**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).
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-mm² 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.

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.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Median</th>
<th>Range</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>99.500</td>
<td>0.000-601.50</td>
<td>0.0044</td>
</tr>
<tr>
<td>PCNA</td>
<td>199.50ab</td>
<td>10.500-957.50</td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>32.000b</td>
<td>0.000-632.50</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Markers</th>
<th>r</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 x PCNA</td>
<td>0.5346</td>
<td>0.09176 – 0.8099</td>
<td>0.0184</td>
</tr>
<tr>
<td>p53 x bcl-2</td>
<td>0.4553</td>
<td>0.01322 – 0.7599</td>
<td>0.0501</td>
</tr>
<tr>
<td>PCNA x bcl-2</td>
<td>0.4584</td>
<td>0.009342 – 0.7615</td>
<td>0.0484</td>
</tr>
</tbody>
</table>

Source: Post graduation Program in Oral Pathology – UFRN


<table>
<thead>
<tr>
<th>Marker</th>
<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>X²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>5 (26.3%)</td>
<td>12 (63.1%)</td>
<td>1 (5.3%)</td>
<td>1 (5.3%)</td>
<td>22.67</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>PCNA</td>
<td>4 (21.1%)</td>
<td>8 (42.1%)</td>
<td>3 (15.7%)</td>
<td>4 (21.1%)</td>
<td>4.14</td>
<td>0.246</td>
</tr>
<tr>
<td>bcl-2</td>
<td>12 (63.1%)</td>
<td>5 (26.3%)</td>
<td>0 (0.0%)</td>
<td>2 (10.6%)</td>
<td>23.23</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

(-) 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).

**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 adenosmas. 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 adenosmas. However, other investigators such as Zhu et al. have reported that the proliferative activity of pleomorphic adenosmas 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 adenosmas 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
Las lesiones mostraron un marcado elevado en el índice de marcación para el marcador p53, en acuerdo con los hallazgos de Marioni et al. y Lazzaro & Cleveland (2000) y Weber et al. (2002). Sin embargo, diferentes resultados se han reportado por Rosa et al. y Alves et al., quienes observaron un bajo porcentaje de expresión de p53 en cualquier caso, sugiriendo que este marcador no está implicado en la patogenia de los adenomas pleomorfos. Sin embargo, en contraste, se observó una mayor expresión del marcador PCNA en 8 (42.1%) casos. Con relación a estos hallazgos, los resultados indicaron que en el grupo de adenomas pleomorfos se observó una correlación significativa entre la expresión de p53 y PCNA, lo cual sugiere que este marcador no participa en la patogenia de los adenomas pleomorfos. Además, se observó una correlación significativa con el índice de marcación para el marcador bcl-2, lo que sugiere que este marcador no es efectivo en la patogenia de los adenomas pleomorfos. En conclusión, los hallazgos observados sugieren que el marcador p53 tiene un papel importante en la proliferación celular y apoptosis, lo cual es crucial para la supervivencia celular. Sin embargo, más estudios detallados son necesarios para confirmar esta hipótesis.


