

# The Prognostic Values of BCL-2, Caspase-3 and GSTP Expressions in Salivary Gland Tumors

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## ABSTRACT

**Objective:** There are numerous diagnostic, biological, and histological manifestations of salivary gland tumors, each of which offers concerns and difficulties in terms of diagnosis, grading, categorization, and therapy. The purpose of this study was to evaluate and compare the immunohistochemical expression of Bcl-2, caspase-3, and GSTP in benign and malignant salivary gland tumors, as well as how they are connected to a variety of clinicopathological variables.

**Methods:** A total of 61 cases of buffered formalin-fixed, paraffin-embedded tissues from previously identified cases of benign and malignant salivary gland tumors were included in this study. The immunohistochemistry staining process was carried out according to the manufacturer's recommendations, employing polyclonal anti-Bcl-2, anti-caspase-3, and anti-GST antibodies.

**Results:** The correlation between mean tumor diameter and Bcl-2 expression was shown to be statistically significant ( $rs=0.258$ ,  $p<0.05$ ). In pleomorphic adenoma tumor tissues, there were statistically significant correlations between the expression levels of Bcl-2 and caspase-3 ( $rs=0.66$ ,  $p<0.01$ ), Bcl-2 and GST ( $rs=0.61$ ,  $p<0.01$ ), and caspase-3 and GST ( $rs=0.73$ ,  $p<0.01$ ) when tumor types were compared. The tissues with pleomorphic adenoma, adenoid cystic carcinoma, and mucoepidermoid carcinoma had the highest staining intensity of Bcl-2 expression, while the lowest staining intensity of GSTP expression was observed.

**Conclusion:** It seems probable to draw the conclusion that salivary gland tumors that resist apoptosis have elevated levels of Bcl-2 expression. The prognosis for salivary gland tumors may be poor due to the positive correlation between tumor diameter and high Bcl-2 expression.

## INTRODUCTION

Salivary gland tumors occur in the head and neck region after squamous cell carcinoma.<sup>[1]</sup> They constitute 3-4% of all head and neck tumors. The most common sites of occurrence are the parotid gland, submandibular gland, and sublingual gland. They are heterogeneous tumoral formations and are difficult to diagnose due to the overlapping microscopic features among tumors and intra-tumor morphological diversity.<sup>[2]</sup> This situation complicates the studies to elucidate the etiopathogenesis of tumors and causes

difficulties in diagnosis. As a result of advances in molecular techniques, various human tumors are being analyzed more extensively, and by virtue of a limited number of molecular studies on malignant salivary gland tumors, some genetic changes have been detected in proto-oncogenes, anti-oncogenes, and apoptotic genes.<sup>[3,4,5]</sup> Expressions of these genes in salivary gland tumor tissues have prognostic value. This information is important for understanding tumor biology, for clinical follow-up, and management of tumors.

Apoptosis, known as programmed cell death, occurs in normal and pathological conditions. As the target of treat-

ment strategies, apoptosis plays an important role in cancer therapy. Changes in the apoptotic pathway are often associated with cancer or a pathological condition.<sup>[6,7]</sup> It is thought that some apoptotic and anti-apoptotic markers play a role in the prognosis of oral tumors and are important in determining the behavior and pathogenesis of these tumors.<sup>[8,9]</sup>

Bcl-2 was carried by patients with the follicular variant of B-cell lymphoma.<sup>[10]</sup> It is expressed in many types of malignant tumors and protects cells from apoptosis-induced DNA damage.<sup>[11]</sup> Its increased expression reduces the susceptibility of cancer cells to apoptosis and is associated with the aggressiveness of tumor cells.<sup>[12]</sup> It also plays a role in maintaining mitochondrial membrane integrity and regulating caspase-3 activation.<sup>[13]</sup>

Caspase-3 is the enzyme responsible for the actual destruction of the cell after activation during apoptosis.<sup>[14]</sup> Caspases are implicated in nuclear changes in apoptosis in both the intrinsic and extrinsic pathways.<sup>[15]</sup> An alteration or loss of function of caspases leads to disruption of the apoptotic process and ultimately to cancer.<sup>[16]</sup>

Glutathione S-transferases (GST) are Phase-II enzymes that are primarily found in the cytosol. They are involved in cancer metabolism by metabolizing certain drug-active components and detoxifying a wide variety of chemical carcinogens in cells. Many human cancers frequently have overactive GST proteins.<sup>[17]</sup> The majority of GST proteins have complicated, multifaceted biology, and recent research has shown that these proteins actively contribute to tumorigenic processes like drug resistance, cell survival, and proliferation.<sup>[18,19]</sup> Among these enzymes, GST-Pi (also known as GSTP or GSTP1-I) is responsible for catalyzing the reaction between glutathione (GSH) and its electrophilic substrates when harmful free radicals are present. Additional investigation has also shown that GSTP is highly expressed in tumor cells and strongly linked to the development of tumors, carcinogenesis, and resistance to chemotherapy.<sup>[20,21]</sup>

This study aims to compare the expression of Bcl-2, caspase-3, and GSTP in benign and malignant salivary gland tumors and to evaluate their role in the development of these tumors by analyzing the clinical and demographic data of the patients.

## MATERIALS AND METHODS

A total of 61 cases of buffered formalin-fixed, paraffin-embedded tissues from previously identified cases of benign and malignant salivary gland tumors is included in this study. The benign tumors, such as pleomorphic adenoma (PA, 31 cases) and malignant tumors, such as mucoepidermoid carcinoma (MEC, 10 cases) and adenoid cystic carcinoma (ACC, 20 cases), were taken for the study.

The clinical and pathological data of the patients were obtained from the hospital electronic database and patient reports. Tissue blocks that best represent the tumor were

selected for this study. New sections were taken from each selected block for histological evaluation and stained with Hematoxylin & Eosin. The diagnoses were confirmed by reviewing the Hematoxylin & Eosin stained slides. After histological evaluation, 4-5 $\mu$  thick thin sections were taken from each selected tumor block for immunohistochemistry.

## Immunohistochemical Procedure

For immunohistochemical staining, 61 formalin-fixed, paraffin-embedded tissue sections, after deparaffinization, were incubated with 3% hydrogen peroxide for 10 minutes. The sections were boiled in a pressure cooker with citrate buffer pH 6.0 for 3 minutes. The sections were then incubated for 10 minutes at room temperature with protein blocking (SHP125; ScyTek Laboratories, West Logan, UT). Sections were incubated with diluted primary antibody anti-bcl-2 (E-AB-64075, Elabscience Biotechnology Inc, USA, dilution 1:50), anti-caspase-3 (E-AB-63602, Elabscience Biotechnology Inc, USA, dilution 1:100), and anti-GSTP (E-AB-40418, Elabscience Biotechnology Inc, USA, dilution 1:500) for 1 hour. The secondary antibody streptavidin-peroxidase complex (SHP 125) (ScyTek Laboratories, West Logan, UT, USA) was applied for 10 minutes. Diaminobenzidine (DAB) was then incubated to monitor peroxidase activity. Hematoxylin was used for counterstaining. Tissue sections were evaluated by two expert pathologists. Immunohistochemical evaluations according to the staining intensities of the tissues under the light microscope were as follows: (0) negative staining (no staining), (+1) mild staining (there is protein expression), (+2) moderate protein expression, (+3) severe staining.

## Statistical Analysis

Analyses were made using RStudio version 1.4.1103. The relationship between age and mean tumor size and the relationship between expression levels for each tumor type were investigated using the Spearman's rho test. The relationship between tumor types and mean expression levels was investigated by the Kruskal-Wallis test.

## RESULTS

Bcl-2, caspase-3, and GSTP protein expressions in salivary gland benign and malignant tumor samples of 61 patients were investigated using the immunohistochemical staining procedure. Some descriptive features of the patients included in the study are shown in Table 1.

Only the correlation between Bcl-2 expression and tumor size is statistically significant ( $r_s=0.258$ ,  $p=0.045<0.05$ ), whereas the correlation coefficient between the expression levels of each protein and age is not statistically significant ( $p>0.05$ ) (Table 2).

When the expression levels of the monitored proteins were compared in each tissue group, statistically significant correlation rates were found only in PA tumor tissues; the Spearman correlation coefficients between Bcl-2 and caspase-3, Bcl-2 and GSTP, and caspase-3 and GSTP are

**Table 1.** Patients and treatment characteristics

|                          | N              | %    |
|--------------------------|----------------|------|
| Gender                   |                |      |
| Female                   | 31             | 50.8 |
| Male                     | 30             | 49.2 |
| Tumor Type               |                |      |
| Adenoid Cystic Carcinoma | 20             | 32.8 |
| Mucoepidermoid Carcinoma | 10             | 16.4 |
| Pleomorphic Adenoma      | 31             | 50.8 |
| Relapse                  |                |      |
| None                     | 50             | 82.0 |
| Yes                      | 11             | 18.0 |
| Age                      | 47.3 (average) |      |
|                          | 61 (range)     |      |
| Tumor Size Mean          | 14.93 mm       |      |

$r_s=0.66$  ( $p<0.01$ ),  $r_s=0.61$  ( $p<0.01$ ), and  $r_s=0.73$  ( $p<0.01$ ), respectively (Table 3).

There was no statistically significant difference between ACC, MEC, and PA tumor groups in terms of Bcl-2 ( $p=0.219>0.05$ ), caspase-3 ( $p=0.451>0.05$ ), and GSTP ( $p=0.142>0.05$ ) scores (Table 4).

## DISCUSSION

Salivary gland tumors have highly variable histological and biological behavior. Since they are not very common tumors in the clinic, they pose difficulties in terms of accurate diagnosis. In addition, while tumors have similar histological features, they could also show different histopathological features within the same tumor, and this fact becomes the source of complexity in their diagnosis. For this reason, molecular-based studies conducted in recent years are important for the diagnosis of tumors and targeted therapies.

Apoptosis is the cell death process that is responsible for the removal of senescent, injured, and altered cells in normal and some specific pathologies such as neoplasia.<sup>[22]</sup> Changes in apoptosis rates can result in oncogenic and pathological changes. Inhibition of the apoptotic pathway in the tumorigenesis process has provided new targets for molecular cancer therapy.<sup>[23]</sup>

Bcl-2 plays a role in protecting normal and neoplastic cells from apoptosis. Its higher expression in tumors is associated with resistance to treatment and tumor aggressiveness.<sup>[24]</sup> It has also been reported that high Bcl-2 expression in salivary gland tumors prevents salivary gland tumors from the apoptotic process.<sup>[25]</sup>

Yin et al.<sup>[26]</sup> studied 71 mucoepidermoid tumor tissues originating from minor salivary glands and compared Bcl-2

**Table 2.** Relationship between age and tumor size with Bcl-2, caspase-3 and GSTP. The values in parentheses indicate p-values

|            | Bcl-2          | Caspase3       | GSTP           |
|------------|----------------|----------------|----------------|
| Age        | 0.185 (0.153)  | 0.039 (0.763)  | -0.041 (0.756) |
| Tumor Size | 0.258* (0.045) | -0.001 (0.994) | 0.112 (0.391)  |

\*: Statistically significant.

**Table 3.** Relationship between the expressions of marker proteins according to tumor types. The values in parentheses indicate p-values

|                          | Marker    | Caspase-3      | GSTP           |
|--------------------------|-----------|----------------|----------------|
| Adenoid Cystic Carcinoma | Bcl-2     | 0.24 (0.32)    | 0.36 (0.12)    |
|                          | Caspase-3 | -              | 0.39 (0.09)    |
| Mucoepidermoid Carcinoma | Bcl-2     | 0.05 (0.90)    | 0.05 (0.90)    |
|                          | Caspase-3 | -              | 0.14 (0.70)    |
| Pleomorphic Adenoma      | Bcl-2     | 0.66* (<0.001) | 0.61* (<0.001) |
|                          | Caspase-3 | -              | 0.73* (<0.001) |

\*: Statistically significant.

**Table 4.** Mean ranks of expression levels ( $\pm$ SD)

|                              | Bcl-2           | Caspase3        | GSTP            |
|------------------------------|-----------------|-----------------|-----------------|
| Adenoid Cystic Carcinoma     | 2.55 $\pm$ 0.69 | 2.20 $\pm$ 0.83 | 0.55 $\pm$ 0.61 |
| Mucoepidermoid Carcinoma     | 2.80 $\pm$ 0.42 | 2.10 $\pm$ 0.74 | 1.10 $\pm$ 0.10 |
| Pleomorphic Adenoma          | 2.32 $\pm$ 0.87 | 1.90 $\pm$ 0.83 | 0.84 $\pm$ 0.58 |
| Kruskall-Wallis test p-value | 0.219           | 0.451           | 0.142           |

expression levels according to the degree of differentiation. They found that low-grade tumors expressed higher Bcl-2 compared to high and intermediate-grade tumors. da Cruz Perez et al.<sup>[27]</sup> also reported that low-grade MECs expressed more Bcl-2 than intermediate and high-grade MECs, implying that Bcl-2 might be used as a prognostic marker.

There is a relationship between Bcl-2 overexpression and salivary gland tumor types, with malignant tumors expressing higher Bcl-2 compared to benign tumors.<sup>[28,29]</sup> When Manjunatha et al.<sup>[30]</sup> compared Bcl-2 expression in a study of 50 benign and malignant salivary gland tumors, they found Bcl-2 expression in 55% of PA, 45% of MEC, and 100% of ACC. In this study, the most intense staining was observed in MEC, and the least intense staining was observed in PA when the average Bcl-2 protein expressions were compared.

It has been reported that Bcl-2 expression varies in epithelial neoplasms such as salivary gland tumors, and this expression difference is related to cell type and degree of differentiation.<sup>[31]</sup> It has been reported that Bcl-2 expression is negative in cells with terminal differentiation, such as salivary gland mucosa cells, normal acinar cells, and duct cells, whereas Bcl-2 expression is positive in basal cells of mucous and intermediate cells and normal oral epithelium.<sup>[31]</sup>

Atlı et al.<sup>[32]</sup> reported statistically significant increases in some of the GST isozymes in salivary gland tumors. In parallel with the current study, Zieper et al.<sup>[33]</sup> reported that the majority of salivary gland tumors, whether benign or malignant, displayed mild GSTP staining. Only MECs had considerably higher GSTP reactivity than other tumors, which could be due to the malignancy of the tumor. They also stated that a significant rise in GSTP activity in mucoepidermoid carcinomas should be relevant in recurrent and metastatic MECs. In our study, the comparatively modest immunostaining reactivity of GSTP in adenoid cystic carcinoma may be explained by the fact that as the size of the gland decreases, the incidence of tumor malignancy in the gland increases.

Caspase-3 is a key regulator of the apoptotic pathway. Some studies showed that high levels of caspase-3 lead to a favorable prognosis.<sup>[34]</sup> Koyun et al.<sup>[35]</sup> showed that the staining intensity of caspase-3 was lower in basal cell carcinoma samples than in normal tissues. Also, Winter et al.<sup>[36]</sup> indicated that low levels of caspase-3 were shown in poorly differentiated prostate cancers. Devarajan et al.<sup>[37]</sup> studied that low expression of caspase-3 may be related to the chemoresistance of breast cancer tissue. In the present study, we did not find any statistical differences in PA, MEC, and ACC salivary gland tumors.

The association of combined Bcl-2, caspase-3, and GSTP staining with salivary gland tumors may provide clinicians with useful information about tumor progression because the immune-reactivity of those proteins was associated with larger tumors, higher histologic grades, and greater invasion extension.

## Conclusion

In the current study, all benign and malignant salivary gland

tumors had high Bcl-2 expression and low GSTP expression. A link was discovered between tumor size and Bcl-2 expression. Increased Bcl-2 expression in benign and malignant cancers may block apoptosis and contribute to tumor cell immortalization. Because the number of patients in this study was modest and it was the first investigation with these tumor groupings, further comprehensive studies with larger sample sizes are required.

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## Ethics Committee Approval

This study approved by the Kartal Dr. Lütfi Kırdar City Hospital Ethics Committee (Date: 10.11.2021, Decision No: 2021/514/213/1).

## Informed Consent

Retrospective study.

## Peer-review

Externally peer-reviewed.

## Authorship Contributions

Concept: M.A., S.O.; Design: M.A., S.O.; Supervision: S.Ç., S.O.; Fundings: S.Ç.; Materials: K.B., S.A., M.G.D., G.K.A.; Data: M.G.D., K.B., G.K.A.; Analysis: F.K., C.Y.; Literature search: M.A., C.Y.; Writing: M.A.; Critical revision: S.O.

## Conflict of Interest

None declared.

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## Tükürük Bezi Tümörlerinde Bcl-2, Kaspaz-3 ve GSTP Ekspresyonlarının Prognostik Değerleri

**Amaç:** Tükürük bezi tümörlerinin çok sayıda tanısıl, biyolojik ve histolojik belirtileri vardır; bunların her biri tanı, tedavi ve tümörlerin kategorize edilmesi açısından zorluklar oluşturmaktadır. Bu çalışmanın amacı, benign ve malign tükürük bezi tümörlerinde Bcl-2, kaspaz-3 ve GSTP'nin immünohistokimyasal ekspresyonlarını incelemek ve çeşitli klinikpatolojik değişkenlerle nasıl bağlantılı olduğunu değerlendirmektir.

**Gereç ve Yöntem:** Bu çalışmaya benign ve malign tükürük bezi tümörü tanısı almış, formalinle tamponlanmış ve parafine gömülü dokular-dan oluşan 61 vaka dahil edilmiştir. İmmünohistokimya boyama işlemi, poliklonal anti-Bcl-2, anti-kaspaz-3 ve anti-GSTP antikorları kullanılarak üreticinin tavsiyelerine göre gerçekleştirildi.

**Bulgular:** Ortalama tümör çapı ile Bcl-2 ekspresyonu arasındaki korelasyonun istatistiksel olarak anlamlı olduğu gösterilmiştir (rs=0.258, p<0.05). Bcl-2 ve kaspaz-3 (rs=0.66, p<0.01), Bcl-2 ve GSTP (rs=0.61, p<0.01), kaspaz-3 ve GSTP (rs=0.73, p<0.01) ekspresyon düzeyleri tümör tipleri ile karşılaştırıldığında, pleomorfik adenoma tümör dokularında ekspresyon düzeyleri arasında anlamlı bir korelasyon bulunmuştur. Pleomorfik adenom, adenoid kistik karsinom ve mucoepidermoid karsinomlu dokularda Bcl-2 ekspresyonu en yüksek boyanma yoğunluğuna sahipken, GSTP ekspresyonu en düşük boyanma yoğunluğuna sahipti.

**Sonuç:** Apoptoza dirençli tükürük bezi tümörlerinin yüksek düzeyde Bcl-2 ekspresyonuna sahip olduğu sonucu çıkarılabilir. Tümör çapı ve yüksek Bcl-2 ekspresyonu arasındaki pozitif korelasyon, tükürük bezi tümörlerinde kötü prognoza neden olabilir.

**Anahtar Sözcükler:** Bcl-2; kaspaz-3; GSTP; tükürük bezi tümörü.