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Inmunohistochemical Expression of PCNA, p53 and bcl-2 in Pleomorphic Adenomas

Expresión Inmunohistoquímica de PCNA, p53 y bcl-2 en Adenomas Pleomórficos

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SUMMARY: The aim of the study was to determine the immunohistochemical expression of the PCNA, p53 and bcl-2 proteins in pleomorphic adenomas. Nineteen specimens of pleomorphic adenomas were selected for analysis by the streptavidin-biotin-peroxidase method with antibodies against p53, PCNA and bcl-2 proteins. It was observed weak labeling for p53 in 12 cases (63.1%) and for PCNA in 8 (42.1%). With respect to the bcl-2 labeling index, no expression of this protein was detected in 12 cases, corresponding to 63.1% of the sample. Based on these findings, it was concluded that p53 and PCNA can favor the proliferative activity of pleomorphic adenomas, whereas bcl-2 probably does not effectively participate in the pathogenesis of this tumor.

KEY WORDS: Pleomorphic adenoma; Salivary gland tumour; p53; PCNA; bcl-2.

INTRODUCTION

Pleomorphic adenoma, also known as a benign mixed tumor, is a benign neoplasm of the salivary gland which shows a remarkable degree of morphological diversity (Jaeger *et al*., 1997). It is the most common tumor of the salivary gland, accounting for approximately 40 to 70% of all salivary gland tumors (Matturri *et al*., 1996; Jorge *et al*., 2002; Neville *et al.,* 2002).

Most pleomorphic adenomas occur in the superficial lobe of the parotid gland; however, when they affect the minor salivary glands, the most frequent site of involvement is the region of the hard palate, followed by the upper lip and buccal mucosa (Neville *et al*.). Clinically, pleomorphic adenoma presents as a slow growing mass, firm on palpation, which is usually encapsulated and may ulcerate in some situations. It affects any age but is more frequent in adults aged 30 to 50 years, with a slight predominance among females (Felix *et al.*, 1999; Louro *et al.*, 2002). Histologically, the tumor is characterized by histomorphologic heterogeneity consisting of an epithelial component that forms ducts and cystic structures as well as cell clusters arranged in islands or nests, whereas the stroma can be mucoid, myxoid or chondroid depending on the product derived from the altered metabolism of epithelial cells (Matturri *et al*.).

Recurrence of pleomorphic adenomas is observed in 10 to 12% of cases and malignant transformation is not rare (Matturri *et al*.). A well-established fact in the literature is that carcinomatous foci occasionally develop in pleomorphic adenomas and that the risk of malignant transformation increases with the duration of these lesions (Ohtaké *et al*., 2002; Marioni *et al*., 2003).

The expression of genes related to cell proliferation and oncogenesis seems to be associated with the prognosis of some oral tumors. Mutation in the tumor suppressor gene p53 is the most common genetic modification found in malignant oral tumors. Additionally, numerous studies involving proliferating cell nuclear antigen (PCNA) and the bcl-2 protein have been conducted to determine the processes related to cell proliferation and, consequently, the susceptibility of some tumors to malignant transformation (Ohtaké *et al*.; Rosa *et al.*, 1997; Yanez *et al.,* 1999; Alves *et al.*, 2002).

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The objective of the present study was to determine the immunohistochemical expression of PCNA, p53 and bcl-2 in pleomorphic adenomas in order to contribute to the understanding of cell proliferation and the possible malignant transformation of this tumor.

MATERIAL AND METHOD

Nineteen representative pleomorphic adenoma specimens were selected for immunohistochemical analysis from the files of the Service of Pathologic Anatomy, Discipline of Oral Pathology, Department of Dentistry, Federal University of Rio Grande do Norte. The material from the files was cut into 5-µm thick slides and stained with hematoxylin-eosin for morphologic analysis by light microscopy.

For immunohistochemistry, 3-µm thick slides were obtained from each specimen and mounted on glass slides with organosilane (3-aminopropyltriethoxy-silane, Sigma Chemical Co., St. Louis, MO, USA). The streptavidin-biotin-peroxidase method (SABC – streptavidin-biotin complex) with antibodies against proliferation (p53 and PCNA) and anti-apoptosis (bcl-2) proteins was used. The sections were deparaffinized, submitted to antigen retrieval and incubated with the anti-p53, anti-PCNA and anti-bcl-2 primary antibodies as shown in Table I. For inactivation of endogenous peroxidase, the sections were immersed twice in hydrogen peroxide (10 volumes) for 5 min each. Between steps, the samples were washed with Tris-HCl, pH 7.4. The clones, dilutions and incubation times used are listed in Table I. The reaction was developed with 0.03% diaminobenzidine (Sigma) as chromogen, and the material was counterstained with Mayer's hematoxylin.

The slides submitted to immunohistochemistry were analyzed regarding the presence or absence of labeling of the antigens studied.

For quantitative analysis of p53, PCNA and bcl-2 positive cells, the cells were counted under a Nikon® light microscope using a 0.25-mm^2 Weibel NGW2® grid at a final magnification of 1000x by two independent examiners. The mean obtained was then used to calculate the labeling index based on the ratio of the number of immunopositive cells per 1000 randomly counted cells per case studied, multiplied by 100 to express the index in percentage. The labeling intensity was not considered in this analysis.

Since the quantitative data showed no normal distribution, the Kruskal-Wallis test was used for comparison at the 5% level of significance. Spearman's correlation coefficient at a level of significance of 5% was applied to determine a possible correlation between the three markers. The data were analyzed using the SPSS 13.0 software for Windows. For qualitative analysis which permitted comparison of the present data with those reported in the literature, the absolute labeling indices were transformed into the following scores according to the criteria of Alves et al.: (-) negative, ≤ 5%; (+) weak, >5 and ≤ 25%; (++) moderate, >25 and ≤ 50%; (+++) strong, >50%. These data were analyzed using the chi-square test for proportions at the 5% level of significance.

Table I. Specificity, clone, dilution, incubation time and previous treatment of the primary antibodies. Natal/RN, 2008.

Clone	Specificity	Source	Dilution	Incubation time	Antigen retrieval
DO-7	p53	Dako[a]	1:25	60 min	Steamer, 30 min – citrate, pH 6.0
PC10	PCNA	Dako[a]	1:50	Overnight (18 h)	Steamer, 20 min – citrate, pH 6.0
124	Bcl-2	Biogenex[b]	1:40	Overnight (18 h)	Steamer, 20 min – Tris-EDTA, pH 9.0

[a]Dako Corporation, Glostrup, Denmark. bBiogenex

RESULTS

The Kruskal-Wallis test revealed a significant difference between markers (p = 0.0044). Application of Dunn's multiple comparisons test showed that this difference was significantly only between p53 and bcl-2 (Table II).

The results of Spearman's correlation analysis between the absolute labeling indices obtained for the three markers are shown in Table III. A significant positive correlation was observed between p53 and PCNA and between PCNA and bcl-2, which was higher for p53 x PCNA. No significant correlation was noted between p53 and bcl-2.

Table IV shows the labeling indices obtained for p53, PCNA and bcl-2 in the pleomorphic adenomas studied. Taking into account the percent distribution of the labeling scores obtained for p53, the chi-square test for proportions revealed a significant difference, with weak labeling

Table II. Distribution of p53, PCNA and bcl-2 labeling in pleomorphic adenomas (n = 19). Natal/RN, 2008.

Marker	Median	Range	p
p53	99.500[a]	0.000-601.50	0.0044
PCNA	199.50[a,b]	10.500-957.50	
bcl-2	32.000[b]	0.000-632.50	

Different letters indicate a significant difference between markers.
Source: Post graduation Program in Oral Pathology – UFRN.

Table III. Correlation coefficient (r), confidence interval (CI) and p value obtained for the markers detected in pleomorphic adenomas (n = 19). Natal/RN, 2008.

Markers	R	95% CI	p
p53 x PCNA	0.5346	0.09176 – 0.8009	0.0184
p53 x bcl-2	0.4553	0.01322 – 0.7599	0.0501
PCNA x bcl-2	0.4584	0.009342 – 0.7615	0.0484

Source: Post graduation Program in Oral Pathology – UFRN

Table IV. Percent distribution of labeling scores for p53, PCNA and bcl-2 in pleomorphic adenomas. Natal/RN, 2008.

Marker	-	+	+ +	+ + +	X^2	p
p53	5 (26.3%)	12 (63.1%)	1 (5.3%)	1 (5.3%)	22.67	< 0.0001
PCNA	4 (21.1%)	8 (42.1%)	3 (15.7%)	4 (21.1%)	4.14	0.246
bcl-2	12 (63.1%)	5 (26.3%)	0 (0.0%)	2 (10.6%)	23.23	< 0.0001

(-) negative; (+) weak; (+ +) moderate; (+ + +) strong.
Source: Post graduation Program in Oral Pathology – UFRN.

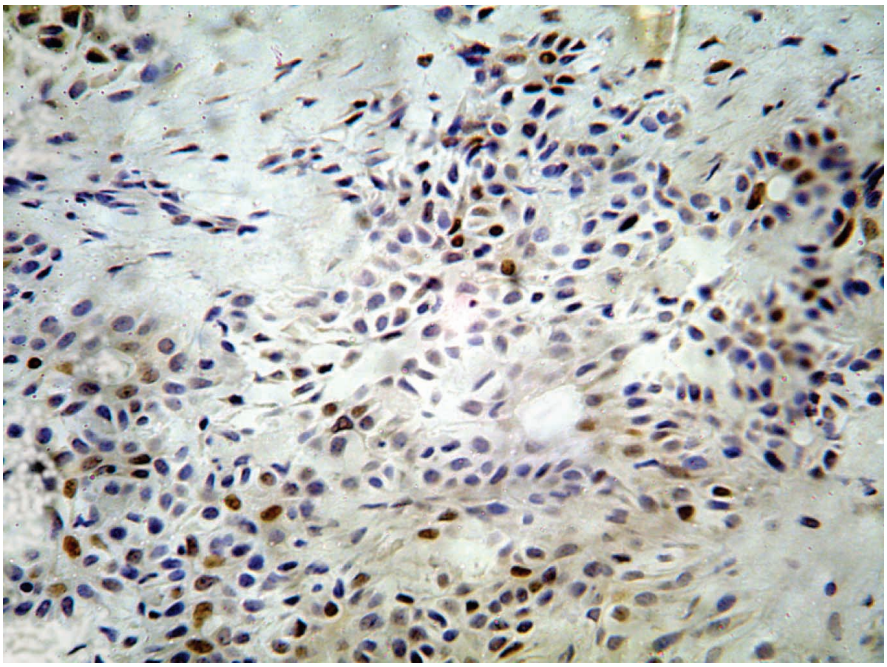

Fig. 1. Pleomorphic adenoma showing weak expression of p53 (p53 immunolabeling, 400x).

predominating in 12 cases, corresponding to 63.1% of the sample (p <0.00001) (Fig. 1). A predominance of weakly labeled cases was also observed for PCNA, although this difference was not significant (p = 0.246). Weak labeling was observed in 8 cases, corresponding to 42.1% of all cases evaluated (Fig. 2). However, some cases shown occasional strong expression of PCNA. A significant difference was observed in the percent distribution of bcl-2 labeling scores (p < 0.0001), with a predominance of bcl-2-negative cases, corresponding to 63.1% of the sample (12

cases). It was observed a weak expression of bcl-2 in 7 cases (Fig. 3). Taking into account the distribution of each score between markers, the proportion test revealed a significant difference for the negative score, which was observed for bcl-2 in most cases (p = 0.0136). With respect to the distribution of the other scores, no significant difference was observed between markers (p = 0.0712, p = 0.152 and p = 0.319, respectively).

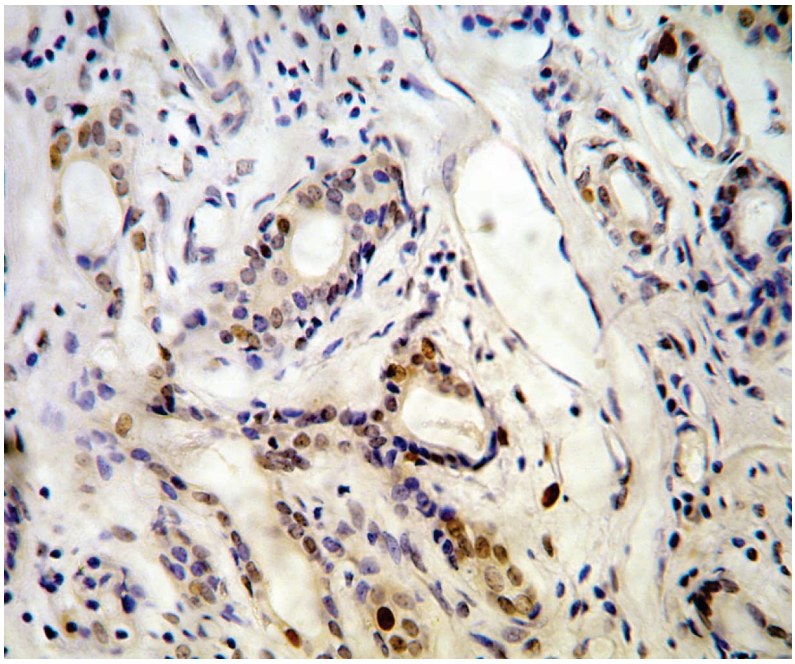

Fig. 2. Pleomorphic adenoma showing weak expression of PCNA (PCNA immunolabeling, 400x)

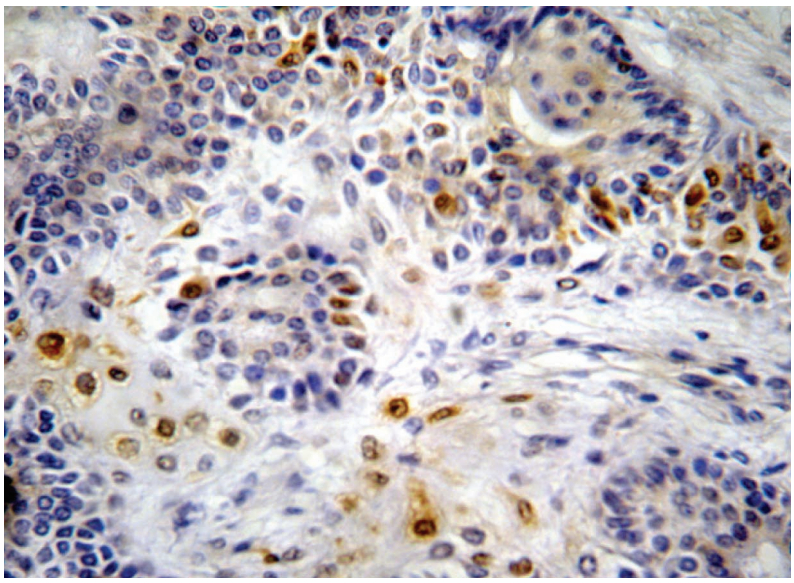

Fig. 3. Pleomorphic adenoma showing weak labeling of cells for bcl-2 (bcl-2 immunolabeling, 400x).

DISCUSSION

Pleomorphic adenoma is the most frequent salivary gland tumor, whose clinical and microscopic characteristics have been well established in the literature. However, the pathogenesis of this tumor is still unknown, a fact that encouraged the present immunohistochemical study of PCNA and bcl-2 in order to determine the role of these proteins in cell proliferation and even to establish a possible relationship of p53 expression with the malignant transformation of pleomorphic adenoma. PCNA is a nuclear protein that plays an important role in DNA synthesis, with a significant participation in cell replication (Zhu *et al.*, 1997). In the present study, most cases showing a predominantly weak labeling for PCNA. This result agrees with the studies of Matturri *et al*. and Alves *et al*., who reported a predominance of weak to moderate labeling of this protein in pleomorphic adenomas.

Strong labeling of PCNA was observed in four of the present cases, indicating a greater proliferative activity of these tumors and suggesting a tendency toward recurrence and possible susceptibility of these lesions to malignant transformation, in agreement with Matturri *et al.* Additionally, Ohtaké *et al.* observed high positivity for PCNA, especially in atypical cells, and suggested a potential of malignant transformation for pleomorphic adenomas. However, other investigators such as Zhu *et al.* have reported that the proliferative activity of pleomorphic adenomas is not significant and have associated their high rate of recurrence with inadequate surgical procedures. The predominance of cases showing weak or no labeling in the present study suggests a less aggressive behavior and possibly lower tendency toward malignant transformation for most pleomorphic adenomas in this sample.

p53 is a tumor suppressor gene located on the short arm of chromosome 17 and is the most commonly mutated gene in tumors (Ohki *et al*., 2001). Many studies have correlated the expression of normal and mutant p53 with the differentiation, aggressiveness and prognosis of salivary gland tumors, but the results are highly controversial. In the present study, most

cases showed weak labeling for p53, in agreement with the findings of Marioni *et al*., Lazzaro & Cleveland (2000) and Weber *et al.* (2002). However different results have been reported by Rosa *et al*. and Alves *et al.,* who observed no expression of p53 in any case, emphasizing that this protein is not implicated in the pathogenesis of pleomorphic adenomas. In contrast, most cases were positive for the p53 protein in the studies of Ohki *et al.* and Ohtaké *et al.* indicating, according to these authors, a tendency toward malignant transformation of pleomorphic adenomas. This statement is also supported by Li *et al*. (1997) who observed that the expression of p53 was frequently associated with deletion of the p53 gene detected by fluorescence in situ hybridization test (FISH), favoring a possible installation of carcinoma ex-pleomorphic adenoma.

Based on the present findings, especially the significant correlation between the expression of PCNA and p53, we may infer that this correlation it could be associated with a more evident proliferative profile in some cases of pleomorphic adenoma.

The bcl-2 protein is responsible for the inhibition of apoptosis and is therefore important for greater cell survival. According to Yanez *et al*., bcl-2 plays an important role in the development of pleomorphic adenomas since all cases analyzed by the authors were positive forthis protein. Aoki *et al.* (2004) also detected a high percentage of bcl-2-positive cases, with immunoreactivity being observed in 33 (94.3%) of 35 cases analyzed. The present findings are in contrast with these results since most cases were negative for bcl-2 protein, suggesting that this protein is not involved in the pathogenesis of pleomorphic adenoma. In addition, a significant correlation was observed between bcl-2 and PCNA, i.e., in most cases in which PCNA was expressed bcl-2 was also identified. These findings demonstrate a concomitant proliferative and apoptotic activity of pleomorphic adenoma cells, a profile expected for benign neoplasm.

We concluded that the correlation of p53 and PCNA expression can favor the proliferative activity of pleomorphic adenomas and that bcl-2 did not effectively participate in the pathogenesis of this tumor in the cases studied here. Considering that the incomplete surgical removal is told in the literature as main cause of recurrence for the pleomorphic adenomas, thefindings of this study in relation to the highest cellular proliferative activity evidenced by the labeling for PCNA and p53 can to suggest that in some cases of pleomorphic adenoma with this profile, the recurrence risk becomes relatively higher. However, more detailed studies are necessary to confirm this assumption.

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RESUMEN: El objetivo del estudio fue determinar la expresión inmmunohistoquímica de las proteínas PCNA, p53 y bcl-2 en adenomas pleomórficos. Fueron seleccionados 19 especímenes de adenomas pleomórficos para análisis a través del método de la estreptavidina-biotina-peroxidasa con anticuerpos contra las proteínas p53, PCNA y bcl-2. Fue observada leve marcación para p53 en 12 casos (63,1%) y para PCNA en 8 (42,1%). Con relación al índice de marcación para bcl-2, no fue detectada en 12 casos (63,1%) expresión de esta proteína . En base a los resultados, se concluyó que las proteínas p53 y PCNA pueden favorecer la actividad proliferativa de adenomas pleomórficos, y por otro lado, la bcl-2 probablemente no participaría efectivamente de la patogenia de este tumor.

PALABRAS CLAVE: Adenoma Pleomórfico; Tumor de Glándula Salival; p53; PCNA; bcl-2.

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