Immunohistochemical expression of OCT4 and CD44 in major and minor salivary gland neoplasms

Abstract: The aim of this study was to identify tumor parenchyma cells exhibiting immunohistochemical profile of stem cells by evaluating the immunoreactivity of OCT4 and CD44 in a number of cases of salivary gland neoplasms. The sample consisted of 20 pleomorphic adenomas, 20 mucoepidermoid carcinomas, and 20 adenoid cystic carcinomas located in major and minor salivary glands. The expression of OCT4 and CD44 was evaluated by the percentage of positive cells and the intensity of expression. All studied cases showed positive expression of OCT4 and CD44 and higher values than the control groups. For OCT4, luminal and non-luminal cells were immunostained in the case of pleomorphic adenomas and adenoid cystic carcinomas. Moreover, the immunoreactivity of CD44 was particularly evident in the non-luminal cells of these lesions. In mucoepidermoid carcinomas, there was immunoreactivity for both markers in squamous and intermediate cells and absence of staining in mucous cells. For both markers, a significantly higher immunostaining was verified in neoplasms located in the major salivary glands compared with lesions in minor salivary glands (p<0.001). In the total sample and in minor salivary glands, malignant neoplasms exhibited higher immunoreactivity for OCT4 than pleomorphic adenoma. A significant moderate positive correlation (r = 0.444 and p ≤ 0.001) was found between OCT4 and CD44 immunoexpression in the total sample. The high expression of OCT4 and CD44 may indicate that these proteins play an important role in identifying tumor stem cells.

Keywords: Immunohistochemistry; Neoplastic Stem Cells; Salivary Glands.

Introduction

Salivary gland tumors are a relatively uncommon and complex group of human neoplasms, accounting for 2 to 6.5% of all head and neck tumors. Most of these tumors affect the major salivary glands (MaSGs), particularly the parotid gland in 60 to 80% of cases, while the minor salivary glands (MiSGs) are involved in 9 to 23% of cases.1,2

Among benign tumors, pleomorphic adenoma (PA) is the most common tumor of the MaSGs and MiSGs,3,4 while the most common malignant tumor is mucoepidermoid carcinoma (MEC), which is
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characterized by its variable clinical behavior. Another common malignant tumor is the cystic adenoid carcinoma (CAC), which deserves special attention because of its microscopic features and poor prognosis. Salivary gland tumors are a diagnostic challenge due to their complex histopathological features and considerable variation with regard to clinical characteristics, biology, and clinical behavior. The etiopathogenesis of these tumors remains unknown, but emerging evidence suggests the existence of a tumorigenic population of cancer cells that exhibit stem cell-like properties, called tumor stem cells, which are able to initiate and maintain tumor formation and progression. In addition to tumor initiation, tumor stem cells play an important role in tumor relapse and resistance to chemotherapy. Several studies have shown that this population of cells is resistant to traditional chemotherapy and radiation treatments. Several markers have been used for the identification of tumor stem cells in different neoplasms, including ALDH, CD44, Bmi-1, CD133, OCT4, Nanog, SOX2, CD24, Snail, Twist, Keratin 14, Msi-1, Lgr5, and c-Met. OCT4 is a transcription factor expressed in embryonic stem cells, germ cells, and human adult stem cells, and it has been associated with a high proliferative potential and tumor progression. The expression of this marker is observed in different types of cancer, including breast and colorectal cancer, as well as head and neck cancer. CD44, a cell surface marker, has been used to identify tumor stem cells. In addition, CD44 is an early proliferation marker of these cells. Several markers have been used for the identification of tumor stem cells in different neoplasms, including ALDH, CD44, Bmi-1, CD133, OCT4, Nanog, SOX2, CD24, Snail, Twist, Keratin 14, Msi-1, Lgr5, and c-Met. OCT4 is a transcription factor expressed in embryonic stem cells, germ cells, and human adult stem cells, and it has been associated with a high proliferative potential and tumor progression. The expression of this marker is observed in different types of cancer, including breast and colorectal cancer, as well as head and neck cancer. CD44, a cell surface marker, has been used to identify tumor stem cells. In addition, CD44 is an early proliferation marker of these cells.

Studies have investigated the expression of tumor stem cells biomarkers in different pathological processes, including proliferation, invasion, and metastasis, and in the prognosis of different types of tumors. However, little is known about the expression of these markers in salivary gland tumors. Within this context, the objective of this study was to identify and quantify neoplastic cells in the tumor parenchyma of PA, MEC and CAC of the MaSGs and MiSGs by evaluating the immunexpression of OCT4 and CD44, and assess the correlation between the expression of these proteins and the biological behavior of the tumors studied.

Methodology

Twenty PAs, 20 MECs and 20 CACs, with 10 cases each located in the MaSGs and 10 in the MiSGs, were used. For comparison, five biopsies of mucus extravasation phenomenon were selected for the MiSG tumors and five samples of remnant parenchyma of a normal salivary gland for the MaSG tumors.

The histopathological features of PAs, MECs, and CACs were analyzed descriptively by two pathologists. In PA, the cells were divided into subgroups according to the classification proposed by Seifert et al.: subgroup 1 or classical, subgroup 2 or myxoid, subgroup 3 or cellular, and subgroup 4 with focal monomorphic differentiation in the epithelial component. Microscopic parameters of the MEC cases were evaluated, and the tumors were classified as low, intermediate, and high histological grade of malignancy. In the CAC cases, the predominant histological pattern was evaluated, and the tumors were classified as tubular, cribriform, or solid according to Barnes et al. The study was approved by the Research Ethics Committee of UFRN, Natal, Brazil (Protocol No. 1.097.098).

Immunohistochemistry

The paraffin-embedded specimens were cut into 3-µm histological sections, which were mounted on slides with 3-aminopropyltriethoxy-silane adhesive (Sigma Chemical Co., St. Louis, USA). The material was submitted to the immunoperoxidase technique, with detection by the EnVision™ + Dual Link System-HRP (Dako North America, Inc., Carpinteria, USA), using the following primary antibodies: anti-OCT4, diluted 1:250 (clone Ab19857, Abcam, Cambridge, MA, USA) and anti-CD44, diluted 1:250 (clone Ab51037, standard, Abcam, Cambridge, MA, USA). For both antibodies, antigen retrieval was performed with Trilogy (Cell Marque™ Corporation, Natal, Brazil) in a Pascal cooker (DakoCytomation, Natal, Brazil) for 3 minutes. The specimens were incubated with the primary
antibodies overnight at 4ºC. The positive controls for the anti-OCT4 and anti-CD44 antibodies consisted of human tonsil and bladder sections, respectively. For the negative control, the primary antibodies were replaced by 1% bovine serum albumin in buffer solution.

**Immunohistochemical analysis**

Each specimen was analyzed by a previously trained examiner under a light microscope (Eclipse E200, Nikon Co., Tokyo, Japan). The immunoexpression of OCT4 and CD44 was evaluated semi-quantitatively using an adaptation of the method described by Fu et al.19 and Huang et al.13, respectively.

Each slide was examined throughout its extension and five histological fields were selected randomly at 100x magnification. These five fields were then analyzed at 400x magnification. For the anti-OCT4 antibody, all cells in the tumor parenchyma that exhibited nuclear and/or cytoplasmic brown staining were classified as positive. For the anti-CD44 antibody, all cells in the tumor parenchyma that exhibited membrane and/or cytoplasmic brown staining were defined as positive.

For the evaluation of OCT4 and CD44, an initial score was attributed corresponding to the estimated proportion of positive tumor cells. The percentage of positive cells (PP) was then calculated and the intensity of expression (IE) in the five selected fields was evaluated for each specimen. The PP was classified as follows: 0 (0% positive cells), 1 (< 25% positive cells), 2 (25–50% positive cells), 3 (51–75% positive cells), or 4 (> 75% positive cells). The IE was classified as 0 (no expression), 1 (weak expression), 2 (intermediate expression), or 3 (strong expression).

For each specimen analyzed, scores were attributed to PP and IE in each of the five fields and the score that occurred most often (mode) in the five fields was considered for analysis. The scores of PP and IE were summed to obtain the total score, which ranged from 0 to 7. The total immunostaining score (TIS) was calculated as: TIS = PP + IE. In the control cases consisting of normal salivary gland tissue, the same assessment for OCT4 and CD44 as described above was performed.

**Statistical analysis**

The results were exported to the SPSS 22.0 program (Statistical Package for the Social Sciences). The Mann-Whitney test was used for analysis. Spearman’s test was applied to evaluate the correlation between the immunoexpression of the markers. A level of significance of p < 0.05 was adopted.

**Results**

**Clinical and morphological analysis**

There was a predominance of females in the total sample (n = 35; 58.3%), with a female/male ratio of 1:1 in PAs, 1.8:1 in MECs, and 1.5:1 in CACs. The mean age of the patients at the time of diagnosis was 44.7 (±18.8) years. With respect to anatomical location of the tumor, the MaSGs affected were the parotid (n = 18; 60%), submandibular (n = 10; 33.3%), and sublingual glands (n = 2; 6.7%). The most common site of MiSGs was the palate (n = 14; 46.7%), followed by the lip mucosa (n = 6; 20%).

Among the PA cases studied, 9 (45%) were of the cellular subtype, 6 (30%) of the classical subtype, and 5 (25%) of the myxoid subtype. Mucoepidermoid carcinoma was classified as low grade in 10 (50%) cases, intermediate grade in 3 (15%), and high grade in 7 (35%). Regarding CAC, the solid pattern was observed in 10 (50%) cases, the cribriform pattern in 6 (30%), and the tubular pattern in 4 (20%).

**Immunohistochemical analysis**

Immunoexpression of OCT4 was predominantly found in the nucleus and, to a lesser extent, in the cytoplasm. With respect to the type of immunostained cell in the tumor parenchyma, epithelial and myoepithelial cells were positive for OCT4 in PAs and CACs (Figures 1A and 1B). In MECs, immunostaining was observed in intermediate and epidermoid cells, while no expression of this marker was found in mucous cells (Figures 1C and 1D).

When OCT4 staining was evaluated semi-quantitatively regardless of the type of tumor (PA, MEC or CAC) and anatomical location (MaSG or MiSG), the most frequent PP score was 4 (n = 40; 66.6%) and the most frequent IE score was 3 (n = 30;
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The mean TIS for OCT4 was 5.96 (±1.24) and median 6.00.

**Immunoeexpression of CD44**

CD44 staining was predominantly detected in the cell membrane, although cytoplasmic staining was also observed in some cases. Regarding cell type, in PAs and CACs, CD44 was preferentially expressed in myoepithelial cells in the tumor parenchyma but immunostaining was also detected in epithelial cells (Figures 2A and 2B). In MECs, immunostaining was observed in intermediate and epidermoid cells, but no expression of this marker was found in mucous cells (Figures 2C and 2D).

The most frequent PP score was 4 (n = 24; 40%) and the most frequent IE score was 3 (n = 29; 48.3%), regardless of the type (PA, MEC or CAC) and location of the tumor (MaSG or MiSG). The mean TIS for CD44 was 5.47 (±1.51) and median 5.00.

**Comparison of OCT4 and CD44 immunoexpression**

The mean and median TIS obtained for the two markers differed significantly between the MaSG and MiSG control groups and the groups of tumors (p<0.05). Higher TIS values for OCT4 and CD44 were observed in MaSG tumors and this difference was statistically significant (Table 1). However, when TIS was analyzed irrespective of location, comparison of the immunoexpression of OCT4 or CD44 revealed no significant difference between morphological subtypes (OCT4/CD44: p = 0.665/p = 0.186 for PA, p = 0.844/p = 0.77 for MEC, and p = 0.718/p = 0.528 for CAC).

With respect to tumor behavior, malignant tumors (MEC and CAC) exhibited significantly higher expression of OCT4 than benign tumors (PA), regardless of location (Table 2). This difference was clearly evident and significant in the MiSGs...
In contrast, no significant differences were observed in the immunoexpression of CD44 between malignant and benign tumors, regardless of location (Table 2). However, the mean and median TIS for CD44 were lower in PA compared to MEC and CAC (Table 3).

Analysis of the correlation between OCT4 and CD44 immunoexpression in the total sample revealed a significant moderate positive correlation ($r = 0.444$ and $p \leq 0.001$). A weak and nonsignificant correlation was found in the MiSG group ($r = 0.171$ and $p = 0.365$), as well as in the MaSG group ($r = 0.099$ and $p = 0.943$).

**Discussion**

Several factors have been studied in an attempt to better understand the histogenesis, biological behavior, therapeutic potential, and prognosis of salivary gland tumors, including proteins associated with tumor stem cells. These cells were first identified...
In acute myeloid leukemia by Bonnet and Dicke. In solid tumors, tumor stem cells were detected for the first time in a breast tumor by Al-Hajj et al. and exhibit characteristics of self-renewal, differentiation, increased membrane permeability, migration capacity, and antiapoptotic activity. Thus, a specific subset of tumor cells with stem cell characteristics may play a role in tumor initiation, progression, and recurrence.

Tumor stem cells and associated proteins have been investigated in some oral neoplasms. Recent studies on head and neck squamous cell carcinoma indicated an important role of tumor stem cells in tumor development and progression. However, research on the participation of these cells in benign and malignant salivary gland tumors is still in an early stage and studies investigating the expression of OCT4 and CD44 in these tumors are scarce.

In this study, evaluation of the immunohistochemical expression of OCT4 revealed immunostaining in all cases, with most PAs, MECs, and CACs exhibiting strong predominantly nuclear expression in cells of the tumor parenchyma. These data agree with the findings of Habu et al. and Qiao et al. who demonstrated predominantly nuclear expression of OCT4 in squamous cell carcinomas of the head and neck. In contrast, Zhang et al. studying salivary gland MECs, only found expression of OCT4 in the nucleus of the cell line evaluated. Immunostaining for CD44 was observed in all cases of this study and most of them exhibited high expression, predominantly in membrane. These results agree with the findings of Adams et al. who found CD44 immunoreactivity in all cases of salivary gland MECs, and of Fujita and Ikeda who demonstrated a higher frequency of membrane staining in the CAC cases studied. In the present study, cytoplasmic staining for OCT4 and CD44 was also observed in some cases. Previous studies also demonstrated that OCT4 and CD44 are expressed to a lesser extent in the cell cytoplasm of some tumors. Detection of OCT4 and CD44 in the cytoplasm may be attributed to an increased synthesis of these proteins, which results in their cytoplasmic accumulation.

It should be noted that CD44 was preferentially expressed in myoepithelial cells in PAs and CACs, although immunostaining of epithelial cells was observed in some cases, similar to results reported by Ianez et al. In contrast, Soave et al. emphasized that expression of CD44 in CACs was only observed in myoepithelial cells. According to these authors, the multiple histological features of salivary gland tumors can be attributed to the presence of myoepithelial cells. Another important finding was the absence of OCT4 and CD44 expression in mucous cells of MECs, suggesting a higher

### Table 2. Evaluation of OCT4 and CD44 immunoexpression according to lesion behavior, regardless of location.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tumor behavior</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>Q25–75</th>
<th>Ranks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>Benign</td>
<td>20</td>
<td>5.4</td>
<td>5.5</td>
<td>5.5–7.0</td>
<td>24.3</td>
<td>0.039*</td>
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<tr>
<td></td>
<td>Malign</td>
<td>40</td>
<td>6.2</td>
<td>6.5</td>
<td>6.5–7.0</td>
<td>33.6</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>Benign</td>
<td>20</td>
<td>5.4</td>
<td>6.0</td>
<td>4.2–7.0</td>
<td>30.4</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>Malign</td>
<td>40</td>
<td>5.4</td>
<td>6.0</td>
<td>6.0–7.0</td>
<td>30.5</td>
<td></td>
</tr>
</tbody>
</table>

*statistically significant difference (Mann-Whitney test).

### Table 3. Evaluation of OCT4 and CD44 immunoexpression according to lesion behavior in MiSGs.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tumor behavior</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>Q25–75</th>
<th>Ranks</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>OCT4</td>
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<td>6.0</td>
<td>5.0–7.0</td>
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<tr>
<td>CD44</td>
<td>Benign</td>
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<td>4.2</td>
<td>4.5</td>
<td>3.0–5.0</td>
<td>13.7</td>
<td>0.404</td>
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<tr>
<td></td>
<td>Malign</td>
<td>20</td>
<td>4.7</td>
<td>5.0</td>
<td>3.2–6.0</td>
<td>16.4</td>
<td></td>
</tr>
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</table>

*statistically significant difference (Mann-Whitney test).
degree of differentiation and a less aggressive phenotype of these cells, which do not exhibit the behavior of tumor stem cells. On the other hand, epidermoid and intermediate cells seem to be more undifferentiated as indicated by the high expression of these markers.

Comparison of OCT4 and CD44 expression between MaSG and MiSG tumors and their respective control groups revealed lower expression of these markers in the latter. These findings agree with those reported in the study of Adams et al.\(^9\) in which normal salivary glands expressed low levels of tumor stem cells markers (CD44, ALDH, CD24, and CD10) compared to the MEC cases evaluated. These results highlight the importance of immunoexpression of tumor stem cells markers for tumor behavior prediction.

A significantly higher expression of OCT4 and CD44 was found in MaSG compared to MiSG tumors. A hypothetical explanation for this differentiated expression are the differences in the histological features between salivary glands and/or characteristics inherent to embryonic development through the epithelial-mesenchymal transition. This result agrees with the observation of Soave et al.\(^15\) who also demonstrated high expression of CD44 in malignant MaSG tumors, suggesting a lower participation of tumor stem cells in MiSG tumors, a finding that could have therapeutic implications.

In the present study, OCT4 and CD44 were more expressed in malignant tumors compared to benign tumors. For OCT4, regardless of tumor location (MaSG or MiSG), this difference was statistically significant. For CD44, higher expression was found in malignant MiSG tumors. From these results, it is possible to suggest that the immunoexpression of tumor stem cell markers may be related to the biological behavior of salivary gland neoplasms.

The correlation between OCT4 and CD44 immunoexpression was also analyzed and a moderate positive correlation was found for the total sample. These findings indicate that tumor stem cells biomarkers are related to tumorigenesis in salivary gland tumors and that they might be important for maintaining the properties of quiescence and self-renewal of these cells. The identification of a specific marker that defines the phenotype of tumor stem cells is desirable so that the clinical relevance of the marker in each type of tumor can be evaluated, thus permitting to establish effective therapeutic targets.

**Conclusion**

The results of the present study suggest that OCT4 and CD44 are important for the identification of tumor stem cells. However, further investigation with other proteins and methods for the detection of tumor stem cells is needed to establish the role of these cells in the development and progression of salivary gland tumors.

**Acknowledgement**

National Council for Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education Personnel (CAPES)

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**References**


