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# Quantitative analysis of argyrophilic nucleolar organizer regions and epidermal growth factor receptor in ameloblastomas

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**OBJECTIVE: The aim of this study was to evaluate the proliferation activity by means of the quantification of the argyrophilic nucleolar organizer regions (AgNORs) and the patterns of expression of the epidermal growth factor receptor (EGFR) in ameloblastomas.**

**METHOD: The methods of evaluation included the H/E stain for the morphologic analysis, the silver impregnation technique for quantification of the AgNORs and the immunohistochemical stain with anti-EGFR antibody in 11 cases of ameloblastoma.**

**RESULTS: The results did not show a significant statistical difference as per quantification of the AgNORs. The expression of the EGFR on the epithelial islands of ameloblastoma was not uniform, and the location of the expression was also variable. The predominant expression was that of cytoplasm and the islands with an expression of membrane only were rare and generally smaller in size.**

**CONCLUSION: The tumor presents an irregular growth. Smaller islands are associated with a higher proliferation activity and therefore could be responsible for tumor infiltration.**

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**Keywords:** ameloblastoma; EGFR; AgNORs

## Introduction

Ameloblastomas are locally invasive (1) and destructive (2) benign odontogenic tumors. Their recurrence rate is high (3, 4) even for patients that undergo surgical excision of the tumor and of a safety tissue margin around it (5). The mechanism of such local aggressiveness is still unknown (6).

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For a better understanding of the aggressive behavior of ameloblastomas, their expression of metalloproteinases and growth factor receptors and their proliferative activity have been investigated using histochemical and immunohistochemical methods (7–15).

Among the methods of evaluation of proliferative activity is the argyrophilic nucleolar organizer region (AgNOR) technique, which uses silver to stain the proteins associated with the active nucleolar organizer regions (NORs). NORS, which transcribe ribosomal RNA (rRNA), are DNA segments found in the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22 of the human species (16–18). Active NORs are associated with non-histone and argyrophilic proteins, which are known as AgNORs when stained with silver (18). The number and size of AgNORs change according to the rhythm of ribosomal RNA transcription. Usually, in malignant tumors the AgNORs are smaller and more numerous than in benign tumors. The nuclei of cells with physiological rRNA production have one single and large AgNOR, whereas the nuclei of cells with intense rRNA production have a large number of small AgNORs (19). The AgNOR counting method reflects the rate that the cell follows the cell cycle. Therefore, the AgNOR technique may be used in association with other methods of growth fraction evaluation to provide information about the rate of tumor proliferative activity (17).

Proliferative activity, as well as cell response to several extracellular stimuli, is associated with the activation of growth factor receptors (20). Several studies have investigated mechanisms to control these receptors and cell proliferation (21–25). Among growth factor receptors, the epidermal growth factor receptor (EGFR) stands out because, although also found in normal epithelial cells, it is greatly expressed by a wide range of different tumors (20, 23). EGFR is a 170-kD transmembrane glycoprotein that consists of an external domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction (20, 25–28). The binding of specific growth

factors (EGF and transforming growth factor-α) to EGFR results in recruitment of signal transducers (22, 28–30), which may lead to cell proliferation and to processes associated with tumor progression, such as angiogenesis, apoptosis inhibition, motility, adhesion, and invasion (21).

Several studies have investigated proliferative activity in ameloblastomas (2, 14–17). However, few have distinguished the epithelial cell types of the tumor (7, 8, 14). Studies that evaluated EGFR expression in ameloblastomas reported different and, at times, contradictory results (15, 31, 32). The purpose of this study was to investigate proliferative activity using AgNOR quantification and EGFR expression in 11 cases of ameloblastoma.

## Methods

*Selection and preparation of samples*

Eleven cases of ameloblastoma were selected from the files of the Oral Pathology Laboratory of the School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Inclusion criteria were histopathologic diagnosis of ameloblastoma, no calcified tissue in the sample, and enough material for at least three consecutive sections.

Three 3-μm sections were obtained from each paraffin block: one was stained with hematoxylin–eosin for morphologic analysis; one was silver stained to investigate proteins associated with active NORs, according to the method described by Ploton et al. (33); the other was labeled with monoclonal mouse anti-human EGFR (clone H11-DAKO, DakoCytomation, Carpinteria, CA, USA), at 1:25 dilution and antigen retrieval with 0.2% trypsin, using the Envision + ™ detection system (DakoCytomation). Positive and negative controls for the immunohistochemical reaction were oral squamous cell carcinoma; for the negative control, the primary antibody was omitted.

This study was approved by the Ethics and Research Committee of the School of Dentistry, Universidade Federal do Rio Grande do Sul.

*Quantitative and descriptive analysis*

Digital image analysis was used to quantify AgNORs. The selected epithelial islands were examined under a binocular microscope (Standard 20; Zeiss, Oberkochen, Germany) at 400× magnification. Images were captured with a video camera (JVC™, 1CCD, TK-C620U, color: JVC, Tokyo, Japan) connected to a computer (Aquanta DX; UNISYS™) using Microsoft VidCap Windows 95 (Microsoft Corp., Redmond, WA, USA) software. Images were transferred to the Imagelab™ 2.3 software (Sistema de Processamento e Análise de Imagens, Softium-Sistemas de Informática, São Paulo, Brazil) at a 440 × 330 pixel resolution. AgNORs were counted with the aid of the manual count tool.

Epithelial cell fields with a histopathology typical of ameloblastoma were captured in numbers that allowed for counting of 200 epithelial cells, 100 pre-ameloblast-like cells, and 100 stellate reticulum-like cells.

Argyrophilic nucleolar organizer regions were quantified by counting the number of dots per nucleus, measuring the area of AgNOR dots, and calculating the percentage of AgNORs per nucleus (pAgNOR ≤ 2 and pAgNOR > 2 per nucleus). The positivity criterion was the visualization of a single dot or multiple black dots inside the yellow nucleus (Fig. 1). Dots that could not be distinguished from each other were counted as a single dot, following the standardized approach described by Crocker et al. (34).

The analysis of EGFR expression consisted of description of labeling on each slide visualized under a microscope (Eclipse E200; Nikon, Tokyo, Japan) at 400× magnification. The epithelial cells in the tumor sites chosen for analysis were peripheral pre-ameloblast-like cells and stellate reticulum-like cells inside the islands. The positivity criteria were brown color (DAB) only in plasma membrane (membrane labeling) (Fig. 2), granular pattern of distribution restricted to cytoplasm (cytoplasmic labeling) (Fig. 3), or both (membrane and cytoplasmic labeling) (Fig. 4). Normal oral mucosa, when found on the slide, was also evaluated.

*Calibration*

Before the study, the observer was trained by an advising professor to standardize positivity and counting criteria. During the study, intraobserver calibration for AgNOR counting was conducted by recounting one of every 10 fields and analyzing results with a *t*-test ($\alpha = 0.05$). For EGFR labeling, five slides were randomly selected after the end of EGFR positivity description for a new reading to check the degree of observer agreement; results showed no statistical differences between the two readings.

*Statistical analysis*

Statistical analysis was conducted by means of tables, graphs and descriptive statistics (mean and standard deviations). The Student's *t*-test was used for

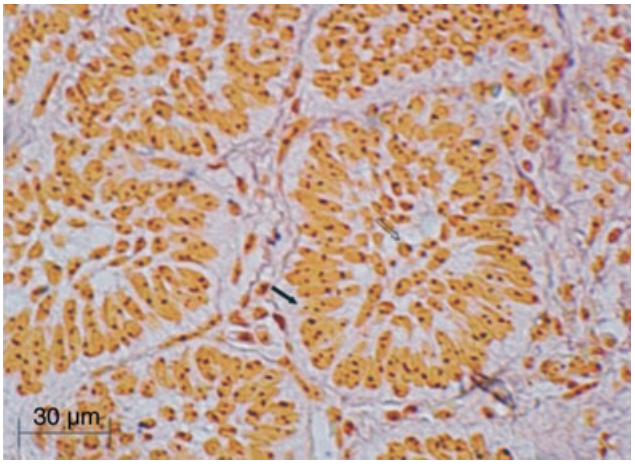


**Figure 1** Photomicrograph of silver-stained ameloblastoma follicular islands for argyrophilic nucleolar organizer region (AgNOR) quantification. A pre-ameloblast-like cell with three AgNORs (black arrow) and a stellate reticulum-like cell with one AgNOR (white arrow) are seen. Silver staining technique – original magnification: 400×.

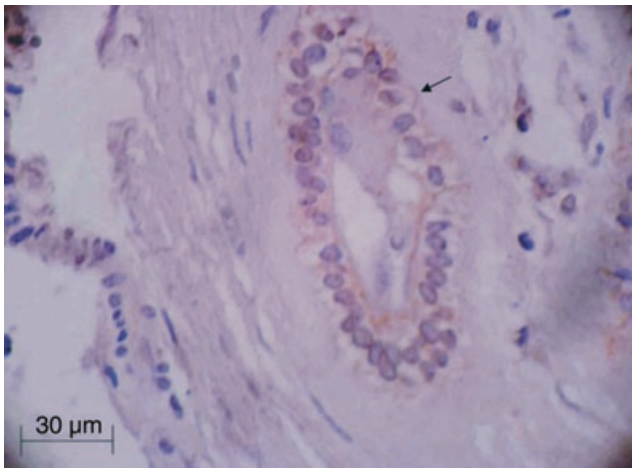


**Figure 2** Photomicrograph of a smaller island containing pre-ameloblast-like cells with antibody anti-epidermal growth factor receptor membrane labeling (→). Original magnification: 400×.

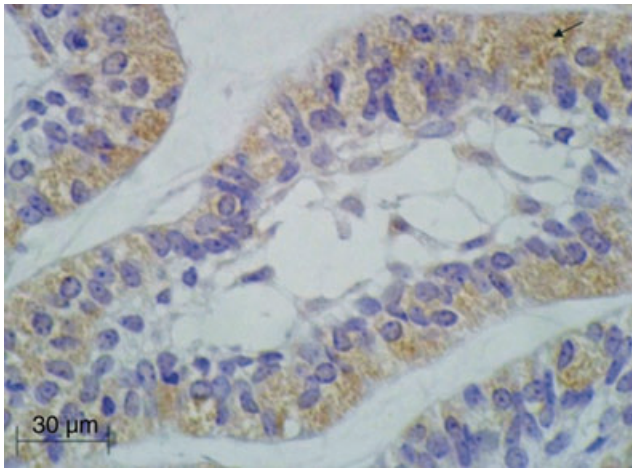


**Figure 3** Photomicrograph of an island containing pre-ameloblast-like cells with antibody anti-epidermal growth factor receptor cytoplasmic labeling (→). Original magnification: 400×.

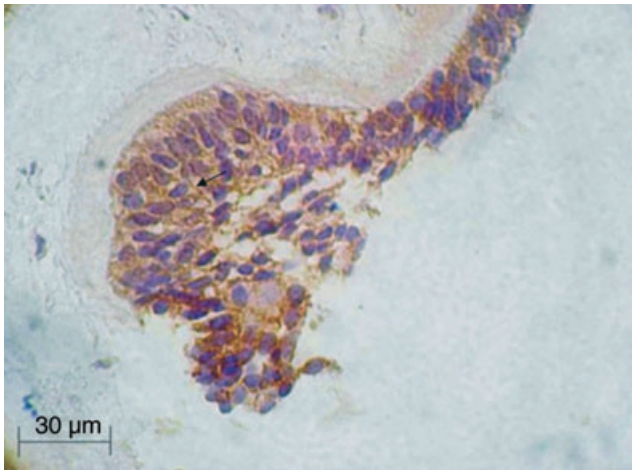


**Figure 4** Photomicrograph of epithelial cells containing anti-epidermal growth factor receptor antibody membrane and cytoplasmic labeling (→). Original magnification: 400×.

comparison of means of paired samples. The SPSS 10.0 software was used for analysis and processing of data.

## Results

*Quantitative analysis of AgNORs*

No statistically significant differences were found for mean number of AgNORs (Table 1), area measurement (Table 2), or percentage of AgNORs per nucleus (pAgNOR ≤ 2 (Table 3) and pAgNOR > 2 (Table 4) between the cell types under study. However, the description of data showed a trend toward greater mean

**Table 1** Mean and standard deviation (SD) of number of AgNORs per nucleus in pre-ameloblast-like cells and stellate reticulum-like cells (Porto Alegre, 2005)

| *Type of cell* | *n* | *Mean* | *SD* | *P-value* |
|---|---|---|---|---|
| Pre-ameloblast-like cells | 11 | 1.51 | 0.19 | 0.08 |
| Stellate reticulum-like cells | 11 | 1.39 | 0.16 | |

Source: Oral Pathology Laboratory, School of Dentistry, Universidade Federal do Rio Grande do Sul. AgNOR, argyrophilic nucleolar organizer region.

**Table 2** Mean and standard deviation (SD) of AgNOR area per nucleus ($\mu m^2$) in pre-ameloblast-like cells and stellate reticulum-like cells (Porto Alegre, 2005)

| *Type of cell* | *n* | *Mean* | *SD* | *P-value* |
|---|---|---|---|---|
| Pre-ameloblast-like cells | 11 | 0.64 | 0.19 | 0.29 |
| Stellate reticulum-like cells | 11 | 0.59 | 0.17 | |

Source: Oral Pathology Laboratory, School of Dentistry, Universidade Federal do Rio Grande do Sul. AgNOR, argyrophilic nucleolar organizer region.

**Table 3** Mean and standard deviation (SD) of percentage of cells with pAgNOR ≤ 2 AgNORs per nucleus in pre-ameloblast-like cells and stellate reticulum-like cells (Porto Alegre, 2005)

| *Type of cell* | *n* | *Mean* | *SD* | *P-value* |
|---|---|---|---|---|
| Pre-ameloblast-like cells | 11 | 92.18 | 5.87 | 0.43 |
| Stellate reticulum-like cells | 11 | 93.27 | 5.28 | |

Source: Oral Pathology Laboratory, School of Dentistry, Universidade Federal do Rio Grande do Sul. pAgNOR, percentage of argyrophilic nucleolar organizer region.

**Table 4** Mean and standard deviation (SD) of percentage of cells with pAgNOR > 2 AgNORs per nucleus in pre-ameloblast-like cells and stellate reticulum-like cells (Porto Alegre, 2005)

| *Type of cell* | *n* | *Mean* | *SD* | *P-value* |
|---|---|---|---|---|
| Pre-ameloblast-like cells | 11 | 7.82 | 5.86 | 0.44 |
| Stellate reticulum-like cells | 11 | 6.73 | 5.27 | |

Source: Oral Pathology Laboratory, School of Dentistry, Universidade Federal do Rio Grande do Sul. pAgNOR, percentage of argyrophilic nucleolar organizer region.

number of AgNORs per nucleus, pAgNOR > 2 per nucleus, and greater mean AgNOR area per nucleus in pre-ameloblast-like cells. Stellate reticulum-like cells had a greater mean only in the evaluation of pAgNOR ≤ 2 per nucleus (Table 3).

*Descriptive analysis of EGFR expression*

The labeling of EGFR expression in the epithelial islands of ameloblastoma was not uniform, and labeled and unlabeled islands were seen on all slides that were examined. The location of labeling also varied in the different epithelial islands. Membrane labeling (Fig. 2), cytoplasmic labeling (Fig. 3) and membrane and cytoplasmic labeling (Fig. 4) were found, but the predominant finding was cytoplasmic labeling. Few islands, usually the smaller ones, had membrane labeling. Most islands did not show variation in EGFR expression labeling for the different cell types that were analyzed. However, a variation in labeling was observed inside some islands, regardless of cell type. A difference in labeling intensity was found not only on different slides, but also in different islands on the same slide.

When present, the oral mucosa showed membrane and cytoplasmic labeling, particularly in the epithelial cells of proliferative layers (basal and parabasal), but labeling decreased away from these layers and closer to the intermediate and surface layers.

## Discussion

The mean number of AgNOR dots per nucleus in both cell types evaluated in this study was compatible with the values found for benign lesions, that is a mean value below 3 AgNOR dots per nucleus (19, 34), in agreement with findings reported by Rosa et al. (2) and Eslamini et al. (13). The mean value found is compatible with the slow growth of ameloblastomas and suggests that the increase in proliferative activity of tumor cells may not be the only factor responsible for the infiltrative behavior of the lesion, as suggested by Do Carmo and Silva (12).

A greater mean area was found in pre-ameloblast-like cells of ameloblastomas, but this difference was not statistically significant. This result is different from those reported by Rosa et al. (2), who evaluated AgNORs in ameloblastoma and basal cell carcinoma, and found that ameloblastomas had a greater mean area and a smaller mean number of AgNOR dots than basal cell carcinoma. However, our results confirm the findings of Pinheiro et al. (6), who reported a greater mean area in cells with a greater number of AgNOR dots in ameloblastomas. Such divergent findings may be explained by the use of different software or hardware, which may affect the accuracy of dot area measurement.

The analysis of AgNOR percentage per nucleus revealed that most cells had one AgNOR per nucleus, which indicates proliferation at a rate similar to the physiologic rate, according to a study by Xie et al. (35), who reported 70% of normal epithelial cell nuclei with one or two AgNORs. The low percentages found for nuclei with pAgNOR > 2 dots indicate low tumor proliferative activity. However, the pre-ameloblast-like cells had greater than two pAgNORs, as well as a mean number of dots and a mean AgNOR area greater than stellate reticulum-like cells. Although this difference was not statistically significant, it may suggest that the proliferation rate of pre-ameloblast-like cells is greater than that of stellate reticulum-like cells.

The analysis of the results indicates that it is not necessary to use more than one method of AgNOR quantification for ameloblastomas because all the methods yielded similar results for tumor proliferative activity. However, the use of percentage of AgNORs per nucleus is suggested because this method indicates the exact portion of cells that have proliferative activity greater than the physiological rate.

All the 11 samples of ameloblastoma, regardless of their histologic classification, were positively labeled in the analysis of EGFR expression. Few studies in the literature evaluated EGFR expression in ameloblastomas (10, 15, 31, 32), and their results are divergent: from all cells being positively labeled (10) to the total absence of labeling (32). These differences may be explained by the different positivity criteria adopted, such as only membrane labeling (31), membrane and/or cytoplasmic labeling (15), and membrane or membrane and cytoplasmic labeling associated with labeling intensity (10, 32). In addition, different types of antibody clones have been used, which may result in binding to different receptor domains and thus affect the location of labeling. However, these results confirm the need for studies to provide detailed descriptions of antibodies, detection systems, and labeling criteria and their interpretation so that a consistent evaluation and comparison of results may be conducted, as suggested by Ciardiello and Tortora (25).

Although positive labeling was seen on all slides, it was not uniform. We found islands with labeling, islands without labeling, and islands where labeling was found in one half but not in the other. Similar findings were reported by Ueno et al. (15), who found that 88% of the islands were labeled. This may be explained by the lack of homogeneity in fixation, or by the fact that some islands were in fact not expressing the receptor, or not expressing the receptor in the entire island at the same time. This type of expression may be involved in the non-uniform growth of lesions and, therefore, justify irregular tumor infiltration.

Membrane, cytoplasmic, and membrane and cytoplasmic EGFR labeling were noted, and the most frequent was cytoplasmic labeling, which was seen in seven of our 11 cases. This labeling pattern suggests the internalization of the receptor (36, 37). These cells may, therefore, react more slowly to stimuli (38), which may affect the proliferative activity of the lesion.

Few islands had only membrane labeling, and these islands were usually the smaller on the slide under analysis. This type of labeling is frequently found in cells with intense proliferative activity, such as in carcinomas (39), and this suggests that these islands may respond more rapidly to the proliferative stimulus. The largest islands may already be in a more 'mature' state, and,

consequently, at a less proliferative stage with a slower response to stimulus. This reduced mitotic capacity is typical of benign cells, which have a proliferative threshold different from that of malignant cells with an unlimited proliferative capacity (40).

Previous studies demonstrated a greater recurrence rate (1, 5) and a greater proliferative activity for follicular tumors (14, 41). The results of this study suggest that the greater proliferative activity of follicular lesions may be associated with its constitution, as follicular tumors are often formed by smaller islands than those found in plexiform lesions.

Most of the cases did not show a difference in the EGFR labeling pattern in the different cell types that were analyzed. However, expression varied in terms of receptor location regardless of cell type in some of the islands, which may indicate that, even in the same island, a different growth pattern may occur. This result is in disagreement with the findings of Li et al. (32), who showed that follicular ameloblastomas had intense labeling in peripheral cells with a decrease toward the center of the tumor islands, and with those by Ueno et al. (15), who reported that follicular ameloblastomas usually showed cytoplasmic labeling in stellate reticulum-like cells, but seldom in pre-ameloblast-like cells.

## Conclusions

A varied pattern of EGFR expression was found in the different cell groups, which suggests an irregular tumor pattern. Membrane EGFR expression was usually seen in the smaller islands, a pattern compatible with a more rapid response to proliferative stimulus, which suggests that these islands have the greatest proliferative rate and, thus, contribute to tumor infiltration. The analysis of predominance of smaller islands on histopathologic diagnostic slides may help define the proliferative activity of each lesion.

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