Abstract: The aim of this study was to evaluate the role of PCNA and AgNOR in the assessment of salivary gland tumor proliferation using a double staining technique. Ten cases of pleomorphic adenoma (PA) and seventeen cases of adenoid cystic carcinoma (ACC) were examined. Numeric and morphometric parameters of AgNOR were evaluated and compared in PCNA-positive and PCNA-negative nuclei. There were statistically significant differences in AgNOR numbers, perimeters and contour indices between PCNA-positive and -negative nuclei in the PA samples. The ACC samples demonstrated significant differences only in the AgNOR areas. Our results show that in salivary gland tumors there is not always a relationship between proliferative activity evaluated by AgNOR numeric and morphometric parameters and PCNA immunostaining. (J. Oral Sci. 46, 87-92, 2004)

Key words: AgNOR; adenoid cystic carcinoma; double staining technique; PCNA, pleomorphic adenoma; proliferation markers.

Introduction

PCNA (proliferating cell nuclear antigen) and AgNOR (silver-staining nucleolar organizer region) have been used as additional aids to obtaining prognostic information about salivary gland tumors besides the classical clinicopathological indicators (1-3). In the majority of previous studies, these markers were evaluated separately. PCNA is a nonhistone nuclear protein that functions as a cofactor for DNA polymerase-delta and plays a role in the initiation of cell proliferation (4). PCNA immunohistochemistry appears to have predictive significance in the evaluation of salivary tumors (2,5) although some conflicting results have been reported (6).

The AgNORs are a set of proteins associated with segments of DNA that transcribe to ribosomal RNA. These proteins are defined as markers of “active” ribosomal genes (7,8). Previous studies of AgNORs as indicators of precise proliferative status have sometimes resulted in ambiguity (9). In salivary gland tumors, studies have most frequently addressed the prognosis of neoplasms with the aim of using AgNORs to distinguish between benign and malignant tumors (10-13). However, AgNORs appear to be of little value in estimating the prognosis of salivary gland malignancies (14).

Pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC) are common tumors which involve both the major and minor salivary glands. Although both tumors originate in the intercalated duct with the same cellular derivation (15) and secretion of proteins of the extracellular
matrix (16), they exhibit the characteristic behavior of benign and malignant tumors, respectively, coupled with distinct proliferative activity (17-20)

The objective of this study was to evaluate the role of PCNA and AgNOR in the assessment of salivary gland tumor proliferation using a double staining technique.

**Materials and Methods**

Formalin-fixed paraffin-embedded tissue specimens from 10 pleomorphic adenomas and 17 adenoid cystic carcinomas of the minor oral salivary glands were selected from the files of the Oral Pathology Department of the Federal University of Minas Gerais, Federal University of Uberlândia and Federal University of Pelotas (Brazil). Histopathological diagnosis was undertaken based on hematoxylin-eosin-stained sections. There were five solid, six tubular and six cribriform ACCs.

**Double staining technique**

Sections were cut at 3 µm thickness. The slides were deparaffinized, hydrated and immersed in citric acid (10 mM) at pH 6.0, then heated in a microwave oven at 700 W in three cycles of 5 min each for antigenic retrieval.

The primary antibody, anti-PCNA (PC10, Dako Corporation; Glostrup, Denmark), was incubated with the sections at 1:100 dilution for 18 h at 4°C. The sections were then thoroughly rinsed and exposed to the secondary antibody (Biogenex, San Ramon, CA, USA) for 30 min at room temperature. After further washing, the sections were exposed to alkaline phosphatase-conjugated streptavidin (Biogenex, San Ramon, CA, USA) for 30 min at room temperature. Freshly made fuchsin (New fuchsin: Biogenex, San Ramon, CA, USA) was used as the chromogen for 30 min in the dark at room temperature. No nuclear counterstaining was done. Negative controls were incubated in buffer without primary antibody.

Silver staining was applied according to the method of Ploton et al. (21), with some modifications. In brief, the final working solution was freshly made by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. Slides were incubated in this solution for 25 min in the dark at room temperature, washed with deionized water at 45°C, washed in running deionized water for 15 min, dehydrated, cleaned and mounted in Permunt® (Fischer Scientific, Fair Lawn, NJ, USA).

Quantitative and morphometric parameters of the AgNORs were performed using image-processing software (KS-300; Kontron Elektronik/Carl Zeiss). The sections were examined at ×1000 magnification. For each case, 100 PCNA-positive cells and 100 PCNA-negative cells were examined. The nucleus was considered positive for PCNA only if there was an unequivocal red reaction product. The morphometric parameters considered were area, perimeter and contour index (CI) for each AgNOR.

Mean numbers per nucleus, area, perimeter and contour index of AgNORs were calculated for PCNA-positive and PCNA-negative nuclei. The contour index of an AgNOR is an expression of shape irregularities (the minimum value of 3.544 corresponds to a perfect circle). Student’s paired t-test was used to compare the AgNOR parameters of PCNA-positive and -negative cells when the sample was normal, and the Mann-Whitney test when the sample was not normal, using the Sigma Stat Program. Differences at \( P < 0.05 \) were considered significant.

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**Fig. 1** PCNA/AgNOR double staining in pleomorphic adenoma (A) in adenoid cystic carcinoma: tubular (B), cribriform (C) and solid (D). The AgNORs are shown as black dots within the nuclei of PCNA-positive (arrows) and PCNA-negative cells.

**Fig. 2** PCNA/AgNOR double staining in normal salivary gland (arrow). AgNOR counts vary between one and two per nuclei.
Results

A positive reaction to the PCNA antibody was revealed as intense red staining with a granular pattern limited to the cell nucleus. The AgNORs were interpreted as black dots within the cell nucleus (Fig. 1). In normal salivary gland tissue present in the specimens (internal control), PCNA-positive cells were rare and the AgNOR counts per nucleus varied between one and two (Fig. 2).

The AgNOR count per nucleus in the PA samples was higher in PCNA-positive than in PCNA-negative cells. The same result was found for the ACC samples; however, significant differences were observed only for AP (Table 1).

In the PA samples, the perimeter and area of the AgNORs were higher in PCNA-positive than in PCNA-negative cells. The CI was smaller in cells immunolabelled by PC10. There was also a significant difference in the perimeter and CI of AgNORs between PCNA-positive and PCNA-negative nuclei in the PA samples. In the ACC samples significant difference between PCNA-positive and -negative cells was found only in the AgNOR areas (Table 1).

In the ACC samples, double staining showed the same pattern among solid, tubular and cribriform types, although a higher number of PCNA-positive cells were demonstrated in some cases of solid ACC.

Discussion

Prior to this investigation, studies employing PCNA or AgNOR techniques for evaluation of the proliferative activity of salivary gland tumors have assessed these markers separately. The co-localization of AgNOR and PCNA in the tissue through double labeling allows them to be analyzed in the same cell and provides a method to directly assess the relationship between these markers, overcoming the problem of neoplasm heterogeneity (22).

The present study showed a higher mean number of AgNORs in PCNA-positive than in PCNA-negative cells in the PA samples. Others studies have also shown concordance between PCNA and AgNORs (22-24). This result is also in agreement with others studies showing the capacity of both PCNA and AgNOR number of discrimination of a higher proliferative activity in solid/ductal areas than that in myxomatous/chondroid areas in AP (25-27).

However we did not find any positive relationship between PCNA and AgNORs when we analyzed only the numeric index of AgNORs in the ACC samples. Other studies have also noted a lack of correlation between these indices when analyzed separately in other kinds of tumors (28-32). The inadequacy of statistical correlations between the PCNA index and AgNOR counts has been attributed to the fact that these methods quantify different aspects of proliferative activity.

PCNA is correlated with the S-phase of the cell cycle and plays a critical role in the initiation of cell proliferation (4,33,34). However, the long half-life of the protein leads to labeling of cells that have already left the cell cycle (35). PCNA staining may also occur in non-S-phase cells engaged in nucleotide excision DNA repair (36). Despite the diverse roles played by the PCNA protein, there is evidence for deregulated overexpression in some types of

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<th>Table 1 Mean AgNOR scores in PCNA-positive and PCNA-negative cells</th>
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Min: Minimum
Max: Maximum
SEM (standard error of mean)
*Significant difference (P < 0.05)
salivary gland tumors with a prognostic significance (5).

On the other hand, quantitative analysis of interphase NORs has proved to be valuable in tumor pathology for distinguishing malignant from benign lesions of the same origin (37,38) and has been used to discriminate between benign and malignant salivary gland tumors (10-13). Vuhuhula et al. (1) correlated the mean AgNOR count with the biological behavior of ACC and speculated that AgNOR may be more indicative of the biological nature of the condition and hence the degree of malignancy, rather than its proliferative status. Fonseca et al. (14) concluded that in ACC, AgNOR counts do not seem to be a prognostic indicator, in contrast to the usefulness of this method in other tumor types.

The “paradoxical” AgNOR results, i.e. the absence of a relationship between AgNOR number and the PCNA index in ACC, should be also related to the demonstration of cellular metabolic activity of the cells by silver staining. The AgNOR number in ACC could reflect metabolic activity rather than changes in proliferative activity. In 2002, Dayan et al. (39), studying the effects of age on the proliferative capacity of acinar and ductal cells, demonstrated this intricate relationship.

Morphometric and quantitative analyses of AgNORs in malignant tumor cells show large numbers in the nucleus, small in size, and with a scattered distribution and irregular shape, compared with benign tumors cells (40). The disorganized AgNORs may be associated with active NORs or dispersion of multiple nucleoli, resulting in bizarre shapes and large irregular NOR aggregates. In this work, for the PA samples, the perimeter and area of the AgNORs were higher and the CI was smaller in PCNA-positive than in PCNA-negative cells. However, a statistically significant difference was observed only for perimeter and CI. These differences may be the result of a more clustered distribution of NORs in PCNA-positive than in negative ones.

The unique parameter of AgNORs in the ACC samples that showed a statistically significant difference between PCNA-positive and PCNA-negative cells was the mean AgNOR area, which was significantly higher in the immunolabelled cells. Other studies that used a double staining technique for AgNOR and Ki-67 (MIB-1) also found higher mean AgNOR areas in the Ki-67-positive cells (41,42). Some authors have pointed out that measurement of AgNOR area using an image analysis method is the best indicator and the most reproducible parameter of the AgNOR index (43-45) and could be a substitute for AgNOR quantification that should be restricted to cases that exhibit cells with distinct silver stained dots.

Leek et al. (9), studying cellular proliferation in different tissues, showed that an increase in the size of AgNOR clusters rather than their number correlated positively with elevated labeling by BrdUrd. In the ACC samples, the increased AgNOR area may also reflect an increased rate of proliferative activity rather than AgNOR number.

Although this work seems to suggest different roles for PCNA and AgNOR in the evaluation of cellular proliferation in salivary gland tumors, other studies are necessary to confirm this view.

Acknowledgments

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