ORIGINAL ARTICLE

Comparative morphometric study of AgNORs in variants of ameloblastoma

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ABSTRACT

Aims: To compare the area and number of AgNORs (silver stained nucleolar organizer regions) by morphometry between follicular and plexiform variants of ameloblastoma in order to analyze their cell proliferation rates. **Materials and Methods:** This retrospective study was carried out on 30 cases each of follicular and plexiform ameloblastoma. The sections were obtained and stained with silver staining technique to identify the nucleolar organizer regions. AgNORs were quantified using two parameters; manual tag for the number of AgNORs and area measurement using the image analyzer software, Image-Pro-Express. **Results:** Morphometric area measurements of AgNOR were significantly higher for Plexiform ameloblastoma (0.831μm^2) than follicular ameloblastoma (0.528μm^2). Enumeration of the number of AgNORs showed a significantly higher number of AgNOR for follicular ameloblastoma (1.71) than plexiform ameloblastoma (1.43). Among the groups studied, follicular ameloblastoma was more aggressive than plexiform ameloblastoma, as it showed smaller AgNOR area and higher AgNOR number. **Conclusion:** The combination of counting the number and measuring the area of AgNOR dots showed a significant overall difference between AgNOR profiles of follicular and plexiform variants of ameloblastoma.

Key words: Agnor, Follicular ameloblastoma, Morphometry, Plexiform ameloblastoma

INTRODUCTION

Ameloblastomas are locally invasive, highly destructive benign tumors of odontogenic origin. Their recurrence rate is high even for patients who undergo surgical excision of the tumor with a safety tissue margin around.[1] The mechanism of such local aggressiveness is still not known. For a better understanding of the aggressive behavior of ameloblastomas, investigations were carried out using biochemical and immunohistochemical methods to study the expression of metalloproteinases, growth factor receptors and their proliferative activity.[2] Among the various methods used for evaluation of proliferative activity, argyrophilic nucleolar organizer region (AgNOR) uses silver to stain the proteins associated with the active nucleolar organizer regions (NORs).

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Nucleolar organizer regions (NORs) are loops of DNA that transcribe to ribosomal RNA and direct ribosome formation and protein synthesis.[3] NOR sites are located on the short arm of five acrocentric chromosomes, 13, 14, 15, 21 and 22.[4] NOR-associated acidic proteins related to sites of r-RNA transcription can be demonstrated in interphase nuclei in histologic sections by silver staining, which is due to reactivity of sulphydryl groups and can be visualized as black dots, referred to as AgNORs.[4]

The number of AgNORs rises with increase in proliferative activity of cells. AgNORs have been used in histopathologic diagnosis of various benign and malignant lesions.[5] Recent studies show that the number and/or the size of the AgNOR dots in a nucleus are correlated positively with the ribosomal gene activity and therefore with cellular proliferation. A rapidly dividing tumor population shows a greater percentage of cells in the early G1 stages, with a consequently high number of AgNORs. On the contrary, nuclei of tumors with a reduced number of cells actively proliferating are more likely to show a single NOR.[6] It has been consistently shown that AgNORs quantification represents a valuable parameter of cell kinetics and, therefore cell proliferation.[7]Automated image analysis

helps to reduce inter and intra-examiner variability and allows both enumeration and quantification of AgNOR size and type.[4]

The aim of this study was to compare the area and number of AgNORs between the follicular and plexiform variants of ameloblastoma in order to analyze their cell proliferation rates. Morphometric data obtained from images of silver-stained histological sections were used in this study to compare the NOR pattern between the two variants of ameloblastoma to observe if any consistent change existed between these lesions.

MATERIALS AND METHODS

The retrospective study was carried out on tissue sections obtained from histopathologically diagnosed cases of follicular and plexiform ameloblastomas. The study group comprised of 30 cases of follicular and 30 cases of plexiform ameloblastoma. Neither cystic areas of ameloblastoma nor the unicystic variant of this neoplasm were included.

Equipment and Armamentarium used for the study were Silver staining solution, Carl Zeiss trinocular microscope, Digital camera (Nikon) with photomicrography adaptor and Image analysis software: Image Pro-Express Media Cybernetics, USA.

The AgNOR staining solution was obtained by mixing solution A and solution B in the ratio of 2:1. Solution A was prepared by dissolving 25 grams of silver nitrate in 50 ml of de-ionized water. After dissolving silver nitrate crystals the solution was filtered and stored in dark container, well protected from light. Solution B was prepared by dissolving 500 mg of gelatin in 25 ml of de-ionized water. The solution was heated slightly to dissolve the gelatin completely. 0.25 ml of 90% formic acid was then added to the solution and mixed well.[8]

From each paraffin embedded block 4μm thick section was cut using rotary microtome (Leica R.M.2245, Germany). The sections were deparaffinized, rehydrated and then washed in running de-ionized water for 10 minutes. Slides were then stained with silver colloidal solution freshly prepared by mixing solution A and solution B in the ratio of 2:1 and incubated in the dark at 30˚C for 45 minutes. The sections were then washed in running de-ionized water, dehydrated, cleared and mounted in synthetic resin medium. The staining procedure was standardized after a pilot study was carried out in the department, using internal controls such as the development of silver dots on lymphocytes.

For each case, 50 nuclei of tumor cells from five microscopic fields were examined under 100X oil immersion lens (ten cells were selected randomly from each microscopic field). Images were captured using a 3 chip digital camera attached to a Carl Zeiss trinocular microscope with a 100X oil immersion objective lens. The images were classified, transferred and stored in the computer [Figures 1 and 2]. Only well-defined and sharply stained intra-and extra-nucleolar AgNOR dots were included in the counting regime and morphometry. AgNORs which were located within the nuclei at the border of the microscopic field or superimposed nuclei were not included in the study. Counting was done as recommended by Crocker *et al.,*[8] where all silver stained structures were counted but when lying in clusters, each cluster was counted as one unit.

Statistics

The analysis of AgNOR dots was done using two parameters, namely area measurement of AgNORs [Figure 3] and manual tag for the number of AgNORs [Figure 4] using the image analyzer software Image-Pro-Express (Media Cybernetics, USA). The data collected was analyzed statistically by Student's *t-test* and results were expressed as mean ± standard error of mean. ANOVA test was carried out to find out the significant difference between the lesions.

RESULTS

AgNORs were located within the cell nucleus and were distinctly stained in black as dots and blebs; the rest of the nucleus stained yellow-brown. By careful control of the silver technique during preliminary experiments, it was possible to reduce the background staining which does not interfere with the identification and/or counting of AgNORs. Lymphocytes in the tumor stroma serve as internal controls, as these most often demonstrate single or double AgNOR dots per cell. ANOVA test showed significant difference between the group with respect to the mean number and area of AgNORs ($P<0.05$).

ANOVA test showed a significant difference in AgNOR area and number between follicular and plexiform ameloblastoma ($P < 0.05$). The results of the morphometric analysis of AgNOR dots from both the variants of ameloblastoma are shown in Table 1. Plexiform ameloblastoma showed a higher mean AgNOR area of 0.831 μm^2 than the follicular variant with the mean area of 0.528 μm^2, whereas follicular ameloblastoma showed a higher mean AgNOR number of 1.71 than plexiform variant with the mean number of 1.43. The average number of AgNOR dots per nucleus in plexiform variant was significantly lower than in the follicular variant [Graph 1], whereas mean value for area of AgNOR was significantly higher for plexiform ameloblastoma than follicular variant [Graph 2].

DISCUSSION

Follicular and plexiform variants are the most frequently encountered histologic types of ameloblastoma.[9] Clinically they have very similar growth patterns, with local invasiveness and rare metastasis, but have different recurrence rates,[10]

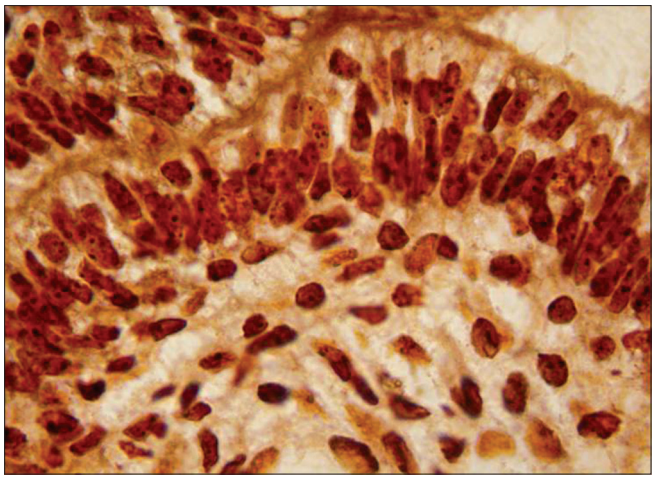


Figure 1: AgNOR stained follicular ameloblastoma (100×)

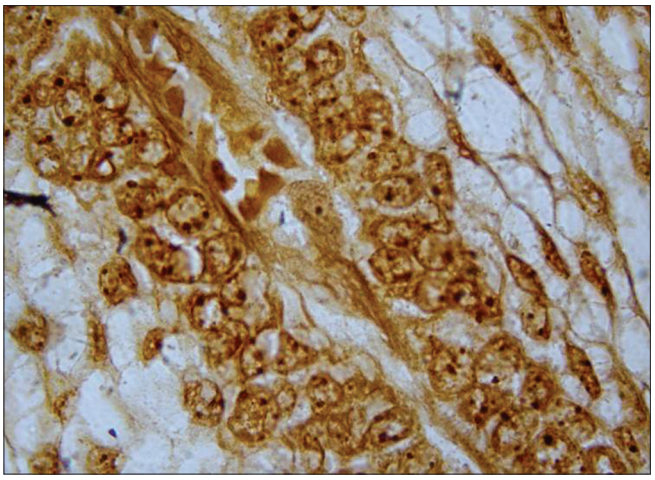


Figure 2: AgNOR stained plexiform ameloblastoma (100×)

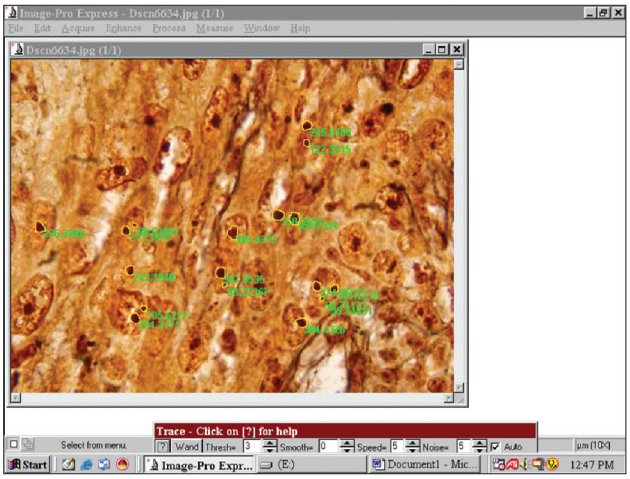


Figure 3: Morphometric analysis of area measurement of AgNORs using the image analyzer software image-proexpress

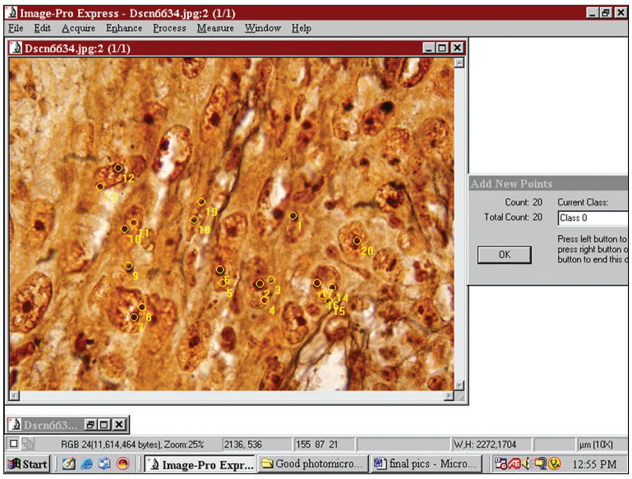
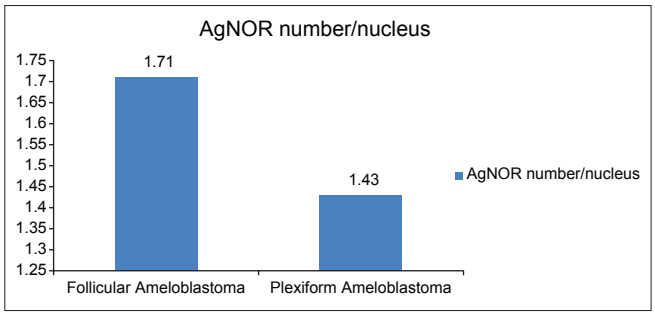
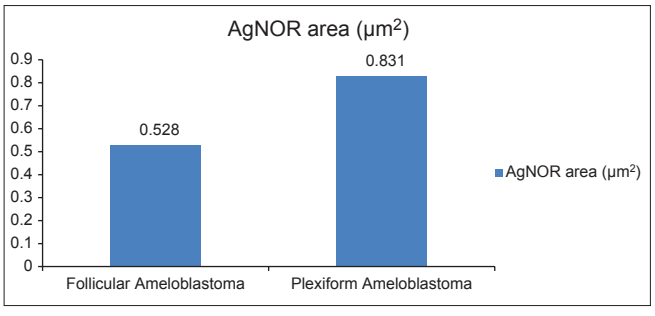


Figure 4: Counting of AgNORs using manual tag for the number of AgNORs using the image analyzer software image-proexpress



Graph 1: Bar graph showing the comparison of mean number of AgNORs in follicular and plexiform ameloblastoma



Graph 2: Bar graph showing the comparison of mean area of AgNORs in follicular and plexiform ameloblastoma

for this reason we performed qualitative and quantitative analysis of AgNORs in these two variants of ameloblastoma to compare their cell proliferation rates.

The variation in NOR distribution within the nucleus provides information regarding the cell cycle. Interphase cells present NORs tightly aggregated in one or two nucleoli. During mitosis, however a nucleolar fragmentation occurs resulting in the dispersion of NORs throughout the nucleus. NOR counting has been used as a method to assess the rate of cellular proliferation.[2] Thus, NORs are more likely to be observed in greater numbers in proliferative conditions.[11] This assumption is confirmed by studies showing a correlation between the number of NORs and other cellular proliferation parameters, such as Ki-67 antigen and DNA flow cytometry.[12] Cytokinetics of the lesions assessed by bromodeoxyuridine labeling index

Table 1: Morphometric analysis of AgNORs in follicular and plexiform ameloblastoma

Parameter	Follicular ameloblastoma (mean ± SD)	Plexiform ameloblastoma (mean ± SD)
AgNOR area (μm²)	0.528 ± 0.179	0.831 ± 0.148
AgNOR number/nucleus	1.71 ± 0.35	1.43 ± 0.43

showed a linear correlation with AgNOR counts.[13] AgNOR number is related to degree of tumor histologic differentiation and pathologic stage.[14] For this reason, several authors have applied this technique and suggested that AgNOR enumeration can be useful in estimating the proliferation potential of neoplasms in the practice of surgical pathology.[15] Eusebi *et al.,* studied AgNORs and other parameters of breast carcinoma and found that only AgNOR counts showed an important relationship with clinical behavior.[16] Ohno *et al.,* analyzed the AgNOR counts in osteosarcomas, and found that osteosarcomas with a better prognosis have lower AgNOR numbers than those with a poorer prognosis.[17]

The mean number of AgNOR dots per nucleus in both the variants of ameloblastoma evaluated in this study was compatible with the values found for benign lesions, that is a mean value below 3 AgNOR dots per nucleus,[8,18] in agreement with findings reported by Rosa *et al.*[14] and Eslamini *et al.*[5] 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.[19]

Plexiform ameloblastoma showed a significantly smaller average number of AgNOR dots per nucleus than follicular variant. In our study, we observed that the mean area of AgNORs (mean ± SD) in plexiform ameloblastoma was 0.831±0.148μm², and in follicular ameloblastoma was 0.528±0.179μm². The mean number of AgNOR dots per nucleus in follicular ameloblastoma was 1.71 ± 0.35 and in plexiform variant was 1.43 ± 0.43. Our results were similar to the study by Do Carmo and Silva (1998) where the mean AgNOR number in plexiform ameloblastoma was 1.49 ± 0.15 and in follicular ameloblastoma was 1.87 ± 0.39.[19] Previous studies by Ueno *et al.,*[10] and Reichart *et al.,*[1] demonstrated a greater recurrence rate and a greater proliferative activity for follicular ameloblastoma than plexiform ameloblastoma.

A significantly higher number of AgNOR dots per nucleus were found in follicular ameloblastoma than in plexiform ameloblastoma cells. Most of the plexiform variant contained one or two AgNOR dots per nucleus, while most of the follicular variant contained two or three AgNOR dots per nucleus. In addition, plexiform ameloblastoma cells contained no more than three AgNOR dot per nucleus, while follicular ameloblastoma cells contained upto five AgNOR dots per nucleus. Morphometric area of AgNOR dots yielded significantly higher mean values for the plexiform ameloblastoma than those for the follicular variant. AgNORs in follicular ameloblastoma were smaller, more numerous and more widespread than in plexiform ameloblastoma, which possibly demonstrates a higher degree of cellular proliferation in follicular variant of ameloblastoma.

Rise in AgNOR material denotes a rise in protein synthesis.[20] Therefore, we may infer that almost half of the ameloblastoma cells would be synthesizing protein and this protein might participate in a mechanism that would contribute in part to the growth pattern of this neoplasm. This secretory activity could involve proteolytic enzymes linked to an invasive phenotype such as the metalloproteinase collagenase IV.[21] This hypothesis is supported by the detection of collagenase IV in different stages of developing tooth.[22] Further studies demonstrating an increased production of metalloprotenases in ameloblastoma are necessary to confirm this hypothesis. Furthermore, the combination of counting and area measurement defined a significant overall difference between AgNOR profiles in these two variants of ameloblastoma.

CONCLUSION

In conclusion, we have determined that although follicular and plexiform ameloblastoma are epithelial tumors exhibiting similar clinical behavior they are composed of tumor cell populations with statistically significant differences in AgNOR patterns that reflect differences in cellular and nuclear activities.

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