BCL2L12 Is a Novel Biomarker for the Prediction of Short-Term Relapse in Nasopharyngeal Carcinoma

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BCL2-like 12 (*BCL2L12*) is a new member of the apoptosis-related *BCL2* gene family, members of which are implicated in various malignancies. Nasopharyngeal carcinoma is a highly metastatic, malignant epithelial tumor, with a high prevalence in Southeast Asia and North Africa. The purpose of the current study was to quantify and investigate the expression levels of the *BCL2L12* gene in nasopharyngeal carcinoma biopsies and to assess its prognostic value. Total RNA was isolated from 89 malignant and hyperplastic nasopharyngeal biopsies from Tunisian patients. After testing the quality of the extracted RNA, cDNA was prepared by reverse transcription. A highly sensitive real-time polymerase chain reaction (PCR) method for *BCL2L12* mRNA quantification was developed using SYBR® Green chemistry. *GAPDH* served as a reference gene. Relative quantification analysis was performed using the comparative C_T ($2^{-\Delta\Delta CT}$) method. Higher *BCL2L12* mRNA levels were detected in undifferentiated carcinomas of the nasopharynx, rather than in nonkeratinizing nasopharyngeal tumors ($P = 0.045$). *BCL2L12* expression status was also found to be positively associated with the presence of distant metastases ($P = 0.014$). Kaplan-Meier survival analysis demonstrated that patients with *BCL2L12*-positive nasopharyngeal tumors have significantly shorter disease-free survival ($P = 0.020$). Cox regression analysis showed *BCL2L12* expression to be an unfavorable and independent prognostic indicator of short-term relapse in nasopharyngeal carcinoma ($P = 0.042$). Our results suggest that mRNA expression of *BCL2L12* may constitute a novel biomarker for the prediction of short-term relapse in nasopharyngeal carcinoma.

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INTRODUCTION

Apoptosis, the most common form of programmed cell death, plays critical roles in many physiologic processes during fetal development and in adult tissues. Morphological features of apoptosis usually entail chromatin condensation, DNA fragmentation, membrane blebbing and disruption of the maintained integrity of organelle structures along with formation of apoptosomes (1,2). Dysregulation of apoptotic mechanisms contributes significantly to the pathogenesis and progression of cancer, as well as to response of tumors to therapeutic intervention (3,4). In recent years, the molecular machinery underlying apoptosis has been elucidated, thus revealing several proteins that are responsible, directly or indirectly, for the morphologic and biochemical changes that characterize this phenomenon (5,6).

The BCL2 family is an apoptosis-related family, consisting of pro- and antiapoptotic members, which are implicated in many types of cancer and leukemia (7). BCL2-family proteins share structural homology, since they all contain at least one BCL2-homology domain, namely BH1, BH2, BH3 and/or BH4 (8). The proapoptotic proteins of the BCL2 family, such as BAX, BAD, BID and $BCLX_S$, facilitate apoptosis, whereas the antiapoptotic members, such as BCL2, $BCLX_L$ and BCLW, exert an opposite action, therefore inhibiting initiation of the apoptotic machinery and eventually impeding this form of programmed cell death (9). Not surprisingly, the relative ratios of pro- and antiapoptotic BCL2-family proteins dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli, including growth factor deprivation, hypoxia, irradiation, anti-cancer drugs, oxidants and Ca^{2+} overload (5). This observation presumably explains why expression of a variety of BCL2-family proteins has an important prognostic value for many types of cancer and leukemia treated by chemotherapy (7,10).

The *BCL2-like 12* (*BCL2L12*) gene was discovered and cloned by Scorilas *et al.* (11) in 2001, and its protein is a newly identified member of the BCL2 family, containing a highly conserved BH2 do-

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main, 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 and another one resulting from alternative splicing and giving rise to a protein of 176 amino acids. 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 the alternative splicing variant, named *BCL2L12-A*, lacks exon 3 and is mainly expressed in fetal liver, spinal cord and skeletal muscle (11).

Nasopharyngeal carcinoma (NPC) is a malignancy of the head and neck region that arises from the epithelial cells that line the nasopharynx. This disease was initially reported in 1901 and characterized clinically in 1922 (12). It is a rare malignancy in the United States, accounting for 2% of all head and neck squamous cell carcinomas, with an incidence of 0.5–2 per 100,000. However, it is endemic to many geographical regions, including South China and Southeast Asia, where the observed incidence rates vary between 15 and 50 per 100,000 individuals. An intermediate incidence has been reported in Alaskan Eskimos and the Mediterranean Basin (North Africa, South Italy, Greece and Turkey), ranging from 15 to 20 per 100,000 individuals (13).

In Tunisia, NPC represents the most frequent head and neck cancer with a bimodal pattern of age distribution. In Southeast Asia there is only one peak of incidence at about 50 years of age, while in North Africa, an additional minor peak of incidence occurs between 10 and 20 years of age, including about 25% of all NPC patients (14–16). In regions where it is highly prevalent, NPC appears as a complex disease, arising through a multistep carcinogenic process, involving several factors, such as Epstein-Barr virus chronic infection, genetic and epigenetic alterations and environmental factors (17–19). Primary assessment of NPC is currently based on microscopic examination of cells and tissues, and therapeutic decision-making is supported by a limited set of clinical, histological and biological features. This classification system has allowed important advances in cancer treatment, yet it is not always accurate (20).

The above data encouraged us to analyze *BCL2L12* mRNA expression in 89 nasopharyngeal tissue specimens by an ultrasensitive quantitative real-time polymerase chain reaction (PCR) (qRT-PCR) method, using the SYBR® Green chemistry, and to evaluate its potential prognostic significance and clinical application as a novel tissue biomarker for NPC.

MATERIALS AND METHODS

NPC Tissue Biopsies

We collected 89 nasopharyngeal tissue specimens from patients having undergone biopsy for primary NPC at the Habib Bourguiba University Hospital of Sfax in the South of Tunisia. Sample collection took place from 2000 to 2007. The selection criteria for the specimens included the availability of sufficient tissue mass for RNA extraction and assay. The patients represented approximately 45% of new cases of nasopharyngeal carcinoma diagnosed and treated at the above institution during the accrual period. All biopsies were histologically confirmed by a pathologist.

Patients had not received any treatment before surgery. Biopsy samples were frozen in liquid nitrogen immediately after resection and stored at –80°C until further use. The study was performed in accordance with the ethical standards of the 1975 Declaration of Helsinki as revised in 1996 and was approved by the Habib Bourguiba University Hospital (Sfax, Tunisia) institutional review board. Moreover, informed consent was obtained from all patients participating in the current study.

The clinical stage was designated according to the tumor, node, metastasis (TNM) classification system of the American Joint Committee on Cancer/International Union Against Cancer (21,22) (Table 1). Furthermore, the histological type of nasopharyngeal biopsies was determined according to the World Health Organization criteria. Out of 79 nasopharyngeal tumors examined, 40 (50.6%) were of undifferentiated type and 38 (48.1%) were nonkeratinizing carcinomas. The histological type remained undetermined for one patient. We also collected 10 hyperplastic nasopharyngeal samples from patients with clinical symptoms indicative of NPC but without any neoplastic cells, as revealed by nasopharyngeal biopsies.

Table 1. TNM classification of the 79 NPC patients' biopsies included in the study.

T—primary tumor extent	
T0	0 (0)[a]
T1	7 (8.9)
T2	21 (26.6)
T3	17 (21.5)
T4	34 (43.0)
N—regional lymph nodes	
N0	7 (8.9)
N1	12 (15.2)
N2	28 (35.4)
N3	32 (40.5)
M—distant metastasis	
M0	55 (69.6)
M1	24 (30.4)
Stage grouping	
I	1 (1.3)
II	8 (10.1)
III	19 (24.1)
IV A	10 (12.7)
IV B	17 (21.5)
IV C	24 (30.4)

[a]Data are n (%).

Patient age at the time of diagnosis varied between 10.0 and 80.0 years, with a mean ± SE of 43.4 ± 1.9 and a median of 44.0 (Table 2). Follow-up information was available for 49 patients and included survival status (alive or deceased from NPC) and disease status (disease free or recurrence/metastasis), along with dates of the events and cause of death.

Human Acute Promyelocytic Leukemia Cell Line HL-60

The human acute promyelocytic leukemia cell line HL-60 was maintained

Table 2. Distribution of numerical variables of the study in 79 cancerous and 10 hyperplastic nasopharyngeal tissue biopsies.

	Mean ± SE[a]	Range	Percentile		
			25th	50th (median)	75th
BCL2L12 in tumors (c/c[b])	123.6 ± 55.9	0.01–3970.2	0.69	2.14	15.4
BCL2L12 in noncancer tissues (c/c[b])	267.1 ± 137.8	9.01–1483.7	58.6	149.3	224.6
Patient age (years)	43.4 ± 1.9	10.0–80.0	28.0	44.0	56.0
DFS (months)	31.1 ± 3.4	2.0–130.0	13.5	29.0	43.5
OS (months)	35.1 ± 3.2	2.0–130.0	20.0	31.0	48.0

[a]Standard error of the mean.
[b]*BCL2L12* mRNA copies/*GAPDH* mRNA copies.

in RPMI 1640 medium, adjusted to contain 10% fetal bovine serum (FBS), 100 kU/L penicillin, 0.1 g/L streptomycin and 2 mmol/L L-glutamine. Cells were seeded at a concentration of 4×10^5 cells/mL and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2, before being collected for further use.

Total RNA Extraction and cDNA Synthesis

Frozen hyperplastic and malignant tissue biopsies were pulverized with a scalpel on dry ice. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions, and then stored at –80°C until use. The concentration and purity of total RNA were assessed spectrophotometrically at 260 and 280 nm.

First-strand cDNA was synthesized from total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA), according to the manufacturer's instructions. The reaction mixture contained 2 μg total RNA diluted in diethylpyrocarbonate-treated water, 2 μg oligo(dT)$_{18}$ primer, 4 μL reaction buffer (5×, 250 mmol/L Tris-HCl, pH 8.3, at 25°C, 250 mmol/L KCl, 20 mmol/L $MgCl_2$, 50 mmol/L dithiothreitol), 2 μL 10 mmol/L dNTP mix, 20 U RiboLock™ RNase Inhibitor (40 U/μL, Fermentas), and 200 U RevertAid™ M-MuLV Reverse Transcriptase (200 U/μL, Fermentas). The final reaction volume was 20 μL. The reaction mixture was incubated at 42°C for 60 min, and the reverse transcription was terminated by heating the mixture at 70°C for 5 min.

Quantitative Real-Time PCR

On the basis of the information of the *BCL2L12* and *GAPDH* cDNA sequences (GenBank accession number: NM_138639.1 and NM_002046.3, respectively), two pairs of gene-specific primers were designed. The sequences of the *BCL2L12* real-time PCR primers were as follows: 5′-CCCTCGGCCTTGCTCTCT-3′ and 5′-GGGCCACCAAAGCATAGAAG-3′, producing an 86-bp PCR amplicon, whereas the sequences of the *GAPDH* real-time PCR primers were as follows: 5-ATGGGGAAGGTGAAGGTCG-3′ and 5′-GGGTCATTGATGGCAACAATATC-3′, resulting in a 107-bp PCR amplicon.

Quantitative real-time PCR was performed using the SYBR® Green chemistry, in MicroAmp® Optical 96-Well Reaction Plates (Applied Biosystems, Foster City, CA, USA). PCR runs and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems). The increase in fluorescence emission (R_n) was measured during the course of PCR amplification, and the difference (ΔR_n) between the fluorescence emission of the product and the baseline was calculated by the Sequence Detection System software (Applied Biosystems) and plotted versus the cycle number (Figure 1A). Threshold cycle values were then calculated by determining the point at which the emitted fluorescence exceeded the threshold, determined as 10× the standard deviation of the baseline from cycles 3 to 15 (23).

The reaction mixture contained 10 ng cDNA diluted in 2.5 μL diethylpyrocarbonate-treated water (Applied Biosystems), 5 μL *Power* SYBR® Green PCR Master Mix (2×) (Applied Biosystems) and 2 μL gene-specific primers (final concentration: 50 nmol/L each), in a final reaction volume of 10 μL. The reaction conditions were as follows: denaturation of the template and activation of AmpliTaq Gold® DNA Polymerase LD, at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, for denaturation of the PCR products, and 60°C for 60 s, for primer annealing and extension. Each real-time PCR was performed in duplicate to evaluate data reproducibility.

To distinguish the main PCR products from primer-dimers and/or other nonspecific products, dissociation curves of the PCR products were generated after amplification, by heating the reaction mixtures from 60°C to 95°C with a heating rate of 0.1°C/s and continuously acquiring fluorescence emission data (Figure 1B). The melting temperatures (T_m) of the *BCL2L12* and *GAPDH* amplicons were 81.7°C and 80.1°C, respectively, whereas primer-dimers and/or other nonspecific products were characterized by a much lower T_m (up to 75.0°C).

Calculations and Validation of the Comparative C_T ($2^{-\Delta\Delta CT}$) Method for *BCL2L12* mRNA Quantification

Calculations were made using the comparative C_T ($2^{-\Delta\Delta CT}$) method. The application of this method is based on the assumptions that the PCR amplification efficiencies of the target and the reference genes are similar to each other and close to 100%, as well (24). The prerequisites for the application of the $2^{-\Delta\Delta CT}$ method were checked in a validation experiment, in which C_T values of *BCL2L12* and *GAPDH* were measured in a dilution series of control cDNA over a 10^6-fold range and then plotted against log cDNA dilution. Real-time PCR efficiency (*E*%) for amplification of each gene was calculated using the following formula: $E\% = [-1 + 10^{(-1/\alpha)}] \cdot 100$,

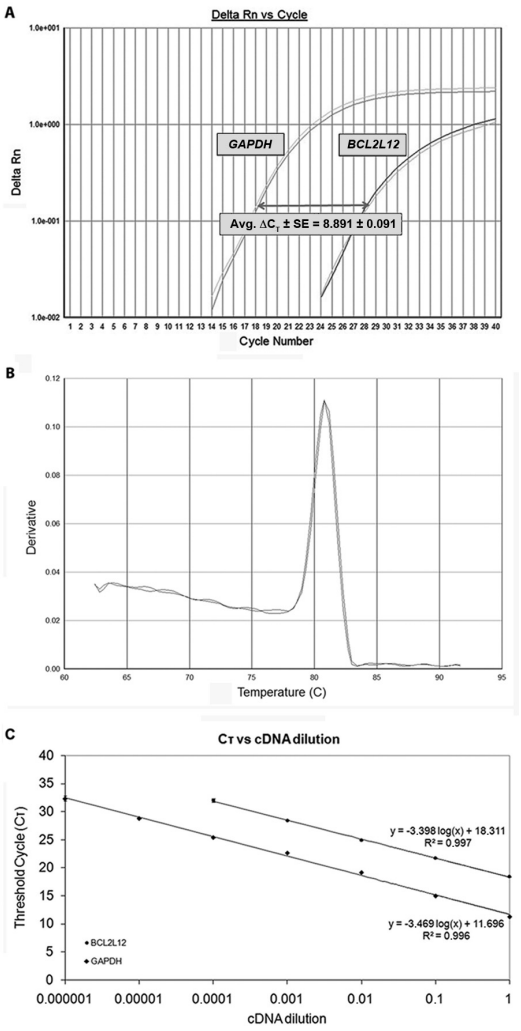

Figure 1. Real-time PCR quantification of *BCL2L12* gene expression in NPC tissue biopsies. (A) Amplification plot of *BCL2L12* and *GAPDH* cDNAs, showing ΔR_n plotted against the cycle number. *BCL2L12* mRNA expression was detected by real-time quantitative PCR using SYBR® Green chemistry, with *GAPDH* as a reference gene. Calculations were made using the comparative C_T ($2^{-\Delta\Delta CT}$) method. (B) Dissociation curves of the *BCL2L12* amplicon, demonstrating the specificity of primers used for the real-time PCR amplification and quantification of *BCL2L12*. Neither primer-dimers nor other nonspecific products were observed after melting of the PCR products. (C) Validation of the comparative C_T ($2^{-\Delta\Delta CT}$) method to assess the efficiency of amplification of the target gene (*BCL2L12*) and the internal control (*GAPDH*). Using reverse transcriptase, cDNA was synthesized from 2 μg total RNA isolated from human HL-60 cells. Serial dilutions of cDNA over a 10^6-fold range were amplified by real-time PCR using gene-specific primers. The most concentrated sample contained cDNA derived from 100 ng total RNA. *BCL2L12* and *GAPDH* average C_T values were calculated for each cDNA dilution and plotted against log cDNA dilution. All data were fit using least-squares linear regression analysis. The absolute values of the slopes of resulting plots are approximately equal, thus implying similar amplification efficiencies for both genes.

where α is the slope of the corresponding amplification plot.

GAPDH was used as a reference gene to normalize all PCRs for the amount of RNA added to the reverse transcription reactions. Moreover, the leukemic cell line HL-60, in which *BCL2L12* is expressed, was used as a calibrator, thus allowing PCR comparison for distinct runs (24). Normalized results were expressed as the ratio of *BCL2L12* mRNA copies to *GAPDH* mRNA copies (c/c), calculated for each nasopharyngeal tissue specimen, in relation to the same ratio calculated for HL-60 cells.

Statistical Analysis

Owing to the non-Gaussian distribution of the expression levels of *BCL2L12* in the NPC patients, analyses of the differences in *BCL2L12* profiles between cancerous and noncancerous nasopharyngeal tissues were performed with the nonparametric Mann-Whitney *U* test.

It is often useful in laboratory medicine to categorize continuous variables to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median and determination of the cut point that maximizes effect difference between groups. If the latter method (the so-called "optimal *P* value" approach) is used, a dramatic inflation of type-I error rates can result (25). A recently developed algorithm, X-tile, allows determination of an optimal cut point while correcting for the use of minimum *P* value statistics (26). Because there are no established cut points available for *BCL2L12* expression in NPC, the X-tile algorithm was used to generate an optimal cut point for categorization of *BCL2L12* expression levels. Thus, an optimal cutoff of 1.95 c/c for *BCL2L12* mRNA expression levels was produced, which is equal to the 47th percentile.

According to the above-mentioned cutoff, *BCL2L12* mRNA expression was classified as positive or negative, and associations between *BCL2L12* status and other qualitative clinicopathological variables were analyzed using either the χ^2 or the Fisher exact test, where appropriate. Furthermore, univariate and multivariate unconditional logistic regression models were developed to evaluate the association between the prognostic markers and the relative risks for relapse and death of patients. Only patients for whom the status of all variables was known were in-

Table 3. Relationship between *BCL2L12* status[a] and other clinicopathological variables.

	Total	Number of patients (%)		*P*
		BCL2L12-negative	*BCL2L12*-positive	
Cases	79	37 (46.8)	42 (53.2)	
Sex				
Male	51	19 (37.3)	32 (62.7)	0.033[b]
Female	28	18 (64.3)	10 (35.7)	
Age (years)				
≤30	25	11 (44.0)	14 (56.0)	0.81[b]
>30	54	26 (48.1)	28 (51.9)	
Tumor histology[c]				
Undifferentiated	40	23 (57.5)	17 (42.5)	0.045[b]
Nonkeratinizing	38	13 (34.2)	25 (65.8)	
Unknown	1			
Tumor extent[d]				
T1/T2	28	17 (60.7)	11 (39.3)	0.099[b]
T3/T4	51	20 (39.2)	31 (60.8)	
Regional lymph node status				
N0	7	6 (85.7)	1 (14.3)	0.17[e]
N1	12	6 (50.0)	6 (50.0)	
N2	28	12 (42.9)	16 (57.1)	
N3	32	13 (40.6)	19 (59.4)	
Distant metastasis				
M0	55	31 (56.4)	24 (43.6)	0.014[b]
M1	24	6 (25.0)	18 (75.0)	

[a]Cutoff point: 1.95 c/c, equal to the 47th percentile.
[b]Calculated by χ^2 test.
[c]Histology classification system, established by the World Health Organization.
[d]Staging system, established by the American Joint Committee on Cancer (21,22).
[e]Calculated by Fisher exact test.

cluded in the multivariate regression models, which incorporated *BCL2L12* mRNA expression and all other variables for which the patients were characterized. The multivariate models were adjusted for tumor extent and histology. Survival analyses were also performed by constructing Kaplan-Meier disease-free survival (DFS) and overall survival (OS) curves. The differences between the curves were evaluated by the log-rank test. The level of significance was defined at a probability value of <0.05 ($P < 0.05$).

RESULTS

Relative Quantification of *BCL2L12* mRNA Expression in Nasopharyngeal Tissues

For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and reference genes must be approximately equal. As illustrated in Figure 1C, the slopes of *BCL2L12* and *GAPDH* amplification plots are similar (–3.398 and –3.469, respectively), which clearly means similar efficiencies for the corresponding amplicons. Indeed, *BCL2L12* and *GAPDH* PCR efficiencies are 96.9% and 94.2%, respectively, as calculated using the above-mentioned formula.

BCL2L12 mRNA levels in NPC biopsies ranged from 0.01 to 3970.2 c/c (*BCL2L12* mRNA copies/*GAPDH* mRNA copies) with a mean ± SE of 123.6 ± 55.9, whereas *BCL2L12* mRNA expression in hyperplastic nasopharyngeal tissues varied between 9.01 and 1483.7 c/c, with a mean ± SE of 267.1 ± 137.8 (Table 2).

Association of *BCL2L12* Expression Status with Clinicopathological Variables

BCL2L12 expression was classified into two categories (positive or negative), as described in "Materials and Methods." Therefore, of 79 NPC biopsies examined, 42 (53.2%) were classified as positive for *BCL2L12* expression and 37 (46.8%) as negative.

Table 3 presents the correlation between *BCL2L12* mRNA expression status of the nasopharyngeal tumors with various clinicopathological parameters, as well as with patients' sex and age. *BCL2L12* mRNA levels were higher in NPC biopsies originating from male rather than female patients. Furthermore, *BCL2L12* positivity was less frequently observed in undifferentiated carcinomas of the nasopharynx rather than in nonkeratinizing nasopharyngeal tumors ($P = 0.045$). *BCL2L12* expression status was also found to be positively related to the presence of distant metastases ($P = 0.014$). Remarkable associations were not observed between *BCL2L12* mRNA expression status and tumor extent, regional lymph node status and patients' age at the time of diagnosis.

Relationship between *BCL2L12* and Nasopharyngeal Carcinoma Survival

Follow-up information was available for 49 NPC patients, among whom 17 (35%) had relapsed and 17 (35%) had died from the disease. Univariate analysis reveals the strength of the association existing between each clinicopathological parameter and DFS, as well as OS. In the present study, univariate analysis indicated that mRNA expression of the *BCL2L12* gene constitutes a significant predictor of disease-free survival ($P = 0.017$), since *BCL2L12* mRNA-positive patients have a higher risk of relapse (hazard ratio 6.82). When all clinicopathological parameters were included in the Cox multivariate regression model, *BCL2L12* mRNA expression retained its negative prognostic significance for disease-free survival of NPC patients (hazard ratio 4.78 and $P = 0.042$) (Table 4).

To evaluate *BCL2L12* mRNA expression in terms of predicting survival outcome, we also performed Kaplan-Meier survival analysis and found that NPC pa-

tients with *BCL2L12*-positive tumors have remarkably shorter disease-free survival ($P = 0.020$), compared with individuals with *BCL2L12*-negative nasopharyngeal malignancies (Figure 2A). With regard to overall survival, *BCL2L12* expression was not shown to possess any statistical significance (Figure 2B). Because the prognosis of NPC patients with initially distant metastasis is extremely poor, we also analyzed separately the group of patients without initially distant metastasis and constructed the corresponding Kaplan-Meier OS curve, but again no statistical significance for overall survival of NPC patients was observed.

Table 4. *BCL2L12* mRNA expression and nasopharyngeal carcinoma survival.

	DFS			OS		
	Hazard ratio[a]	95% CI[b]	*P*	Hazard ratio[a]	95% CI[b]	*P*
Univariate analysis						
BCL2L12 mRNA						
Negative	1.00			1.00		
Positive	6.82	1.62–28.57	0.017	2.72	0.77–9.52	0.11
Tumor extent[c] (ordinal)	1.41	0.77–2.54	0.27	1.17	0.66–2.07	0.58
Histology[d] (undifferentiated/nonkeratinizing)	0.31	0.085–1.06	0.063	0.65	0.19–2.17	0.49
Multivariate analysis[e]						
BCL2L12 mRNA						
Negative	1.00			1.00		
Positive	4.78	1.056–21.67	0.042	2.36	0.61–9.23	0.21
Tumor extent[c] (ordinal)	1.18	0.63–2.23	0.59	1.06	0.59–1.91	0.84
Histology[d] (undifferentiated/nonkeratinizing)	0.44	0.11–1.74	0.24	0.86	0.23–3.11	0.825

[a]Hazard ratio, estimated from Cox proportional hazard regression model.
[b]Confidence interval of the estimated hazard ratio.
[c]Staging system, established by the American Joint Committee on Cancer (21,22).
[d]Histology classification system, established by the World Health Organization.
[e]Multivariate models were adjusted for sex, age, tumor extent and histology.

DISCUSSION

Nasopharyngeal carcinoma is an epithelial malignancy, with high prevalence in Southeast Asia and North Africa. This type of cancer, although highly metastatic, is sensitive to radiotherapy, and a combination of radiotherapy and chemotherapy is typically used for advanced-stage disease treatment. Nonetheless, the prognosis for advanced-stage NPC is poor, even with this combined treatment, and the disease has a high recurrence rate (27). Thus, supplementary work is needed to develop new effective therapeutic strategies against NPC.

To date, several genes and their products have been studied as potential prognostic and/or predictive biomarkers for NPC, including factors related to key cell functions such as cell proliferation, apoptosis, autophagy and necrosis. A recent

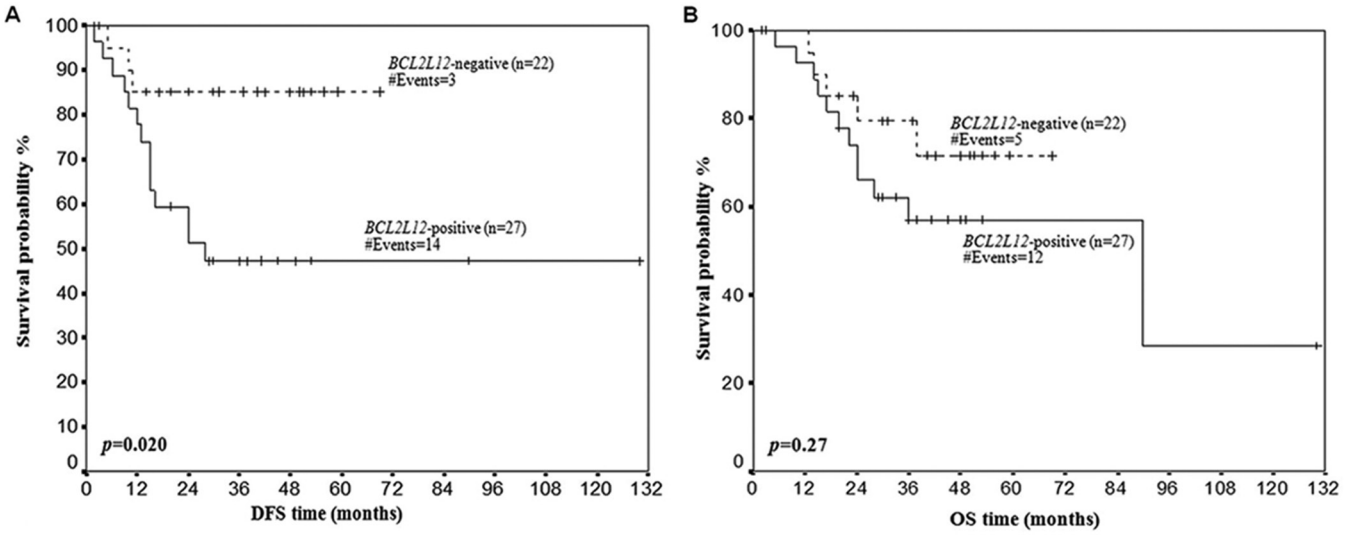

Figure 2. Kaplan-Meier curves for DFS and OS of patients with *BCL2L12* mRNA-positive and mRNA-negative nasopharyngeal tumors. *BCL2L12* mRNA expression has an unfavorable prognostic value for nasopharyngeal carcinoma, since patients with *BCL2L12* mRNA-positive nasopharyngeal tumors have significantly shorter DFS ($P = 0.020$) (A) and OS ($P = 0.27$) (B), compared with NPC patients with *BCL2L12* mRNA-negative tumors.

study demonstrated the prognostic significance of *PTGS2* (*COX2*) mRNA expression, suggesting that mRNA overexpression of the *PTGS2* gene may be useful for identifying NPC patients with lymph node metastasis (28). Additionally, it has been shown that aberrant promoter methylation of *RASSF1*, *RARB* and *DAPK1* is an important indicator of advanced tumor stage and lymph node metastasis in NPC (29). Recently, cytoplasmic heterogeneous nuclear ribonucleoprotein K (HNRNPK) and thymidine phosphorylase (TYMP) have been proposed as independent prognostic and therapeutic markers for NPC (30). Similarly, *BCL2* mRNA expression represents an unfavorable and independent prognostic marker in NPC (31). Moreover, BCL2 protein levels seem to constitute an important predictor in advanced-stage NPC (32). Not surprisingly, it has also been proposed that BCL2 modulates lymph node metastasis of NPC cells (33). Still, another study showed a better clinical outcome for BCL2-positive NPC patients, implying that this outcome may depend on the histological type of tumor, since BCL2 protein expression was significantly associated to undifferentiated NPC (34). Furthermore, primary tumor volume has been shown to possess significant prognostic value, especially in advanced-stage NPC (35–37). However, the necessity of discovering novel, reliable tissue molecular markers for NPC remains high, since no such markers have been established and used in clinical practice yet.

BCL2L12 is a new member of the *BCL2* family, members of which are involved in several malignancies. Although it is clear that BCL2L12 is involved in apoptosis, it remains somewhat obscure or even controversial whether its role is pro- or antiapoptotic. Interestingly, *in vitro* experiments have shown that BCL2L12 can bind $BCLX_L$, most likely through its BH3-like motif (38). Mechanistically, unlike typical BCL2 family proteins, cytoplasmic BCL2L12 does not affect cytochrome C release or apoptosome-driven caspase-9 activation, but instead it is likely to inhibit postmitochondrial apoptosis signaling at the level of effector caspase activation, at least in murine cortical astrocytes and human glioma cell lines (39–41). Besides that, nuclear BCL2L12 interacts with the tumor suppressor protein p53 and impedes the capacity of the latter to bind some of its target gene promoters. Thus, BCL2L12 attenuates endogenous p53-directed transcriptomic changes after genotoxic stress and inhibits p53-dependent DNA damage-induced apoptosis (42). However, in mouse embryonic fibroblasts, Bcl2l12 functions as a proapoptotic factor upon genotoxic stress, sensitizing ultraviolet-irradiated cells to apoptosis (43,44).

Expression analysis of *BCL2L12* revealed that this apoptosis-related gene may be considered as a favorable prognostic biomarker for various malignancies, including breast (45,46), colon (47), and gastric cancer (48). It is worth mentioning that an association between *BCL2L12* and *BCL2* mRNA expression has been noticed in breast tumors (45). In addition, we previously reported that expression of *BCL2L12-A*, an alternative transcript of *BCL2L12*, appears to be of importance in colon cancer, since it is associated with Dukes' stage and lymph node status (49). Remarkable modulations of *BCL2L12* mRNA levels have also been observed in HL-60 (50–54) and SHI-1 (55) leukemia cells, MCF7 breast adenocarcinoma cells (56,57), OVCAR-3 ovary adenocarcinoma cells (58), AGS (59) and SGC7901 (60) gastric cancer cells, after treatment with various antineoplastic agents. These important alterations in *BCL2L12* expression seem to depend on the apoptotic pathway that is triggered by each apoptotic inducer, implying a strong relationship between changes in *BCL2L12* mRNA levels and apoptosis. Recently, it was proposed that *BCL2L12* and *BCL2L12-A* may play an important role in cisplatin-induced apoptosis in MDA-MB-231 breast adenocarcinoma cells (61,62).

In the current study, we investigated the expression of the novel apoptosis-related gene *BCL2L12* in nasopharyngeal carcinoma and in hyperplastic nasopharyngeal tissue. Our results revealed that *BCL2L12* mRNA expression is significantly associated with various clinicopathological parameters, including histological type and presence of distant metastases. NPC patients with *BCL2L12*-negative nasopharyngeal tumors were more likely to have undifferentiated tumors. The undifferentiated subtype of NPC, which is the most common form, typically contains many noncancerous lymphocytes and is strongly associated with Epstein-Barr virus infection (63). Therefore, it is tempting to speculate that *BCL2L12* is downregulated by Epstein-Barr virus latent membrane protein 1 (LMP1), a particularly essential viral protein inducing BCL2 expression in Epstein-Barr virus–infected B-lymphocytes (64).

In addition, our results seem to indicate that *BCL2L12* mRNA expression is associated with more aggressive forms of NPC, whereas low *BCL2L12* mRNA levels are related to early-stage disease, thus suggesting an antiapoptotic role for BCL2L12 in NPC. Similarly, recent oncogenomic studies revealed that the BCL2L12 oncoprotein is robustly expressed in the majority of primary glioblastoma multiforme (GBM) specimens, at a significantly higher level than in oligodendrogliomas and oligoastrocytomas (65), and/or surrounding normal brain tissue (40). Moreover, increased expression of the Bcl2l12 protein in murine cortical astrocytes confers apoptotic resistance to various agents and stimulates a necrogenic response, and, conversely, its RNAi-mediated knockdown sensitizes human glioma cell lines to apoptosis (40).

To the best of our knowledge, this is the first study examining the expression and prognostic value of *BCL2L12* in nasopharyngeal carcinoma. However, another notorious antiapoptotic gene, *BCL2*, has recently been investigated as a potential prognostic marker in this epithelial malignancy. Expression of this gene, both at the mRNA (31) and protein level

(32,33), was shown to be associated with poor prognosis in NPC patients. Our survival analysis uncovered the potential of *BCL2L12* as an unfavorable prognostic factor, since NPC patients with *BCL2L12*-positive tumors had an increased risk of relapse. Cox proportional hazard regression analysis also demonstrated that *BCL2L12* is a significant, independent predictor of disease-free survival.

In conclusion, our results demonstrate that *BCL2L12* mRNA expression is related to unfavorable prognosis in NPC patients and may represent a novel, useful tissue biomarker for the prediction of short-term relapse in NPC. *BCL2L12* overexpression may also account for resistance of NPC patients with advanced-stage disease to chemotherapeutic and irradiation treatment. Future studies are required to clarify the role of *BCL2L12* in nasopharyngeal tumors, to evaluate the potential prognostic significance of the BCL2L12 protein in nasopharyngeal carcinoma and to further define the utility of this biomarker in NPC patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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