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# Bcl-2 Protein Expression in the Oral Mucosa of Smokers: An Immunohistochemical Comparison with Non-Smokers

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**Abstract:** ***Tobacco use is a major risk factor for oral cancer, known to disrupt cellular apoptosis, DNA repair, and proliferation mechanisms. This study investigates the immunohistochemical expression of Bcl-2 protein in the oral mucosa of Saudi cigarette smokers, aiming to explore its potential as a prognostic biomarker. A total of 200 buccal mucosa samples were collected, 100 from smokers and 100 from non-smokers-between January and May 2025. Using immunohistochemical techniques, the samples were analyzed for Bcl-2 expression and cytological abnormalities. Results showed that 48% of smokers had abnormal cytological findings compared to 20% of non-smokers. Additionally, smokers demonstrated a higher Bcl-2 expression (12%) compared to non-smokers (5%). The findings underscore the harmful cellular effects of smoking and suggest that Bcl-2 may serve as a useful biomarker in assessing oral health risks in tobacco users.***

**Keywords:** oral mucosa, Bcl-2 expression, tobacco use, immunohistochemistry, cytological abnormalities

## 1. Introduction

Smoking presents serious health hazards and is a leading cause of illness and early death worldwide. Smoking-related diseases claim the lives of five million people annually, the majority of whom reside in underdeveloped nations [1]. Every year, the United States spends approximately $167 billion on smoking-related health issues [2]. In a similar vein, Saudi Arabia spends over $160 million on tobacco each year [3].

Many studies have looked at the changes in cells in the mouth mucosa of smokers using different methods, such as cytological assessment, DNA damage detection, and histological investigations. These studies show that smoking causes many changes, such as changes in cell differentiation, greater cell proliferation, and increased epithelial thickness. In many cases, these changes occur because tobacco smoke damages DNA and causes changes in genes that either prevent or cause tumors [4].

The process of carcinogenesis doesn't involve increased cell proliferation alone but also decreased apoptosis, which allows increased cell survival and accumulation of genetic damage [5]. Apoptosis is controlled by various proteins including bcl-2. Bcl-2 is an anti-apoptotic protein that regulates the apoptotic pathway by inhibiting post-mitotic differentiation, thereby preventing apoptosis. [6,7].

A lot of people use immunohistochemistry (IHC) to find and measure protein expression in organs. Certain antibodies bind to the target protein, while a chromogen stains the antibody-bound protein to reveal it under a microscope [8]. This study is significant as it contributes to understanding how Bcl-2 expression may serve as a diagnostic biomarker for early detection of smoking-related oral pathologies.

This study aims to investigate the cellular alterations and immunohistochemical expression of Bcl2 in the oral mucosa among smokers in Saudi Arabia, providing insights into the potential biomarkers for early detection of smoking-related oral pathologies.

## 2. Materials and methods

**Study Design and Participants From:**
From January to May 2025, 200 randomly selected healthy volunteers participated in this cross-sectional study, including 100 cigarette smokers and 100 nonsmokers serving as the control group. The Epi Info Software Package Version 7.2 (Centers for Disease Control and Prevention, Atlanta, Georgia) determined the sample size based on a 95% confidence level and a 5% margin of error. All participants provided two buccal smears each, with the study adhering strictly to safety protocols. The study included Saudi nationals aged 18 to 85 years who were in good general health, regardless of smoking status. Exclusion criteria included non-Saudi citizens and individuals under the age of 18.

**Sample Collection:**
A buccal smear is a medical procedure that involves collecting cells from inside the buccal mucosa for examination. Using a wooden tongue depressor, we obtained exfoliative cells from the oral mucosa, specifically from the tongue dorsum and both cheeks. Subsequently, we proceeded to evenly distribute the cells onto two pristine glass slides and promptly preserved them in 95% ethyl alcohol while they were still damp. We dispatched the buccal smears to the histopathology lab at Rayyan College of Medicine in Saudi Arabia for staining and diagnosis.

**Papanicolaou's Staining:**

After fixation in ethanol, we hydrated the smears in a descending series of ethanol concentrations (diluted with distilled water) from 95% to 70% for two minutes each. We treated the smears with Harris hematoxylin for five minutes to stain the nuclei, rinsed them in distilled water, differentiated them in 0.5% aqueous hydrochloric acid for ten seconds, and then rinsed them again in distilled water. Following a four-second blue dyeing process in alkaline water, we dehydrated the smears twice, for two minutes each, using an ascending series of ethanol concentrations from 70% to 95%. We then stained the smears with Papanicolaou Orange G6 solution for two minutes, rinsed them with 95% ethanol, incubated them with Papanicolaou EA50 staining solution for three minutes, and checked for cytoplasmic staining. Following dehydration in 95% pure ethanol, we cleared the smears in xylene and mounted them using dibutylphthalate polystyrene xylene (DPX) [12].

**Immunocytochemistry:**
We subjected the smears to three rounds of rinsing, each lasting three minutes, using phosphate-buffered saline (PBS). To decrease the activity of endogenous peroxidase, we applied a solution of 0.3% hydrogen peroxide in methanol to each slide for a duration of 15 minutes. We then rinsed the slides three times with PBS. Next, we exposed the slides to primary mouse monoclonal Bcl-2 antibodies (Gene Tech Company Limited, Shanghai, China) at a dilution of 1/100 for 30 minutes at a temperature of 37 °C. We treated the slides with a secondary antibody, specifically Chem Mate TM EnVision+/HRP (Gene Tech Company Limited), at room temperature for 30 minutes. We then performed three PBS washes, followed by two additional PBS washes.

We used a 1/100 dilution of diaminobenzidine (DAB) from Gene Tech Company Limited as the final chromogen to measure immunoreactivity. We applied the chromogen for 10 minutes and then washed it with distilled water for three minutes. Ultimately, we applied a hematoxylin counterstain to the sections for a duration of three minutes. Subsequently, we rinsed the sections in running tap water for a period of five minutes, followed by dehydration in a sequence of alcoholic solutions. Afterwards, we cleaned the sections with xylene and finally mounted them using DPX.

**Interpretation Criteria**: Bcl-2 staining were evaluated based on cytoplasmic expression patterns. Positive staining for both markers was determined by the presence of a clear brown coloration in the cytoplasm of the stained cells [9].

**Cytological Evaluation:**
We examined Pap-stained smears for cytopathological abnormalities. We looked for indications of keratinization, atypia, inflammation, and infection. Cytological changes identify features such as uneven growth and bi- or multi-nucleation [9].

**Quantitative Analysis:**
We used IBM SPSS Statistics for Windows, Version 22 (published in 2013; IBM Corp., Armonk, New York), for statistical analysis, setting the significance level at 0.05. We represented categorical data as frequencies or proportions and examined the study topics and data types with chi-square testing.

**Ethical Consent:**
Before collecting specimens, each participant was required to complete a written ethical consent form. The Al Rayyan Medical Colleges (AMC) Ethical Committee designed and approved the informed ethical consent form.

## 3. Results

**Cytological Findings**
Out of the 100 cigarette smokers, 48 (48%) showed cytological abnormalities, including inflammatory cells, infection, atypia, and binucleated/multinucleated cells. In contrast, among the 100 nonsmokers, 20 (20%) exhibited similar abnormalities (P = 0.015), as shown in Table 1 and Figure 1.

**Table 1:** Study group and cytological findings

| Gender | Normal Cells (%) | Abnormal Cells (%) |
|---|---|---|
| Smokers (n=300) | 52 (52%) | 48 (48%) |
| Nonsmokers (n=200) | 80 (80%) | 20 (20%) |

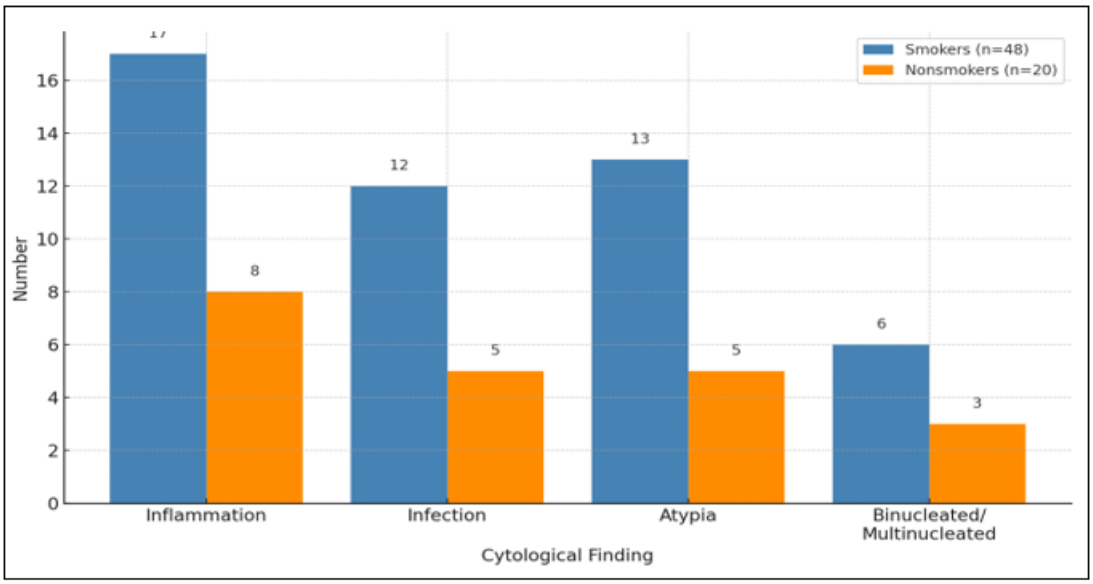


**Figure 1:** Distribution of Cytological Abnormalities

The age distribution of the participants ranged from 18 to 85 years. The study found no significant differences in Bcl-2 expression across different age groups (P = 0.57), as shown in Table 2.

**Table 2:** Age Distribution and Protein Expression

| Age Group (years) | Smokers (n=100) | Nonsmokers (n=100) | Bcl-2 Expression (%) |
|---|---|---|---|
| 18–30 | 30 | 30 | 10 (11.1%) |
| 31–45 | 27 | 25 | 8 (10%) |
| 46–60 | 23 | 20 | 9 (12.9%) |
| 61–75 | 13 | 15 | 5 (12.5%) |
| 76–85 | 7 | 10 | 4 (20%) |

**Table 3:** The gender distribution included 139 males and 61 females. There were no significant differences in Bcl-2 expression between male and female participants (P = 0.27)

| Gender | Smokers (n=100) | Nonsmokers (n=100) | Bcl-2 Expression (%) |
|---|---|---|---|
| Male | 72 | 67 | 25 (11.5%) |
| Female | 28 | 33 | 11 (7.9%) |

The immunohistochemical analysis revealed that smokers had significantly higher expression of Bcl-2 proteins compared to nonsmokers. 12 (12%) smokers and 1 (1%) nonsmoker showed overexpression of Bcl-2 (P = 0.017), as shown in Table 4.

**Table 4:** Immunohistochemical expression and study group

| Parameter | Smokers (n=100) | Nonsmokers (n=100) | P-value |
|---|---|---|---|
| Abnormal Cells | 48 (48%) | 20 (20%) | 0.015 |
| Bcl-2 Expression | 12 (12%) | 1 (1%) | 0.017 |

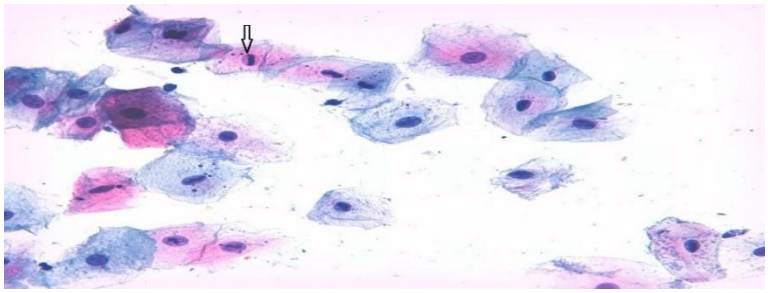

**Figure 3:** Microphotographs of smear samples from buccal mucosa stained with Papanicolaou's method (x40) demonstrate Atypia

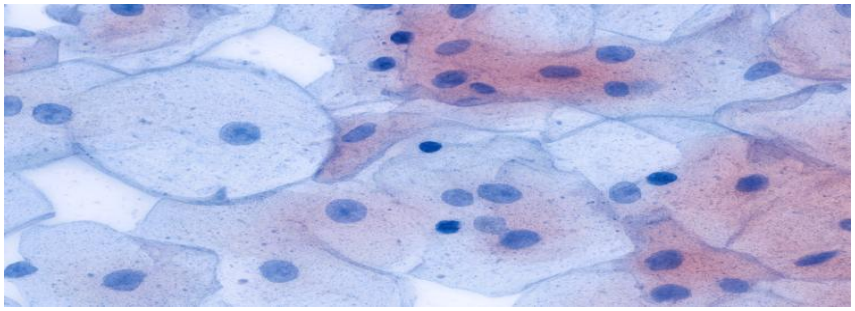

**Figure 6:** Microphotographs of buccal smears from cigarette smokers with immunohistochemical staining (x40) show the expression of Bcl-2 in the brown color of the cytoplasm.

## 4. Discussion

The development of oral cancer is a multistep process, marked by the progressive transformation of normal epithelial cells into malignant ones, often lacking clear boundaries. This transformation is primarily driven by the accumulation of genetic alterations that disrupt normal cellular regulation (10). In healthy oral mucosa, epithelial homeostasis and structural integrity are maintained through a balance between cell proliferation and cell loss by means of desquamation and apoptosis (11). The oral epithelium acts as a protective barrier against environmental carcinogens such as tobacco. In response to these insults, one of the adaptive mechanisms of the cells is the upregulation of epithelial proliferation, observed as epithelial hyperplasia (12,13). Nicotine, a major component of tobacco, has been shown to impair apoptosis, a key defense mechanism responsible for the elimination of damaged or pre-malignant cells. Dysregulation of apoptosis can therefore facilitate the development and progression of oral cancer (14).

This study classified participants by gender, age group, and cytopathological alterations, with male smokers predominantly found in the 31-40 age group and female smokers in the 18-20 age group. The elevated prevalence of smoking among young female adults (18-20) is consistent with global data showing that tobacco use often begins in adolescence or early adulthood, heightening the risk of long-term dependence and subsequent health complications. Abbas et al. (2024) found that 65% of teen smokers had developed significant oral mucosal lesions by age 30. They also found a strong link between starting to smoke early in life and a higher risk of chronic tobacco use and oral cancers later in life [15].

The findings showed a clear correlation between smoking and the development of abnormal alterations in the oral epithelium, which can lead to precancerous or malignant diseases. Similar studies, such as the one by Agabeldour et al. in Sudan, found that 47.1% of regular waterpipe users exhibited cytomorphologically unusual changes, compared to none in the control group [16]. In a different study, cytologic smear samples from the oral mucosa of waterpipe users showed changes that could be seen. The changes were similar for both smoking and hookah use, but smoking had a bigger impact [17]. A study in Jeddah, Saudi Arabia, revealed that 88.8% of smokers had soft tissue lesions and various oral conditions, although premalignant lesions were less common. This high incidence of oral mucosal soft tissue lesions is likely due to tobacco's irritating effect on oral tissues. The higher number of cytological changes seen in this study could be because of the cigar smokers and tobacco users who were there. Both factors have the potential to induce abnormal changes in oral epithelial cells [18].

Additionally, we found a significant association between cigarette smoking and inflammation of the oral mucosa, with 52% of smokers showing signs of inflammation compared to 15% of nonsmokers. This finding aligns with the current understanding that smoking weakens the immune response, increasing susceptibility to infections and inflammation. In our study, smokers were much more likely than nonsmokers to have bacterial infections (12%) in buccal smears. This finding aligns with the findings of Mehta et al. (2023), who discovered that smokers exhibit higher rates of bacterial colonization in their oral cavities when compared to controls. This is likely due to smoking's detrimental effects on oral immune defenses, which foster a pro-inflammatory environment conducive to infections [19].

The immunocytochemical analysis in the present study revealed a positive Bcl-2 expression in all smoker samples. The dysplastic oral epithelium exhibited stronger immunoreactivity, with a significantly higher mean area percentage compared to Group I, indicating a statistically significant difference.

These findings are in agreement with previous research assessing Bcl-2 expression in oral precancerous lesions and epithelial dysplasia [20]. Nair et al. (2011) observed moderate to intense immunoreactivity involving the entire epithelial thickness in cases of oral dysplasia [5]. Similarly, Arya et al. (2016) reported positive Bcl-2 staining predominantly in the basal and parabasal layers of oral precancerous lesions (21). Sadiq et al. (2015) also confirmed positive Bcl-2 expression, demonstrating a statistically significant, progressive increase in Bcl-2-positive cell counts from mild to severe dysplasia (22).

These results support the notion that Bcl-2 has an important role in the initial steps of oral carcinogenesis. Bcl-2 protein prevents apoptosis; thus its overexpression helps cells to escape apoptosis leading to prolonged cell survival which increase the opportunity of accumulation of mutations that consequently enhances the potential of malignant transformation [22].

The significant association between smoking and increased Bcl-2 expression emphasizes the need for targeted public health interventions to reduce smoking prevalence. Regular screening programs for early detection of oral mucosal changes in smokers could help prevent the progression to malignancy. We need future longitudinal research to establish causality and assess the long-term impact of smoking on oral health.

## 5. Limitations

This study's cross-sectional design limits the ability to prove that smoking caused the changes in the studied cells. Also, relying on people's self-reported smoking could lead to information bias. Using biochemical markers to confirm whether someone smokes would make the data more reliable.

## 6. Conclusions

This study demonstrates a considerable effect of smoking on oral cellular health. Smoking increases the expression of Bcl-2 proteins, leading to an increase in cytological problems. These findings highlight the critical need for smoking cessation programs and regular oral health screenings to mitigate risks. Utilizing Bcl-2 as biomarkers in routine assessments can aid early detection and prevention of oral diseases. Public health interventions should prioritize smoking reduction and public education on its detrimental health outcomes. Future research should explore the long-

term impact of smoking on oral health and the underlying molecular mechanisms.

## References

[1] Peto R, Lopez AD. The future worldwide health effects of current smoking patterns. *Tobacco and Public Health: Science and Policy.* New York: Oxford University Press; 2004. p. 281. DOI: 10.1093/oso/9780198526872.003.0016
[2] Marshall L, Schooley M, Ryan H, et al. Youth tobacco surveillance - United States, 2001-2002. *MMWR Surveill Summ.* 2006; 55:1-56.
[3] Ahmad MS. Prevalence and attitude of cigarette smoking among Indian expatriates living in Jeddah, Kingdom of Saudi Arabia. *J Int Oral Health.* 2015; 7:18-21.
[4] Irani S. New insights into oral cancer-risk factors and prevention: a review of literature. *Int J Prev Med.* 2020; 11:202. DOI: 10.4103/ijpvm.IJPVM_403_18
[5] Nair RG, Shameena PM, Varghese I, Sudha S. Immunohistochemical evaluation of bcl-2 oncoprotein in oral dysplasia and carcinoma. *OMPJ*. 2011;2(1):83–8.
[6] Juneja S, Chaitanya NB, Agarwal M. Immunohistochemical expression of Bcl-2 in oral epithelial dysplasia and oral squamous cell carcinoma. *Indian J Cancer*. 2015;52(4):505–10.
[7] Kumamoto H, Ooya K. Immunohistochemical analysis of bcl-2 family proteins in benign and malignant ameloblastomas. *J Oral Pathol Med*. 1999; 28:343–9.
[8] Mohanapriya S, Maheswaran T, Ganapathy N, et al. Evaluation of DNA damage in tobacco-associated human buccal cells using comet assay. Med Pharm Rep. 2021; 94:214-9. DOI: 10.15386/mpr-1692
[9] Huang CC, Lai CY, Tsai CH, Wang JY, Wong RH. Combined effects of cigarette smoking, DNA methyltransferase 3B genetic polymorphism, and DNA damage on lung cancer. *BMC Cancer.* 2021; 21:1066. DOI: 10.1186/s12885-021-08800-w
[10] Brunotto M, Zárate A, Cismondi A, Fernández M, Halac R. Valuation of exfoliative cytology as prediction factor in oral mucosa lesions. *Med Oral Patol Oral Cir Bucal*. 2005;10(2):92–102.
[11] Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. *J Natl Cancer Inst Monogr*. 2001;(29):7–15.
[12] Ten Cate AR. *Oral histology: development, structure and function*. St. Louis: Mosby Company; 2003. p. 329–75.
[13] Karthikeyan R, Reddy BS, Sherlin HJ, Ramani P, Premkumar P, Natesan A. Stromal changes in apparently normal mucosa of smokers and pan chewers – a multiparametric approach. *Braz J Oral Sci*. 2008;7(26):1602–8
[14] Taybos G. Oral changes associated with tobacco use. *Am J Med Sci*. 2003;326(3):179–82.
[15] Abbas Y, Kanotra S, Majeed F, Anjum A, Zehra M. Clinical Profile and Prevalence of Oral Mucosal Lesions in Tobacco Users-A Prospective Study from Jammu, India. Indian J Otolaryngol Head Neck Surg. 2024 Jun;76(3):2373-2380. doi: 10.1007/s12070-023-04433-6. Epub 2024 Jan 24. PMID: 38883546; PMCID: PMC11169173.
[16] Agabeldour AA, Ibrahim MI, Ahmed RA, Aljadoa SA, Ahmed HG. Assessment of oral epithelium cytomorphological changes among water pipe smokers. Eur Acad Res. 2020;3(11):2290-302.
[17] Elmahdi FM, Mostafa HE, Eldib AM, Youssef MH, Alahmadi LS, Alkurdi AA, Hussein HM. Evaluation of cellular changes and immunohistochemistry expression of p53 and p16 in the oral mucosa among Saudi smokers. *Cureus.* 2024;16(2). DOI: 10.7759/cureus.55027
[18] Mehta P, Singh N, George S. Association of cigarette smoking with oral bacterial microbiota and cardiometabolic health. *BMC Microbiol.* 2023; 23:112. DOI: 10.1186/s12866-023-02712-4
[19] Patel KR, Vajaria BN, Begum R, Desai A, Patel JB, Shah FD, et al. Prevalence of high-risk human papillomavirus type 16 and 18 in oral and cervical cancers in populations from Gujarat, West India. *J Oral Pathol Med.* 2014; 43:293-7.
[20] Sudha VM, Hemavathy S. Role of bcl-2 oncoprotein in oral potentially malignant disorders and squamous cell carcinoma: An immunohistochemical study. *Indian J Dent Res*. 2011; 22:520–5.
[21] Arya V, Singh S, Daniel MJ. Clinicopathological correlation of Bcl-2 oncoprotein expression in oral precancer and cancer. J Oral Biol Craniofac Res. 2016 Jan-Apr;6(1):18-23. doi: 10.1016/j.jobcr.2015.12.011. Epub 2016 Jan 19.
[22] Sadiq H, Ahuja P, Gupta P, Singh G, Singh N, Anand P. Immunohistochemical expression of P53 and Bcl-2 in varying grades of oral epithelial dysplasia. *J Pre Clin Dent Res*. 2015;2(4):17–25.