Expression of the Antiapoptotic Proteins Clusterin and Bcl-2 in Laryngeal Squamous Cell Carcinomas

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Abstract
Bcl-2 and clusterin genes have been related to the inhibition of apoptosis, an event that plays a key role in malignant transformation and in invasive disease. In this work, we determine the significance of clusterin and bcl-2 expression in a large series of laryngeal carcinomas. We used immunohistochemical methods and in situ hybridization to examine the expression of these proteins. Non-tumoral epithelial laryngeal tissues did not express clusterin and bcl-2 proteins. However, 9% (14 out of 154) and 25% of these tumors (39 of 154) had positive clusterin and bcl-2 staining, respectively. Clusterin expression was significantly related to the degree of local invasion and higher bcl-2 expression was found in these clusterin-positive tumors (p ! 0.05). Bcl-2 expression was significantly correlated with supraglottic localization, nodal metastases, invasion in depth, and poorly differentiated tumors. However, by multivariate analysis, bcl-2 was shown to be an independent predictor of good prognosis in these tumors (OR = 0.12, 95% CI = 0.02–0.91). These findings indicate that clusterin and bcl-2 are upregulated in laryngeal carcinomas and their expression is related to the invasiveness of these tumors.

Introduction

Apoptotic cell death plays a key role in the pathogenesis, aggressiveness and therapy response of cancer. Besides pathways that accelerate cell division, a disruption of the cell death pathway may contribute to selective expansion of malignant clones. The glycoproteins clusterin and bcl-2 have been reported to present a powerful anti-apoptotic activity in some systems [1, 2]. Clusterin is a heterodimeric highly conserved secreted glycoprotein that is expressed in a wide variety of tissues. In addition to its role in the apoptotic process, clusterin plays an important role in various physiopathological processes, including tissue remodeling, reproduction, lipid transport and complement regulation. Clusterin has also been found to be altered in various human carcinomas [3]. Its

implication in carcinogenesis and progression of some carcinomas [4–6] designates clusterin as an interesting gene to be explored. In fact, clusterin currently is an antisense target in clinical trials for prostate cancer because the suppression of clusterin expression renders human cancer cells sensitive to chemotherapeutic drug-mediated apoptosis [7].

The bcl-2 gene has also been implicated in oncogenesis by its ability to prolong cell survival through the inhibition of apoptosis, without increasing cell proliferation. The expression of bcl-2 has been measured in a variety of human neoplastic tissues including some works on laryngeal carcinomas [8–10]. However, the prognostic significance of bcl-2 protein expression in tumor pathology is contradictory. Some authors have suggested a favorable prognostic role of bcl-2 positivity in breast and non-small cell carcinomas [11–13] while others have found a direct relationship between bcl-2 protein expression and a worse prognosis in prostate carcinomas and meningiomas [14, 15]. However, the prognosis of bcl-2 expression has been reported in laryngeal carcinoma though with variable results [9, 10, 16].

As conventional clinicopathological parameters do not accurately reflect the clinical outcome of patients with laryngeal tumors, the establishment of additional prognostic factors is an essential goal. Therefore, the aim of this study was to examine the expression of the above-mentioned molecules in a well-documented series of laryngeal carcinomas with regard to the clinicopathological parameters and patients' disease-free survival. This is the first report that describes the distribution and possible functional significance of clusterin expression in squamous cell carcinomas of the larynx.

Material and Methods

Patients

We identified 154 consecutive cases of laryngeal carcinomas from the Surgical Department of Otolaryngology of the Virgen del Rocio Hospital and reviewed their clinical records with permission of the institutional review board of the hospital. Total laryngectomy was performed in 123 patients, whereas 31 had partial procedures (supraglottic laryngectomy or cordectomy). Fifty-eight tumors (37.7%) were classified as supraglottic, 45 glottic (29.2%), 4 subglottic (2.6%), 45 transglottic (29.2%) and 20 (13%) were pyriform sinus carcinomas. All patients were male and none of them had received radiotherapy and/or chemotherapy prior to surgery. The youngest patient was 37 years old and the oldest 80 years old (mean age: 60 years). The mean clinical postoperative period for these patients was 40 months (range: 12–96 months). Of the 154 patients, 106 (68%) remained disease-free at the end of the follow-up period. Twenty-seven patients developed tumor recurrence or metastases, which was fatal in 23 (15% of deaths in our series). Twelve cases (7.5%) were lost to follow-up.

Pathological Studies

The Glanz histological grade of malignancy is based upon the tumor cell population (structure, differentiation, nuclear polymorphism, mitoses) and the tumor-host relationship (mode of invasion, vascular invasion, cellular response) [17]. Each of these characteristics was graded in three levels. Cases were also carefully staged to conform to the 1995 criteria of the American Joint Committee for Cancer Staging and End Results Reporting. The T stage was recorded as follows: 5 T1 (3.2%), 20 T2 (13%), 93 T3 (76.6%) and 36 T4 (23.4%). The N stage was 120 N0 (77.9%), 19 N1 (12.3%), 8 N2 (5.2%), 5 N3 (3.2%) and in two cases neck exploration was not avail- able in the clinical notes. The histological and immunohistochem- ical analyses were performed without any knowledge of the clinical stage, treatment, or the further course of the disease.

Histology and Immunohistochemistry

Deparaffinized sections, cut 5 μm thick, were stained with hematoxylin-eosin for histological grading. For the immunohistochemistry, sections were placed in citrate buffer and steamed for 5 min. Primary antibodies employed for staining were bcl-2 (clone 124, Dako, Copenhagen, Denmark) used at 1:40 dilution and e5 (anticlusterin antibody, kindly provided by Dr. Brendan Murphy, St. Vincent's Hospital, Melbourne, Australia) used at 1:1,000 dilution. After PBS washes, the sections were incubated with biotinylated-linked antibody and then with peroxidase-labeled streptavidin. The slides were again washed in PBS and then developed using diaminobenzidine tetrahydrochloride as chromogen. The sections were washed under running tap water and lightly counterstained with hematoxylin, followed by dehydratation and coverslip mounting.

To verify the specificity of the immunohistochemical reaction, a breast cancer sample known to overexpress clusterin and a follicular lymphoma known to express bcl-2 were stained as positive controls. For bcl-2, the intensity of positive staining of lymphocytes was also used as internal positive control. Negative control was performed by omitting the primary antibody. Bcl-2 and clusterin expression was scored as negative if no staining was seen, and positive if more than 10% of tumor cells showed staining. The selection of this cutoff value for clusterin and bcl-2 is based on previous reports [6, 11, 18].

Evaluation of specimens was carried out independently by two investigators, without knowledge of clinical or laboratory information on the patients. Protein expression was analyzed in 20 different fields of the tumors, and the values represent the means of the areas measured.

In situ Hybridization

Human clusterin cDNA was also kindly provided by Dr. B. Murphy. A digoxygenin-labeled antisense RNA probe was obtained using a *Hin*dIII-cut template and T7-RNA polymerase with a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Similarly, a sense probe was prepared for negative control experiments by using a *Nae*I-cut template and SP6-RNA polymerase with the same kit. RNA stability was confirmed by hybridization using digoxygenin-labeled oligo-poly-T as a probe. Details of the technique and sample processing were published elsewhere [6, 19].

Statistical Analysis

The association between bcl-2 and clusterin with the clinical-pathological parameters was determined by the χ^2 analysis and Fisher's exact test. All p values corresponded to two-sided significance testing. The relationships of survival with the expression of proteins were examined using the Kaplan-Meyer method and multivariate survival analysis.

Results

Clusterin expression in normal laryngeal tissue was limited to the mucosecretory gland and lymphocyte infiltrate in some cases. Squamous epithelium did not stain with clusterin. In contrast, epidermoid carcinomas had upregulated clusterin expression in 9% of the cases (14 of 154). The staining in all of these cases showed cytoplasmic location and a granular pattern (fig. 1).

In situ hybridization was examined in 40 cases (including the 14 cases positive for clusterin by immunohistochemistry) to confirm the cellular pattern of clusterin obtained by immunohistochemistry (fig. 1). All cases positive by immunohistochemistry were also positive by in situ hybridization, while only 1 case out of 26 negative for clusterin expression was positive by in situ hybridization. In all cases, the sense probe gave no hybridization signal, demonstrating the specificity of the labeling observed.

The clusterin positivity status was independent of all classical histopathological parameters. However, high clusterin expression was positively linked to the grade of invasion. Thus, 12 out of 14 positive clusterin tumors were grade III (p = 0.03) (table 1).

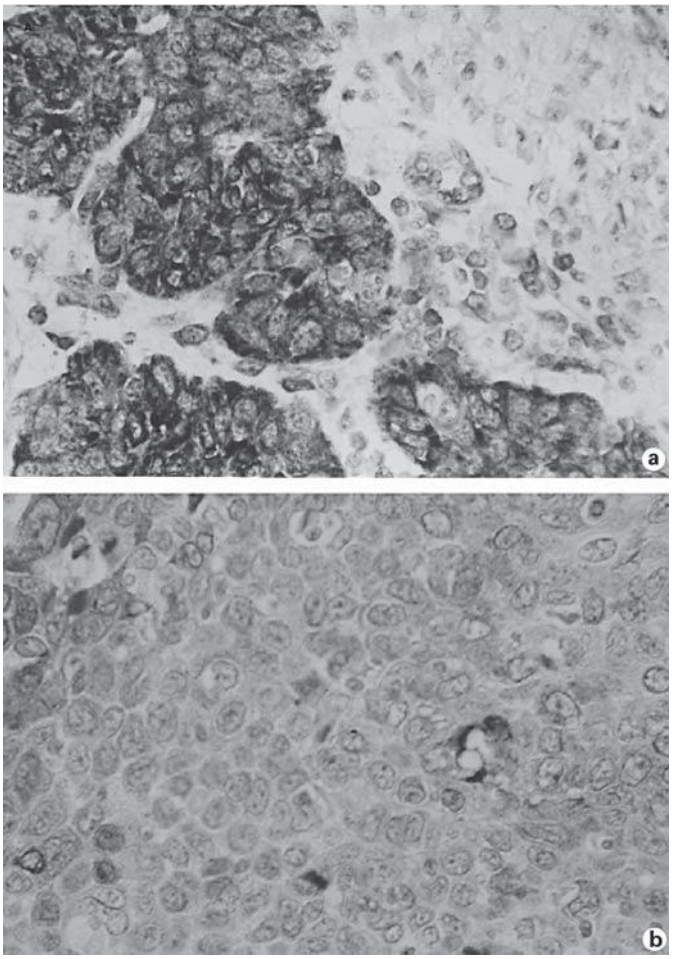

Fig. 1. In situ hybridization of clusterin mRNA in most cells (**a**) and focal staining of bcl-2 protein by immunohistochemistry (**b**) of two moderately differentiated laryngeal carcinomas. x400.

Table 1. Factors related to clusterin expression

Parameter	Clusterin expression		p value
	positive	negative	
Bcl-2			
Positive	7	32	0.04
Negative	7	108	
Invasion in depth			
Grade I and II	2	113	0.03
Grade III	12	27	

Table 2. Factors related to bcl-2 expression

Parameter	Bcl-2 expression		p value
	positive	negative	
Site			0.02
Supraglottic	21	37	
Other sites	18	78	
Neck metastasis			0.01
Yes	15	22	
No	24	93	
Differentiation			0.02
Well to moderately differentiated	28	100	
Poorly differentiated	11	15	
Invasion in depth			0.04
Grade I and II	11	104	
Grade III	28	11	

Normal squamous epithelium was also negative on staining for bcl-2. However, bcl-2 was upregulated in epidermoid carcinomas; thus, of the 154 tumors, 25% (39 of 154) had positive bcl-2 staining (fig. 1). Bcl-2-positive tumors were frequently located in supraglottis (21 out of 39; 53%) compared with bcl-2-negative tumors (37 out of 115; 32%; p = 0.02). Moreover, tumors positive for bcl-2 presented the major grade of invasion in depth (28 of 39; 72%; p = 0.04) (table 2). Importantly, nodal metastases were also found to be significantly related to bcl-2 expression; thus, only 22 out of 115 tumors (19%) negative for bcl-2 expression presented nodal metastases, while 15 out of 39 tumors (38%) positive for bcl-2 expression presented nodal metastases (p = 0.01). Moreover, the expression of bcl-2 was associated with poorly differentiated tumors. Thus, 42% of the poorly differentiated tumors (11 of 26) presented a positive expression for bcl-2, while only 21% of the well- or moderately differentiated tumors (28 of 128) were positive for bcl-2 (p = 0.02) (table 2).

Despite the low number of clusterin-positive tumors in the whole series, these tumors were frequently positive for bcl-2 expression. Thus, 50% clusterin-positive tumors (7 out of 14) were also stained for bcl-2. However, only 29% of clusterin-negative tumors were positive for bcl-2 (p = 0.04) (table 1).

By univariate analysis, the variables associated with short survival were advanced T stage, vascular invasion, invasion in depth, high histological grade, surgical margins affected, tongue base affected, and low bcl-2 expression (table 3, fig. 2). To assess the independent prognostic value of the bcl-2 protein expression, this was included with another prognostic factor in a Cox proportional hazards regression model, where T stage, surgical margins affected and bcl-2 expression were of independent prognostic value.

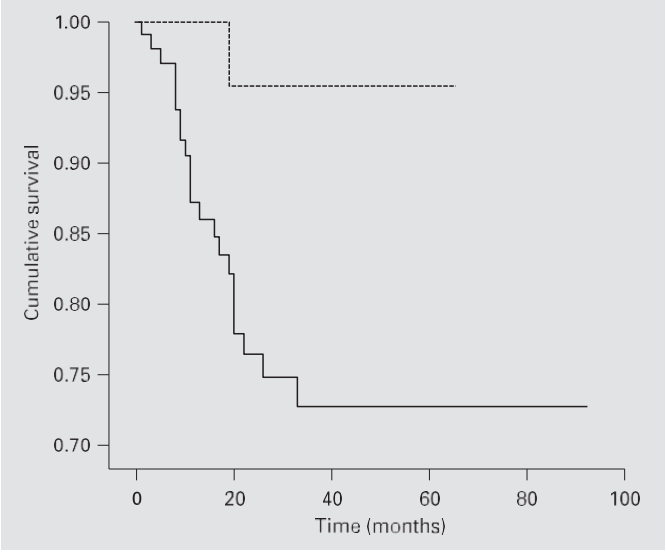

Fig. 2. Overall survival of patients grouped according to bcl-2 expression. The cumulative survival of patients with primary tumors lacking expression of bcl-2 (——) is significantly shorter than that of patients presenting tumors with bcl-2 expression (– – –; p = 0.01).

Discussion

A major finding in our study was the upregulation of clusterin and bcl-2 in a subgroup of laryngeal invasive tumors. Bcl-2 and clusterin share important functions such as regulation of cell death and survival without stimulating cell proliferation [20, 21] and resistance to hormone therapy in prostate carcinomas [22, 23].

Our incidence of positive bcl-2 expression (25%) is similar to most reports on larynx cancer [8, 24, 25]. Bcl-2 expression was found to correlate with tumor grade with a significantly higher incidence of bcl-2 expression in poorly differentiated tumors compared with well-differentiated tumors. The same finding has been reported by others [8–10, 25]. Nodal metastasis was also found to be

Table 3. Variables associated with overall survival

Variable	OR (univariate)	OR (multivariate)
T stage (III–IV vs. I–II)	4.58 (2.01–10.47)	3.96 (1.59–9.85)
Surgical margins: affected (yes vs. no)	5.68 (2.17–14.85)	3.95 (1.45–10.73)
Bcl-2: positive vs. negative	0.14 (0.02–0.97)	0.12 (0.02–0.91)
Tongue base: affected (yes vs. no)	3.36 (1.14–9.96)	–
Vascular invasion (grade III vs. I + II)	3.69 (1.62–8.39)	–
Invasion in depth (grade III vs. I + II)	1.68 (1.03–2.77)	–

95% coefficient interval is given in parentheses. OR = Odds ratio.

associated with the expression of bcl-2, which has been previously reported [8, 10], although in one series the opposite result was found [9]. Our results also showed a higher incidence of bcl-2 expression in supraglottic tumors compared to other locations. Similar findings have been described previously [10, 16]. This association suggests that tumors originating from different sites in the larynx may have a different tumor biology.

Despite the fact that bcl-2 is expressed in a significant number of tumors, its prognostic significance still remains uncertain. In our series, by multivariate analysis, bcl-2 was shown to be an independent predictor of good prognosis in these tumors. Our data are in agreement with several reports that found an association between high expression of bcl-2 and increased survival in larynx cancer [26–28]. However, other reports did not find any relationship between bcl-2 and prognosis [16, 29]. A smaller number of patients included in these latter studies may have influenced this negative result. It cannot be discerned whether bcl-2 is involved directly in contributing to this more indolent phenotype or whether it is simply an epiphenomenon that is a marker for another molecular or biological process. As bcl-2 does not promote cell proliferation, in the absence of additional genetic alterations, bcl-2-positive tumors tend to be relatively nonaggressive. Therefore, bcl-2 expression can be a useful prognostic marker in laryngeal carcinomas. Since it is found in poorly differentiated tumors or with node metastases, bcl-2 might be helpful in distinguishing which tumors with pathological aggressive characteristics might present a better outcome. In fact, in a prior study, a high expression of bcl-2 in locally advanced squamous cell carcinoma of the head and neck predicted increased survival [30].

In addition to bcl-2, our study shows the upregulation of clusterin. Evidence for a role of clusterin in carcinogenesis and progression of some human carcinomas has been accumulated during the last years [4–6]. However, clusterin was upregulated in a low proportion of laryngeal tumors compared with tumors of glandular origin such as prostate or breast [4, 5]. In these tumors, its upregulation is associated with carcinoma progression. The use of antisense oligonucleotides has enhanced androgen sensitivity and chemosensitivity in prostate cancer therapy [31]. Although the clusterin gene is involved in the carcinogenic pathways in some types of tumors, it does not seem to exert a significant role in laryngeal carcinogenesis because its mRNA and proteins are detected in a minority of cases. However, the low proportion of clusterin-positive tumors seems to present an aggressive tumor behavior since their expression was associated with local invasiveness. Studies now being performed in our laboratory should clarify the underlying mechanisms of differential clusterin gene regulation in different tis- sues.

Interestingly, we found a significant association between the expression of clusterin and bcl-2 proteins. In a recent report [32], siRNA-induced clusterin knockdown resulted in downregulation of bcl-2 in two sarcoma cell lines. However, since there is a low number of clusterin-positive tumors in laryngeal carcinomas, additional studies are necessary to verify the results obtained and to elucidate underlying mechanisms.

We found that clusterin mRNA expression correlated well by in situ hybridization with its protein expression by immunohistochemistry. This is in contrast with a prior study in rat brain [33] where a correlation between the clusterin gene and protein expression was absent. In our series of laryngeal carcinomas only one tumor presented RNA expression in cytoplasm without clusterin protein expression, which is in accordance with previous studies in other localizations [5]. Differences in antibody affinity according to cell types or cell state may explain these opposite results.

We can conclude that the antiapoptotic proteins bcl-2 and clusterin are upregulated in a group of laryngeal carcinomas influencing the progression of these tumors.

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