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Expression of Bcl-2 but not Bax has a prognostic significance in tongue carcinoma

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BACKGROUND: Among the molecular mechanisms involved in carcinogenesis, defects in the regulation of programmed cell death (apoptosis) make important contributions to the pathogenesis and progression of cancer. Apoptosis regulatory genes include the anti-apoptotic *bcl-2* gene and the proapoptotic *bax* gene. The aim of this study was to determine the spectrum of Bax and Bcl-2 expression, and to correlate these findings with clinicopathologic variables and prognosis.

METHODS: In this study we have evaluated the immunohistochemical expression of Bcl-2 and Bax proteins in a series of 35 squamous cell carcinomas of the tongue.

RESULTS: Immunoreactivity for Bax was detected in 37.1% and for Bcl-2 in 8.6% of cells, and for both proteins the staining was cytoplasmic and granular. Bcl-2 and Bax expression was mainly seen in peripheral cells of epithelial tumor islands with decreasing immunoreactivity toward the center of the neoplastic nests. Bax immunoexpression was significantly correlated with histologic grading ($P = 0.05$), but not with the remaining clinicopathologic variables. Bcl-2 immunoreactivity was significantly correlated with N-stage ($P = 0.01$) and survival. Patients with Bcl-2-negative tumors [mean survival: 73.97 months; 95% confidence interval (CI): 59–88] vs. Bcl-2-positive ones (mean survival: 17.67 months; 95% CI: 6–29) had a longer survival ($P = 0.01$; odds ratio = 6.9).

CONCLUSIONS: Bcl-2 is associated with aggressive disease, neck lymph node metastasis, and poor prognosis. Whereas Bax is related with histologic grade.

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Introduction

Squamous cell carcinoma (SCC) of the oral cavity represents the sixth most common solid cancer worldwide (1). Locoregional node metastasis, the main prognostic factor in oral cancer, correlates with clinical stage and location of primary tumors. Among the different sites within the oral cavity, carcinoma of the tongue is the most common, and metastasis to cervical lymph nodes is said to occur more frequently from carcinomas of the tongue than from any other intra-oral carcinomas. Frazell and Lucas (2) found that of 1554 patients afflicted of tongue carcinoma, 40% had neck node metastasis at the time of diagnosis.

Among the molecular mechanisms involved in carcinogenesis, defects in the regulation of programmed cell death (apoptosis) may contribute to the pathogenesis and progression of cancer. Dysregulation of oncogenes and tumor suppressor genes involved in apoptosis has been associated with tumor development and progression (3).

Apoptosis regulatory genes include the *bcl-2* gene family, which codes for both proapoptotic and anti-apoptotic proteins (4) such as Bcl-2, Bax, Bcl-x, Bad, Bak, and others. The *bcl-2* oncogene encodes a 26-kDa protein associated the expression of which is topographically restricted to cells in proliferating zones and cells with long lifespans, and is downregulated in terminally differentiating cells (5). In normal stratified squamous epithelia, Bcl-2 expression has been found only in the basal cell layer (6) or in all tissue layers (7). It was recently proposed that Bcl-2 could inhibit cancer progression (8), and it is still unclear whether Bcl-2 expression has any prognostic significance in oral carcinoma.

The Bcl-2-associated X protein (Bax) is a 21-kDa protein that forms homodimers or heterodimers with Bcl-2. Apoptosis depends on the ratio of these two proteins, because it is promoted by Bax and inhibited by the Bax/Bcl-2 heterodimer (9). Furthermore, the relationship between Bcl-2 and Bax is probably modified by the wild form of the gene p53 (9).

The prognosis and clinical outcome of patients afflicted or oral cancer depend mainly on the stage of the disease; however, the widely used tumor node metastasis (TNM) staging system, does not allow for accurate prognosis because it does not incorporate biologic factors. Thus, identification of biologic factors that contribute to the clinical aggressiveness of a cancer can help identify different risk groups. Several prognostic factors are under investigation in oral SCC, including the proteins encoded by *bax* and *bcl-2* genes. However, assessment of the role of biomarkers as indicators of prognosis or response to treatment is complex and not well understood, and thus far the results are heterogeneous. In the present study, we have evaluated the immunohistochemical expression of Bcl-2 and Bax proteins in a series of 35 SCC of the tongue. The aim was to determine the spectrum of Bax and Bcl-2 expression in tongue carcinoma, and to correlate these findings with clinicopathologic variables and prognosis.

Material and methods

Patients

This study is based on 35 patients suffering from a primary tongue carcinoma who were treated at the Department of Oral and Maxillofacial Surgery, Oviedo University Hospital, Oviedo, Spain, from November 1995 to September 2002, and randomly selected from a cohort of 286 consecutive cases. All of them were selected from among the patients having suffered surgically resected tumors for which complete clinicopathologic data and paraffin-embedded tumor specimens were available, and without distant metastases. Clinicopathologic information on each case, including age, gender, tumor size, nodal status, histologic grade, treatment, and presence or absence of tumoral recurrence was obtained from patient files. The characteristics of the 35 patients selected for this study are summarized in Table 1. The average follow-up period was 35.3 months ranging from 5 to 103 months. All patients had been treated surgically with curative intention, and 13 (37%) underwent postoperative radiotherapy, having received 40–70 Gy. The recurrence-free survival and the survival of the patients were quantified. Clinical outcome was measured by two end points: death caused by disease recurrence and non-treatable disease presence at the end of the follow-up time. At the end of this period 14 patients (40%) had died of tumoral recurrence, 20 cases (57%) were alive and free of recurrence, and one case (3%) recurrence-free up to the moment the patient was lost to follow up (at 28 months after the tumor treatment).

Table 1 Patient characteristics

Variable	*Number (%)*
Age [years; mean (range)]	59.8 (27–87)
Gender	
Male	24 (68.6)
Female	11 (31.4)
T-category	
T1	10 (28.6)
T2	10 (28.6)
T3	7 (20.0)
T4	8 (22.9)
N-category	
N0	22 (62.9)
N1	7 (20.0)
N2	5 (14.3)
N3	1 (2.9)
Stage	
I	8 (22.9)
II	7 (20.0)
III	9 (25.7)
IV	11 (31.4)
Histologic grade	
Poor	1 (2.9)
Moderate	6 (17.1)
Well	28 (80.0)
Recurrence	
Yes	14 (40.0)
No	20 (57.1)
Censored	1 (2.9)

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections of the 35 tongue carcinomas mounted on glass slides, using a streptavidin-biotin peroxidase technique. Surgical specimens were fixed in 10% neutral-buffered formalin (pH 7.4) at 4°C for 72 h and then embedded in paraffin. About 4 μm thick tissue sections were mounted on poly-L-lysine-coated slides. Briefly, the sections were dewaxed with xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of slides in methanol with 0.03% hydrogen peroxide for 30 min. The sections were then heated in 10 mM citrate buffer (pH 6.0) three times for 10 min each in a microwave oven at 500 W to retrieve antigenicity. The sections were rinsed in distilled water, and then in phosphate-buffered saline (PBS). Non-specific conjugation was blocked with a solution of 20% rabbit serum (Dako, Copenhagen, Denmark) applied to the sections for 10 min. The sections were incubated with primary antibodies against Bcl-2 (monoclonal anti-Bcl-2, clone 124; Dako, diluted 1:20; isotype IgG_1), both for 30 min; and Bax (monoclonal anti-Bax, clone 2D2; Zymed, South San Francisco, CA, USA, diluted 1:30; isotype mouse IgG_1-κ) overnight at 4°C. They were then rinsed in PBS and incubated with the 1:1000 dilution of the biotinylated antimouse rabbit immunoglobulin (Dako) for 30 min. The detection system was a polymer technology termed EnVision (K4001, Dako, Carpinteria, CA, USA). After washing in PBS, staining was incubated with 3,3′-diaminobenzidine-tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide for 5 min, and then lightly

counterstained with Mayer's hematoxylin. Formalin-fixed, paraffin-embedded tonsil and breast carcinoma tissue was included with each staining procedure and served as positive controls for Bcl-2 and Bax, respectively. For negative control, the primary antibodies were replaced with normal mouse serum. All slides were scored by two investigators, blinded to the histologic diagnosis and the clinical outcome. Occasional disagreements were discussed to reach a consensus. In cases of persistent differences between them, the sections were studied by a third independent observer and the majority decision was thus considered.

A semiquantitative evaluation of staining for Bcl-2 and Bax was performed, with scores ranging from 0, negative (absence of immunopositivity) to 1, low ($<50\%$); and 2, high (more than 50% of positively stained cells). Finally, due to the small number of Bcl-2-positive cases, the results of this protein were assigned to two categories, specifically any score above 0 was considered as expression. Areas with pronounced inflammation or necrosis were excluded.

Statistical analysis

An SPSS for Windows computer program (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The association between Bcl-2 or Bax immunoexpression, T- and N-stages, clinical outcome, and histopathologic grade was analyzed with the chi-square or Fisher's exact test (if $N < 5$). Association of continuous variables was evaluated with the *t*-test. The Kaplan–Meier method was used for the survival analysis and statistical significance was analyzed by the log-rank test. Tests were only considered statistically significant when the *P*-value was <0.05.

Results

Immunohistologic expression of Bax and Bcl-2 in primary tongue carcinoma

Immunoreactivity for Bax was detected in 13 of 35 cases (37.1%). Of these 13 cases, four cases (11.4%) strongly expressed Bax, and nine (25.7%) expressed $<50\%$ of positive cell staining. The remaining 22 cases (62.9%) were negative for Bax. On the contrary, Bcl-2 was only immunoexpressed in three of 35 cases of tongue carcinoma (8.6%) and in the remaining 32 cases was negative (91.4%). In the three Bcl-2-positive cases, the positively stained cells were always more than 50% in number. Cell immunostaining for Bcl-2 and Bax was cytoplasmic and granular (Fig. 1). In the majority of Bcl-2- and Bax-positive cases, the peripheral cells of differentiating epithelial tumor islands were intensely stained, with decreasing immunoreactivity toward the center of the neoplastic nests (Fig. 2). All negative controls were uniformly devoid of any staining, and strong positivity was seen in positive controls. Bax was positive in carcinoma breast cells and depicted a cytoplasmic and granular pattern. In tonsillar tissue, Bcl-2 immunostaining was always negative in follicular germinal centers and positive in mantle zones and in the interfollicular areas.

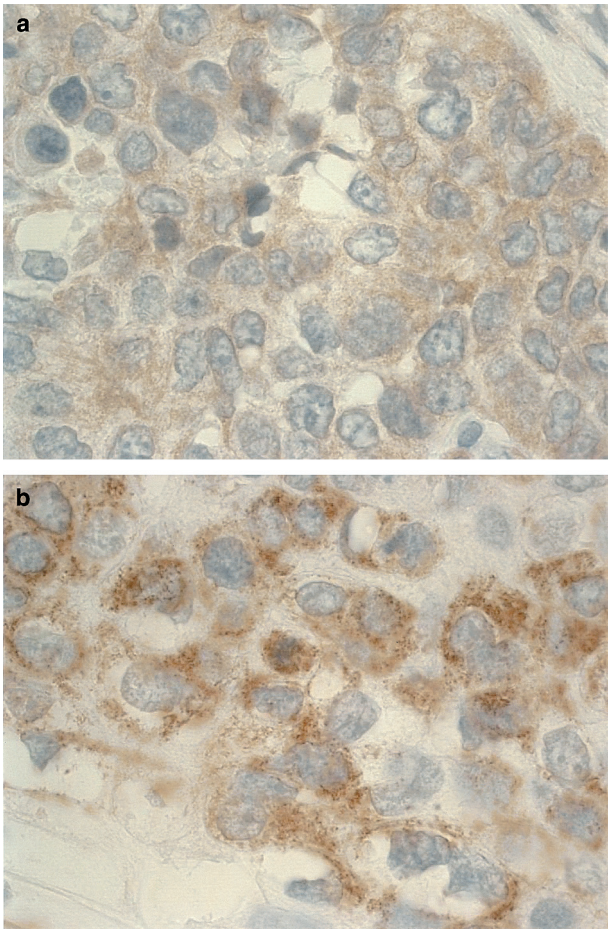

Figure 1 Representative example of cytoplasmic and granular immunostaining for Bcl-2 (a) and Bax (b; original magnification ×500).

Immunohistologic expression of Bax and Bcl-2 in primary oral tongue carcinoma with regard to clinicopathologic variables

Immunoexpression of Bax did not correlate with age or sex, T-size or N-stage. However, Bax immunoreactivity was significantly correlated with histologic grading ($P = 0.05$; Table 2). Performing Kaplan–Meier analysis for Bax expression showed no statistically significant correlation ($P = 0.47$), therefore, Bax immunoexpression does not appear to correlate with survival in our cohort (Fig. 3).

Immunoexpression of Bcl-2 did not correlate with age or sex, tumor size, or histologic grading. However, Bcl-2 immunoreactivity was significantly correlated with both N-stage ($P = 0.01$; Table 3) and survival. Performing Kaplan–Meier analysis, positive Bcl-2 immunostaining correlated with poor survival rates (Fig. 4). Thus, patients with Bcl-2-negative tumors [mean survival: 73.97 months; 95% confidence interval (CI): 59–88] vs. Bcl-2-positive ones (mean survival: 17.67 months; 95% CI: 6–29) had a longer survival ($P = 0.01$ by log-rank test; odds ratio: 6.9).

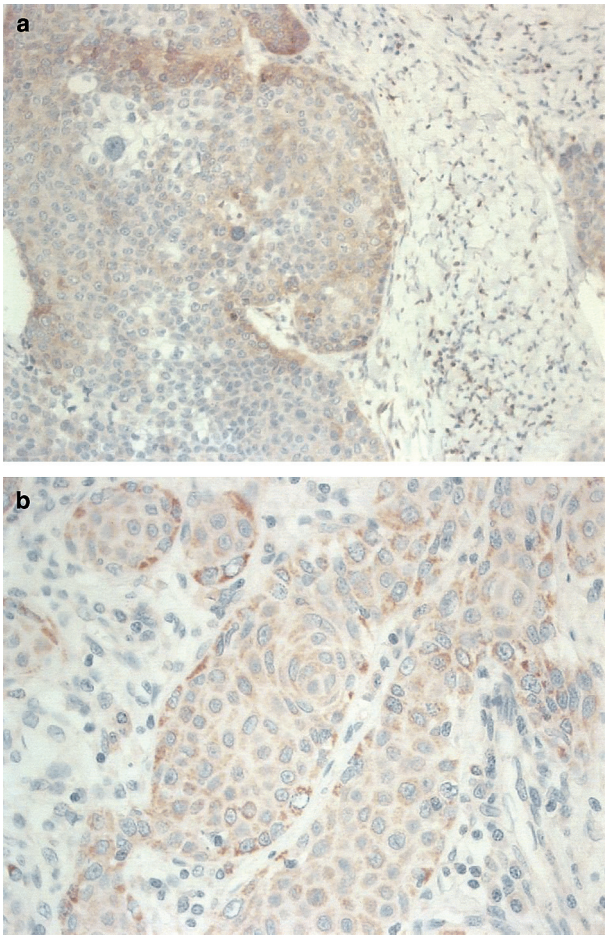

Figure 2 Bcl-2 (a) and Bax (b) immunoreactivity is seen in peripheral cells of islands, cords, and sheets of tongue squamous cell carcinoma, diminishing toward center (original magnification ×100).

Table 2 Association between Bax immunoexpression and histologic grade of differentiation

	Bax immunoexpression			
Histologic grade	–	+	++	*Total*
Poor and moderate	2	2	2	6
Well	20	7	2	29
Total	22	9	4	35

Discussion

The present study describes the expression of the apoptosis-regulating proteins Bcl-2 and Bax in tongue carcinoma. Bcl-2 and Bax proteins have been found in normal oral epithelium (6, 10, 11) and are overexpressed in several human cancers including oral carcinomas (7, 10–14), although their clinical significance and prognostic value is unclear. Bcl-2 protein expression in oral carcinomas varies between 7% and 60% in different studies in developed countries (15). In the present study, the Bcl-2 expression was observed in 8.6% of tongue carcinomas, and was significantly associated with poor outcome and shorter overall survival. Furthermore,

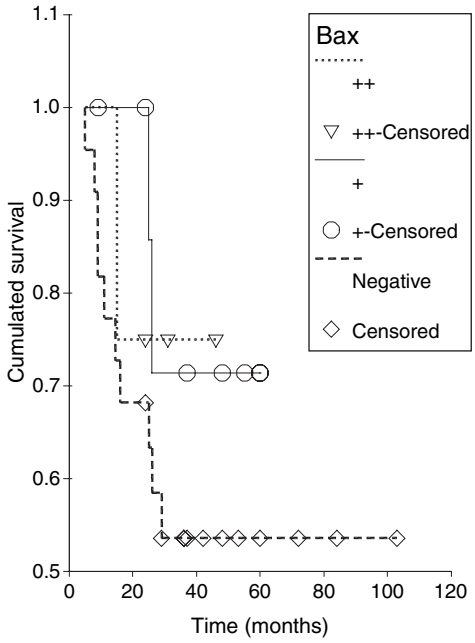

Figure 3 Survival curve for patients with tongue carcinoma according to the immunostaining of Bax ($P = 0.47$).

Table 3 Association between Bcl-2 immunoexpression and neck lymph node status

	Node status		
Bcl-2	*N0*	*N+*	*Total*
Negative	22	10	32
Positive	0	3	3
Total	22	13	35

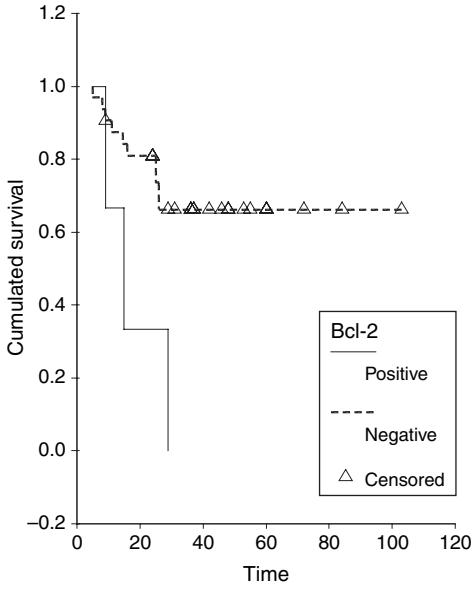

Figure 4 Survival curve for patients with tongue carcinoma according to the immunostaining of Bcl-2 ($P = 0.01$).

Bcl-2 expression correlated significantly with neck node metastasis ($P = 0.01$), indicating association with advanced disease, in accordance with the findings reported by Teni et al. (15). The expression of Bax in the present study was 37.1%, which is within the range of immunoexpression reported (2.2–63%; 7, 15, 16). The differences in Bcl-2 and Bax expression among several studies may reflect genetic, environmental, and methodological differences.

Our results differ from those of other studies (3, 17–20). Thus, Lo Muzio et al. (17) found that patients with absent or low Bcl-2 immunoreactive oral cancers manifested poorer overall survival rates in comparison to patients with moderate or high Bcl-2 immunoreactive tumors; however, the difference was not statistically significant. Yuen et al. (18) found no significant correlation between Bcl-2 expression in oral tongue carcinoma and survival, tumor grade, stage, and nodal metastasis. Stoll et al. (3) found that Bcl-2 was upregulated in 31.8% of SCCs of the oral cavity and oropharynx, and did not find an association with survival or time to recurrence. Staibano et al. (19) found that low positivity for proliferating cell nuclear antigen (PCNA) combined with high positivity for Bcl-2 protein correlated with a better clinical outcome in oral SCC. By converse, high expression of PCNA, Bax, and Bcl-1 appeared to correlate with a worse prognosis. Veneroni et al. (20) failed to demonstrate any prognostic significance of Bcl-2 expression in oral or oropharyngeal SCC.

On the contrary, our findings are consistent with those of Friedman et al. (21) who found that the expression of Bcl-2 in early SCC of the head and neck (SCCHN) predicted a cure rate of 50%, as opposed to the generally expected 90%, showing a statistically significant relationship between poor outcome and expression of Bcl-2. Gallo and Bianchi (22) also found that Bcl-2 expression was a significant predictor of short disease-free interval and decreased overall survival in early stage SCCHN. Our sample was different from the two previous studies, in which tumors were in early stages, and mainly located in larynx whereas in our sample all 35 patients had tumors located in the tongue, and they were homogeneously distributed in the four disease stages (I–IV). It is noticeable that we found only 8.6% of Bcl-2 expression in tongue carcinomas, while Friedman et al. (21) found 36% and Gallo and Bianchi (22) 18.4% in early stages of SCCHN, mainly located in larynx. It could be because 97% of our cases were moderately or well-differentiated tumors, and terminally differentiating (keratinizing) cells showed diminishing immunoreactivity for Bcl-2 (15, 23). It has also been reported that Bcl-2 expression was more frequent in poorly differentiated, non-keratinizing, and early stage carcinomas in comparison with well-differentiated, keratinizing, and advanced stage carcinomas (7). Furthermore, the downregulation of Bcl-2 expression in advanced stage carcinomas with mestastatic potential suggest that this oncoprotein may not be essential for cell survival and that its role may be assumed by other oncoproteins, such as Bcl-x (24). Previous studies have shown the importance of Bcl-2 in early phases of oral carcinogenesis (23). In the present study, the expression of Bcl-2 showed a significant relationship with neck node metastasis and poorer prognosis, which supports the hypothesis that aggressive behavior of the carcinoma is determined in early stages of the disease and that Bcl-2 contributes to it. Our results are also in agreement with those of Costa et al. (25) who observed that all patients with Bcl-2-positive tumors relapsed within 1 year after surgery, whereas a 60% probability of 3-year disease-free survival was observed for patients with Bcl-2-negative tumors. Xie et al. (26), found a correlation between Bax immunoexpression and poor prognosis, and low Bcl-2 expression and favorable clinical outcome in SCC of the tongue. We also observed a significant correlation between positive nodal status and Bcl-2 expression, in accordance with Teni et al. (15), which contributes to support the relationship between Bcl-2 overexpression and aggressive disease. The mechanism by which Bcl-2 overexpression could lead to shorter survival is still poorly understood and quite speculative. Friedman et al. (21) suggest two possibilities. One is that Bcl-2 overexpression prevents spontaneous apoptosis in SCCHN, leading to more rapid accumulation of tumor cells for a given proliferation rate. Another possibility is that Bcl-2 confers resistance to therapy by blocking treatment-related apoptosis, regardless of the type of therapy used.

One interesting finding of this study was that the expression of Bax was associated with poor and moderately differentiated tumors ($P = 0.05$). These data conflict with findings reported by others (27, 28) but it is in accordance with those reported by Teni et al. (15). However, in the present study, Bax immunoexpression did not show a significant prognostic relevance in tongue carcinoma.

In conclusion, in our study of Bcl-2 and Bax immunoexpression in 35 cases of oral tongue cancer, we found that Bcl-2 is associated with a more aggressive behavior of tumor cells and enhances the ability to discriminate between patients with favorable and unfavorable clinical outcome. This would contribute to the possibility of developing new strategies to more accurately determine the prognosis of these tumors. It may be that our findings will have therapeutic implications, perhaps by increasing apoptotic cell death using antisense RNA for Bcl-2 (29).

A study with a larger sample size is required to firmly establish the utility of immunoexpression of Bcl-2 and Bax as molecular prognostic markers in tongue carcinoma. It would be of particular interest to test whether Bcl-2 is confirmed as an independent marker of tongue carcinoma with a dismal prognosis. Furthermore, studies of other oncogenes are needed to dissect the role of Bcl-2 and Bax in cancer progression, as it is a complex process that involves multiple proteins.

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