Digital Analysis of BCL2 Expression in Laryngeal Squamous Cell Carcinoma

ARISTEIDIS CHRYSOVERGIS¹, VASILEIOS S. PAPANIKOLAOU¹, EVANGELOS TSIAMBAS²,
VASILEIOS RAGOS³, DIMITRIOS PESCHOS⁴ and EFTHYMOS KYRODIMOS¹

¹First ENT Department, Hippocraton Hospital, University of Athens, Athens, Greece;

²Department of Immunohistochemistry and Molecular Biology, 401 General Army Hospital, Athens, Greece;

³Department of Maxillofacial Surgery, Medical School, University of Ioannina, Ioannina, Greece;

⁴Department of Physiology, Medical School, University of Ioannina, Ioannina, Greece

Abstract. *Background: Deregulation of apoptosis is critical regarding the development and progression of malignancies, including laryngeal squamous cell carcinoma (LSCC). B-Cell lymphoma 2 (BCL2) (gene locus:18q21.33), located on the outer mitochondrial membrane, acts mainly as an anti-apoptotic factor suppressing and blocking apoptotic signal transduction. Materials and Methods: Fifty (n=50) primary LSCC tissue sections were used. Immunohistochemistry and digital image analysis were implemented for evaluating BCL2 protein expression levels. Results: High BCL2 protein expression levels were observed in 21/50 (42%) LSCC tissue sections, whereas the remaining cases (n=29) demonstrated a low expression. Overall, BCL2 expression was associated with grade ($p=0.046$) and anatomical region of the examined malignancies (transglottic, $p=0.047$). Interestingly, high BCL2 expression levels were strongly associated with radiotherapy-based only regimens ($p=0.01$) in corresponding patients. Conclusion: BCL2 overexpression was found to be correlated with an aggressive phenotype (advanced grade of differentiation) in LSCC, also demonstrating a potential selective anatomic localization (transglottic region). Additionally, BCL2 overexpression appears to be a negative regulator for successful radiotherapy implementation by reducing the apoptotic process in patients.*

Apoptosis refers to genetically programmed cell death mediated by a complex of proteins which positively or negatively influence the apoptotic cascade (1). Two main

pathways are involved in apoptosis: intrinsic and extrinsic. In both these, several proteins are characterized as inducers or inhibitors of apoptosis (2). Concerning the intrinsic pathway, mitochondrial proteins, prominently cytochrome *c* from the inter-membrane space of the organelle, are responsible for regulating apoptotic signal transduction. Cytochrome *c* in cytoplasm activates caspases (especially caspases 3, 8 and 9) under the control of p53 and B-cell lymphoma (BCL) proteins (3). BCL proteins were initially identified and cloned in studies of chromosomal translocations involving chromosomes 14 and 18 [t(14;18)] in follicular lymphomas, but its expression is not specific only to this genetic aberration (4, 5). BCL2 (gene locus:18q21.33, encoded protein product of 25 kDa) is the main member of the BCL protein family. It is located on the outer mitochondrial membrane acting mainly as an anti-apoptotic factor suppressing and blocking apoptotic signal transduction. It consists of two isoforms (1 and 2) which demonstrate specific anti-apoptotic functions. Concerning head and neck squamous cell carcinomas and especially laryngeal squamous cell carcinoma (LSCC), aberrant BCL2 expression seems to modify the chemoradiotherapy response rates in sub-groups of patients combined, or not, with the expression of other proteins, such as p27 and NOTCH2 (6, 7).

In the current study, we analyzed BCL2 protein expression levels in LSCC tissue sections, correlating their digitized staining intensity levels with the corresponding clinicopathological features.

Materials and Methods

Study group. For the purposes of our study, 50 archival, formalin-fixed and paraffin-embedded tissue specimens of surgically resected, histologically confirmed primary LSCCs were used. The specimens were from 45 male and 5 female patients all smokers without a positive DNA test or a clear history of human papilloma virus infection. The hospital Ethics Committee consented to the use of these tissues at the Department of Pathology, Hippocraton

Correspondence to: Evangelos Tsiambas, Cytopathologist, MD, M.Sc., Ph.D., Lecturer Elect in Cytology, Medical School, University of Athens 17 Patriarchou Grigoriou E' Street, Ag. Paraskevi, 153 41 Athens, Greece. Mobile: +30 6946939414, e-mail: tsiambasecyto@yahoo.gr

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Hospital, University of Athens, Athens, Greece for research purposes, according to the World Medical Association Declaration of Helsinki guidelines (2008). According to their clinical status, patients were treated by chemotherapy (cisplatin-based), radiotherapy, or combined chemoradiotherapy regimens. The median survival was 43 months.

The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO) (8). Clinicopathological data of the examined cases are given in Table I.

Antibodies and immunohistochemistry (IHC). IHC for BCL2 marker expression was carried out on 4- μ m serial sections of the corresponding tissue blocks using an automated staining system (I 6000; Biogenex, San Ramon, CA, USA). The corresponding slides were deparaffinized and rehydrated. Sections were enzyme digested for 10 min at 37°C. The EnVisionTM+ detection system (Dako, Glostrup, Denmark) was used for the detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using mouse monoclonal anti-BCL2 (clone NCL-L-BCL-2, 1:100 dilution; Novocastra/Leica Biosystems Ltd, Newcastle, UK) was applied for 30 min at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and slides were incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibody was omitted. Microscopically normal archival tonsil and normal-appearing laryngeal epithelial tissue sections expressing BCL2 protein were considered positive controls. Membranous and diffuse cytoplasmic/peri-nuclear cellular staining patterns were considered acceptable for essential BCL2 expression (Figure 1A-C). Protein expression levels were evaluated quantitatively by implementing a digital image analysis (DIA) protocol.

DIA assay. BCL2 protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels. DIA was performed using a semi-automated system (CX-31 Microscope - Olympus, Melville, NY, USA; Digital camera - Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0 - Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (five optical fields at $\times 100$ magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro assessing diffuse and focal membranous/cytoplasmic protein expression. Based on an algorithm, normal tissue sections (control) were measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey-scale values (0-255) in red-green-blue (RGB) analysis was available for discriminating different protein expression levels (Figure 1D). Values decreasing to 0 represent progressive overexpression of the marker, whereas values increasing to 255 show a progressive loss of staining intensity. Results and DIA values are given in Table I.

Statistical analysis. Descriptive statistics were performed. Associations between variables including protein expression levels, and clinicopathological parameters such as gender, tumor grade and stage, anatomic location, and alcohol consumption were performed

using Pearson chi-square test estimated along with its 99% confidence interval (CI) and Spearman correlation coefficient (SPSS v20; SPSS Inc, IBM, Armonk, NY, USA). Two-tailed values of $p \leq 0.05$ were considered statistically significant. Results and correlations (p -values) are described in Table I.

Results

According to the protein DIA procedure, all the examined cases were evaluated properly demonstrating different BCL2 expression levels. High BCL2 protein expression levels (dark staining intensity) were observed in 21/50 (42%) LSCC tissue sections, whereas the remaining cases ($n=29$) demonstrated low expression (bright staining intensity). Overall BCL2 expression was associated with increased grade ($p=0.046$) and transglottic anatomical location ($p=0.047$). Interestingly, high BCL2 expression levels were strongly associated with regimens based on radiotherapy only ($p=0.01$), whereas overall therapeutic strategies were not correlated with protein expression levels ($p=0.729$). No other statistical correlations were identified concerning the other clinicopathological parameters (gender: $p=0.926$, stage: $p=0.252$, alcohol consumption status: $p=0.392$, overall survival in months: $p=0.08$).

Discussion

Deregulation of apoptotic pathways is a frequent event in head and neck squamous cell carcinoma, leading to increased resistance rates to specific radio-chemotherapeutic regimens in patients combined with advanced stage (lymph node metastases) disease (9). Especially focused on LSCCs, recently published data have shown that mitochondrial-based apoptotic proteins seem critically to affect prognosis in subgroups of patients with specific characteristics. A study group co-analyzed *BCL2* and *BCL2*-associated X (*BAX*) mRNA expression levels by implementing a real-time quantitative polymerase chain reaction protocol (10). They concluded that increased *BAX* expression combined with decreased *BCL2* (high *BAX/BCL2* mRNA expression ratio) was correlated with longer disease-free and overall survival of patients with a low tumor stage (negative lymph nodes at the time of diagnosis). For these reasons, they proposed the *BAX/BCL2* mRNA ratio as a potentially reliable biomarker in predicting biological behavior in patients with LSCC. Additionally, another experimental study analyzing the effect of combined acetazolamide and cisplatin chemotherapy application in Hep-2 laryngeal cell cultures showed increased levels of caspase-3 and *BAX* expression, whereas *BCL2* protein levels were reduced (11).

In the current study, we analyzed BCL2 protein expression by IHC on tissue sections of LSCCs estimating the levels of its staining intensity by implementing a DIA protocol. High BCL2 protein expression levels were observed in a significant

Figure 1. B-Cell lymphoma 2 (BCL2) protein expression patterns in laryngeal squamous cell carcinoma tissues. High (A), medium (B), and low (C) staining levels of the marker. Note membranous and diffuse cytoplasmic/peri-nuclear cellular staining (anti-BCL2, diaminobenzidine-tetrahydrochloride stain; original magnification 100×). D: Implementation of digital image analysis protocol for estimating BCL2 protein expression levels. Progressive digitized snapshot of a microscopic field is analyzed by a macro focused on staining intensity values (0-255 RGB). Red areas show expression levels of the marker. The final value is the result of a synthetic approach of all stained pixels (original magnification 100×).

Table I. Clinicopathological parameters and total B-cell lymphoma 2 (BCL2) expression in patients with laryngeal squamous cell carcinoma (n=50).

Clinicopathological parameter	Frequency	BCL2 expression, n (%)		p-Value
		Overexpression 21/50 (42%)	Low expression 29/50 (58%)	
Gender				
Male	45 (90%)	19/50 (38%)	26/50 (52%)	0.926
Female	5 (10%)	2/50 (4%)	3/50 (6%)	
Anatomical region				
Supraglottis	5 (10%)	0/50	5/50 (10%)	0.047
Transglottis	45 (90%)	21/50 (42%)	24/50 (48%)	
Grade				
1	4 (8%)	1/50 (2%)	3/50 (6%)	0.046
2	19 (38%)	3/50 (6%)	16/50 (32%)	
3	27 (54%)	17/50 (34%)	10/50 (20%)	
Stage				
II	4 (34%)	1/50 (2%)	3/50 (6%)	0.252
III	25 (50%)	8/50 (16%)	17/50 (34%)	
IV	21 (42%)	12/50 (24%)	9/50 (18%)	
Alcohol consumption				
Yes	43 (86%)	17/50 (34%)	26/50 (52%)	0.392
No	7 (14%)	4/50 (8%)	3/50 (6%)	
Treatment				
Chemotherapy	17 (34%)	6/50 (12%)	11/50 (22%)	0.729
Radiotherapy	2 (50%)	2/50 (4%)	0/50	
Combined therapy	31 (42%)	11/50 (22%)	20/50 (40%)	
Survival				
0-30 Months	28 (56%)	14/50 (28%)	14/50 (52%)	0.08
>30 Months	22 (44%)	7/50 (8%)	15/50 (6%)	

Overexpression: Moderate to high expression, staining intensity values ≤ 137 in 100% of the examined nuclei (spectrum between 76 and 137); Low expression: staining intensity values > 144 in 100% of the examined nuclei (spectrum between 145 and 187).

proportion of the examined tissues. Overall, BCL2 expression was associated with increasing grade and transglottic anatomical region of LSCC. Furthermore, high BCL2 expression levels were associated with use of radiotherapy alone. No other statistical correlations were identified concerning the other clinicopathological parameters. Referring to radiotherapy response rates, a recently published molecular study based on a polymerase chain reaction/restriction fragment length polymorphism analysis protocol identified a specific *BCL2* polymorphism (*BCL2*-938C>A) associated with worse disease-specific survival (12). They suggested that this polymorphism should be considered as a sensitive genetic biomarker for predicting the genetic profile of radiotherapy response in these patients. Similarly, epigenetic changes, including methylation modifications and micro-RNA (miR) deregulation, affect BCL2 protein expression. Focused on aberrant miR expression in LSCCs, a molecular study identified a specific *miR-34c* interaction with BCL2 (13). Based on a complex molecular analysis including reverse transcription-quantitative polymerase chain reaction and western blot analysis, they measured *BCL2* mRNA and protein expression levels in transfected M4e cells. They concluded that *miR-34c* induces apoptosis and inhibits the viability of M4e cells by targeting BCL2. Concerning novel genetic factors that modify BCL2 expression by down-regulating its activity and enhancing the apoptotic process in LSCCs, a study group analyzed the human esophageal cancer related gene 4 (*ECRG4*) and considered it a potential tumor suppressor and inhibitor of BCL2 (14). Similarly, another study group investigated the role of Beclin1 protein expression in LSCC cell cultures. They concluded that the molecule's activity is modified by *in vitro* application of cisplatin. In fact, BECLIN1 was found to promote cisplatin-induced apoptosis in Hep-2 laryngeal carcinoma cells by BCL2-modulated autophagy (15). These previous studies underline the significance of BCL2 deregulation at the DNA and mRNA levels, explaining its aberrant protein expression in LSSC tissue sections shown by IHC implementation. Moreover, in this study we performed a DIA protocol, as an innovative, accurate and fast technique for objectively estimating the corresponding levels of BCL2 protein expression, as we have already implemented in evaluating expression of other molecules (16).

Conclusion

BCL2 overexpression is correlated with an aggressive phenotype (advanced grade of differentiation) in LSCC, also demonstrating a potential selective anatomic localization, namely the transglottic region. BCL2 overexpression is a negative regulator for successful radiotherapy implementation by reducing the apoptotic process. For this reason, development of synthetic agents or application of natural anti-

BCL2 agents, such as propolis, which demonstrated strong cytotoxic anticancer activity (17), is a very promising chemotherapeutic strategy for stabilizing and enhancing apoptosis in patients with LSCC.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study. No financial support was granted.

Authors' Contributions

Aristeidis Chrysovergis: clinical advisor (case selection); Vasileios S. Papanikolaou: clinical advisor (case selection); Evangelos Tsiambas: researcher, article writing; Vasileios Ragos: academic advisor; Dimitrios Peschos: academic advisor; Efthymios Kyrodimos: academic advisor, article writing.

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