCL-2 protein over-expression had been described in 72% of gastric cancers [41]. It appeared that inhibition of apoptosis through expression of the BCL-2 protein was particularly related to promoting the intestinal-type gastric carcinoma [41,42]. That finding along with the significantly high percentages of BCL-2-immunopositive tumors with diffuse histology supported the notion of the different mechanism from which, at least partly, the intestinal and diffuse gastric histotypes have arisen [40].

Given the previous findings regarding the expression of the recently identified *BCL2L12* gene [27] in colon [23] and breast tumors [22], we attempted to decipher the possible prognostic role of the classical isoform of this gene in gastric cancer. For this purpose, we developed and carried out a reliable and highly sensitive quantitative real-time PCR in order to analyse *BCL2L12* expression in combination with the clinicopathological characteristics of gastric cancer patients and its potential strength in predicting recurrence/metastases or even death.

In the current work the AGS cancer cells, originating from human gastric adenocarcinoma, were shown to express both the classical and the shorter form of the *BCL2L12* gene. Moreover, AGS cells acted as an efficient calibrator for the relative quantification of *BCL2L12* mRNA levels in the 80 stomach tissues examined.

In light of our findings and as far as we know, this is the first report that provides evidence concerning the expression of the *BCL2L12* gene in cancer of the stomach. According to our results, elevated gene expression levels of *BCL2L12* significantly identified gastric tumors of the early TNM stages (I/II) in opposition to more malignant (III/IV) ones. This could bring up the possibility that over-expression of *BCL2L12* might be related to more benign gastric lesions, as similarly had been suggested in the case of mammary tumors [22]. In accordance with BCL-2 protein immunostaining outcome [41,42], our data possibly define *BCL2L12* down-regulation as a useful predictive marker in differentiating between aggressive (diffuse) and less aggressive (intestinal) stomach phenotypes. On the basis of the overall results of our study, we propose that *BCL2L12* could serve as an independent and favourable biomarker with a statistically significant prognostic impact for DFS and OS of gastric cancer patients. Our data are broadly consistent with previous findings, in which the much promising prognostic power of the gene had already been estimated in the context of different tumor cell types [22,23]. Nonetheless, the evidence we provide here are discordant to those reported by a different research group, whose experimental work suggested an important anti-apoptotic contribution of the BCL2L12 protein in human primary astrocytes and glioma cells [25]. By blocking post-mitochondrial signaling at the level of caspases 3 and 7, BCL2L12 shifts the cell death balance from apoptosis to the alternate cell fate of necrosis [26]. This discrepancy might be attributed to the fact that brain carcinomas comprise a diverse category of aggressive and intensely apoptosis-resistant tumors compared with the mostly epithelial in their morphology stomach adenocarcinomas. Further studies are dispensable for warranting the establishment of the prognostic strength of the novel *BCL2L12* gene in gastric cancer before its extensive application, clinically.

Conclusions

Our data suggest that *BCL2L12* could serve as an emerging independent and favourable prognostic tumor marker for the DFS and OS of patients suffering from gastric carcinomas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.034.

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