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High *BAX*/*BCL2* mRNA ratio predicts favorable prognosis in laryngeal squamous cell carcinoma, particularly in patients with negative lymph nodes at the time of diagnosis

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ABSTRACT

Objectives: Laryngeal squamous cell carcinoma (LSCC), a common type of head and neck cancer, is associated with high rates of metastasis and recurrence. Therefore, accurate prognostic stratification of LSCC patients based on molecular prognostic tumor biomarkers would definitely lead to a better clinical management of this malignancy. The aim of this study was the investigation of the potential combinatorial prognostic value of *BCL2* and *BAX* mRNA expression in LSCC.

Design and methods: Total RNA was isolated from 105 cancerous laryngeal tissue specimens obtained from patients having undergone surgical treatment for primary LSCC. After cDNA preparation, a low-cost, in-house developed, sensitive and accurate real-time quantitative PCR (qPCR) methodology was applied for the quantification of *BCL2* and *BAX* mRNA levels. Then, we carried out a biostatistical analysis to assess the prognostic value of the *BAX*/*BCL2* mRNA expression ratio.

Results: High *BAX*/*BCL2* mRNA expression constitutes a favorable prognosticator in LSCC, predicting significantly longer disease-free survival ($P = 0.011$) and overall survival ($P = 0.014$) of patients. More importantly, the significant prognostic value of the *BAX*/*BCL2* mRNA expression appeared to be independent of the histological grade and size of the malignant laryngeal tumor as well as TNM stage, as revealed by the multivariate bootstrap Cox regression analysis. Kaplan–Meier survival analysis demonstrated also that the *BAX*/*BCL2* ratio can stratify node-negative (N0) LSCC patients into two subgroups with significantly different DFS and OS ($P = 0.021$ and $P = 0.009$, respectively).

Conclusions: The *BAX*/*BCL2* mRNA ratio is a putative molecular tissue biomarker in CLL and hence deserves further validation in larger cohorts of LSCC patients.

1. Introduction

The majority of head and neck malignant tumors are squamous cell carcinoma (SCC), originating from the mucosal lining epithelium of the hypopharynx, larynx, trachea, oral cavity, and oropharynx [1]. SCC comprises about 95% of laryngeal malignancies, the majority of which originate from the supraglottic and glottic regions. Laryngeal SCC (LSCC) occurs most frequently in the sixth and seventh decades of life, yet some cases have also been described to occur in childhood. During the last decades, an increase in the incidence of LSCC has been recorded, both in men and in women [2]. This increase has been related to changes in tobacco and alcohol consumption. Head and neck SCC (HNSCC) is the sixth leading cancer by incidence, as 500,000 new cases per year are diagnosed worldwide, with LSCC being the second commonest type of HNSCC [3]. LSCC is associated with high rates of metastasis and recurrence. Undoubtedly, accurate prognostic stratification of patients, based not only on the current clinical staging system but also on molecular prognostic biomarkers, and prediction of treatment response would contribute to a better clinical management of this type of cancer. Under this perspective, the discovery and evaluation of new tumor biomarkers for LSCC represent a big challenge for researchers [4].

Apoptosis is a strictly controlled form of programmed cell death, playing a pivotal role in normal embryonic development, tissue

Abbreviations: SCC, squamous cell carcinoma; LSCC, laryngeal squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; BH, BCL2-homology; qPCR, quantitative polymerase chain reaction; RQU, relative quantification unit; DFS, disease-free survival; OS, overall survival; CI, confidence interval; HR, hazard ratio; N0, node-negative; miRNA, microRNA.

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homeostasis, and eradication of infected cells or cells with DNA damage [5]. Defective apoptosis constitutes a major reason for the development and progression of cancer. The members of the BCL2 family are key regulators of apoptosis [6]. Based on their function, BCL2 family members are classified in one of two groups: proapoptotic vs. antiapoptotic proteins. The proapoptotic BCL2 family proteins such as BAX, BAD, BID and $BCLX_S$, trigger and/or promote apoptosis, whereas the antiapoptotic members of this family, including BCL2, $BCLX_L$ and BCLW, protect the cell by impeding normal cell death [7]. Interestingly, the relative expression ratios of pro- and antiapoptotic BCL2-family members dictate cell fate and sensitivity (or resistance) of cells to multiple apoptotic stimuli, including growth factor deprivation, hypoxia, exposure to oxidants, irradiation, and treatment with antineoplastic agents [8,9]. As might be expected, several BCL2-family members have already been shown to hold significant prognostic value in many types of cancer, leukemia and/or lymphoma [10].

BCL2 is a proto-oncogene with a pivotal role in apoptosis. This antiapoptotic gene is located at the chromosomal translocation breakpoint between chromosomes 14 and 18, t(14;18), which is common in non-Hodgkin's follicular B-cell lymphoma [11]. BCL2 hinders cell death and hence enhances carcinogenesis [12], while its expression can be crucial for response or tolerance of cells to chemotherapy and radiotherapy [10,13]. Previous research efforts have uncovered the prognostic value of *BCL2* expression in non-small cell lung cancer [14]. Furthermore, deregulated *BCL2* expression was shown to counteract TRAIL-induced apoptosis in H460 cells [15]. The remarkably altered balance in the relative expression levels of some members of the apoptosis-related BCL2 family in breast cancer, including *BCL2*, can affect the progression of the disease [10]. In diffuse large B-cell non-Hodgkin lymphoma, BCL2 and BCLX overexpression in combination with BAX downregulation have been associated with chemoresistance and inferior patient survival [16]. According to our previously published results, high *BCL2* mRNA expression represents a novel molecular tumor biomarker of poor prognosis in nasopharyngeal carcinoma [17].

BAX was the first proapoptotic member of the BCL2 family to be discovered [18]. BAX exerts its proapoptotic action by forming homodimers as well as heterodimers with other members of the BCL2 family, mainly with the antiapoptotic ones such as BCL2 and BAX [19]. Alternative splicing of the *BAX* gene generates several alternative transcripts encoding proapoptotic protein isoforms. The main BAX protein isoform possesses three BCL2-homology (BH) domains – namely the BH1, BH2, and BH3 domains – and has a tertiary structure similar to that of $BCLX_L$ or BCL2 [20]. The BH3 domain of BAX is needed for both its homodimerization and heterodimerization with BCL2 and $BCLX_L$ [21]. The pivotal role of BAX in the regulation of apoptosis is highlighted by the fact that cells highly expressing BAX are sensitized to apoptosis, whereas *BAX*-null cells are resistant to this form or programmed cell death. *BAX* expression has also been linked to the development of solid tumors and hematological malignancies [10,13]. Recently, we provided strong evidence about the independent favorable prognostic value of *BAX* mRNA expression in nasopharyngeal carcinoma [22].

In this study, we sought to investigate the potential combinatorial prognostic value of *BCL2* and *BAX* in LSCC using a cost-effective, in-house developed, sensitive and accurate real-time quantitative polymerase chain reaction (qPCR) methodology to quantify *BCL2* and *BAX* mRNA levels in malignant laryngeal tumors of LSCC patients.

2. Materials and methods

2.1. Collection of LSCC tissue specimens

This study included 105 cancerous laryngeal tissue specimens, obtained from patients having undergone surgical treatment for primary LSCC at Athens General Hospital "Hippokration" (Athens, Greece), between 2005 and 2011. All tumors were histologically characterized and snap-frozen in liquid nitrogen immediately after being resected. Moreover, a detailed database containing clinicopathological and survival data was built. These data included disease staging based on the TNM classification (7th edition), histological grade, and size of the tumor (Table 1). Survival data included relapse events and survival status (alive or deceased), the dates of the events, and the cause of death. This study was approved by the Institutional Review Board of the Athens General Hospital "Hippokration" and conducted in accordance with the ethical standards of the Declaration of Helsinki.

2.2. Cell line culture

The human HNSCC cell line UM-SCC-11 A was subcultured in order to be used as a calibrator in qPCR. UM-SCC-11 A, isolated prior to therapy from a primary tumor of the hypopharynx in a 76-year-old male, grows primarily in islands [23]. This cell line was propagated in complete Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal calf serum, 100 kU/L penicillin, 0.1 g/L streptomycin, and 2 mM L-glutamine. UM-SCC-11 A cells were plated at a concentration of 5×10^5 cells/mL and subcultured for 48 h at 37 °C.

2.3. Total RNA isolation and first-strand cDNA synthesis

TRI® Reagent (Life Technologies Ltd., Carlsbad, CA, USA) was used to extract total RNA from pulverized LSCC tissue specimens and UM-SCC-11 A cells, and the RNA pellet was resuspended in RNAwith Storage Solution (Life Technologies Ltd.), before being treated with RNase-free DNase I (Life Technologies Ltd.) to remove any DNA contamination. The concentration and purity of total RNA extracts were assessed spectrophotometrically at 260 and 280 nm, and their integrity was checked by agarose gel electrophoresis.

2 μg of total RNA were used to construct first-strand cDNA libraries with reverse transcription using the Superscript™ II Reverse

Table 1
Clinical and biological characteristics of LSCC patients.

Total number of patients	105
	Median (range)
BAX/*BCL2* mRNA in LSCC (RQU[a])	0.027 (0.001–43.84)
log(*BAX*/*BCL2* mRNA) in LSCC	−1.57 (−3.00–1.64)
Age (years)	62 (36–87)
DFS[b] (months)	24 (2–82)
OS[c] (months)	33 (5–82)
	Number of patients (%)
Histological grade	
I	14 (13.3%)
II	43 (41.0%)
III	48 (45.7%)
T (tumor invasion)	
T1	15 (14.3%)
T2	22 (20.9%)
T3	34 (32.4%)
T4	34 (32.4%)
N (nodal status)	
N0	85 (80.9%)
N1	15 (14.3%)
N2	5 (4.8%)
TNM stage	
I	15 (14.3%)
II	16 (15.2%)
III	38 (36.2%)
IV	36 (34.3%)

[a] Relative quantification units (*BAX* mRNA copies/*BCL2* mRNA copies).
[b] Disease-free survival.
[c] Overall survival.