Quantitative AgNORs study in ameloblastomas

Análise quantitativa das AgNORs em ameloblastomas

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Abstract

Purpose: Ameloblastomas are tumors that arise from the odontogenic epithelium, including the areas that coat the dentigerous cysts (DC). The aim of the present study was to investigate the proliferative activity present in luminal and mural areas of mural unicystic ameloblastomas (MUA), in conventional ameloblastomas (CA), and in DC, comparing them according to their biological behavior.

Methods: AgNOR quantifications were performed using routine light microscopy under X100 magnification in 10 cases per type of lesion, considering 100 cells of representative areas of the tumor for each case. The AgNORs were observed as black dots within the cell nucleus.

Results: MUA showed significant lower number of NORs/nucleus in the luminal proliferation area than in the area of mural proliferation and in CA ($P<0.05$).

Conclusion: CA exhibit proliferative activity similar to the mural component of MUA, corroborating the pattern of higher aggressive clinical behavior of these tumor variants.

Key words: Ameloblastoma; AgNOR; cell proliferation; dentigerous cyst

Resumo

Objetivo: Os ameloblastomas são tumores originados do epitélio odontogênico, inclusive daquele que reveste os cistos dentígeros (CD). O objetivo do presente trabalho foi avaliar a atividade proliferativa das áreas luminais e murais dos ameloblastomas unicísticos murais (AUM), ameloblastomas convencionais (AC) e cistos dentígeros (CD), comparando seus comportamentos biológicos.

Metodologia: Para tanto foram selecionados 10 casos de AC, 10 casos de AUM e 10 amostras de CD. A análise quantificativa das AgNORs foi realizada através de microscopia ótica de rotina com aumento de 100X nos 10 casos de cada lesão estudada, analisando-se 100 células de áreas representativas do tumor. As AgNORs foram observadas como pontos pretos dentro do núcleo celular.

Resultados: A média de NORs/núcleo da área de proliferação luminal do AUM foi estatisticamente inferior à da área de proliferação mural desta mesma lesão e à do AC ($P<0,05$); nenhuma outra diferença estatística foi encontrada.

Conclusão: Conclui-se que os AC exibem atividade proliferativa semelhante à do componente mural do AUM, o que corrobora o padrão de comportamento clínico mais agressivo dessas variantes clínicas.

Palavras-chave: Ameloblastoma; AgNORs; proliferação celular; cisto dentígero

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Introduction

Ameloblastoma is the most commom and clinically relevant odontogenic tumor. Its prevalence exceeds the prevalence of most tumors grouped, excluding the odontomas (1). Based mainly on clinical behavior and prognosis, there are two clinicopathological variants of intraosseous ameloblastoma: conventional ameloblastoma (CA), also known as solid or multicystic ameloblastoma, and unicystic ameloblastoma (UA) (2).

CA constitutes about 86% of ameloblastomas, presenting several microscopic subtypes, but these different patterns relate poorly with the biological behavior of the tumor (1). The solid ameloblastoma is a locally aggressive tumor that may produce marked facial deformity and serious debilitation. Since it tends to infiltrate the cancelous bone trabeculae, the real margin of the tumor extends itself beyond the radiographic margins. Even if the surgery is done with its marginal resection overcoming the radiographic borders of the tumor, it still yields recurrence rates up to 15% (1).

UA accounts for 13% of all intraosseous ameloblastoma, and has a less aggressive clinical behavior, being frequently treated by enucleation or curettage. It can be originated from *de novo* or from neoplastic transformation of non-neoplastic cyst epithelium, especially from the dentigerous cyst (DC), which can be similar to UA in both clinical and radiographic aspects (1). There are three histopathological variants of UA: luminal, intraluminal, and mural ameloblastomas (3). The luminal unicystic ameloblastoma (LUA) presents a great cystic cavity lined partially or totally by ameloblastic epithelium, and a fibrous wall. The intraluminal unicystic ameloblastoma (IUA) is composed of anastomosing cords and of islands of epithelium, which are both projected into the cystic lumen. The mural unicystic ameloblastoma (MUA) presents islands of epithelium growing inside the connective tissue of the cyst wall. These islands are composed of peripheric collumnar epithelial cells and a centre that is identical to stellate reticulum (4). It is important to highlight that lesions with mural proliferation present a high risk of recurrence, justifying an extended follow-up in these cases (5).

The determination of epithelial proliferative activity constitutes a useful way to investigate the differences among the biological behavior of tumors, defining the strategies of treatment, and their prognostic evaluation. Nucleolar organizer regions (NORs) are loops of DNA that codify the ribosomal RNA, and are considered important for protein synthesis (6). The transcriptional activity of the NORs is associated with non-histonic proteins, which are selectively marked by silver ions. The technique used to visualize the NORs is simple and can be performed in samples processed by routine methods. The NORs stained by silver are named argirophilic nucleolar organizer regions – AgNORs. It has been consistently shown that AgNORs quantification represents a valuable parameter of cell kinectics and, therefore, cell proliferation (7).

The literature has showed an arsenal of instruments used for different odontogenic tumors in the jaws, especially ameloblastomas (8-10) in order to evaluate the biological behavior of these lesions and establish the most appropriate treatment for their distinct pathological variants (11-13). However, there still are many doubts about the correlation between the clinical behavior and the proliferation activity of the ameloblastoma variants.

The specific goal of this study was to investigate the possible correlation between clinical behavior and proliferation activity of two different variants of ameloblastoma: CA and MUA (luminal and mural areas), comparing them with the proliferation activity of DC, using the histochemical method of AgNORs for quantification of NORs per nucleus.

Methods

Case selection and morphological analysis

The present study was approved by the Research Ethics Committee of the Federal University of Pelotas – School of Dentistry. Ten cases of CA, 10 cases of MUA (all with mural and luminal proliferation areas), and 10 cases of DC were selected from the archives of the Department of Oral Pathology, School of Dentistry, Federal University of Pelotas. All cases had been fixed in 10% formalin, routinely processed, and embedded in paraffin wax. Using haematoxylin and eosin-stained sections, the histopathological diagnosis of each case was reviewed, and the quality of the material was checked.

AgNORs histochemical technique

Three-µm sections were obtained, and the AgNOR technique was used according to Ploton et al. (14), modified by Rivero & Aguiar (15), with additional small modifications. To briefly describe the method, after the deparaffinization and rehydration processes, the slides were washed in running deionized water for 5 min. The slides were subjected to treatment with citric acid (Reagen, Rio de Janeiro, RJ, Brazil) 10 mM at pH 6.0, in a microwave oven at 700 W in three cycles of 5 min each, in order to optimize the silver staining. Afterwards the material was cooled in room temperature and then washed in running deionized water for 15 min. The excess of water was removed, and the slides were incubated in a freshly prepared solution made of one part of a solution composed of 2 g of gelatin (Labsynth, Diadema, SP, Brazil) and 1% aqueous formic acid solution mixed to two parts of 50% aqueous silver nitrate solution (Merck, Darmstadt, Germany). The slides were immersed in this solution for 25 min at 35ºC, washed in deionized water at 45ºC, and mounted in Permount® (Fischer Scientific, Fair Lawn, USA).

Quantitative and statistical analysis

AgNORs quantifications were performed using routine light microscopy and AgNORs were evaluated under X100 magnification and oil-immersion using an eye-piece graticule of 0.025 mm^2. The AgNORs were observed as

black dots within the cell nucleus. The number of separate black dots within the nucleus was recorded, and they were considered as one subject when the black dots were closely aggregated. When the examiner was in doubt, the area was not recorded for analysis. For calibration, two observers previously performed the counting procedure.

For each type of lesion, the AgNORs of 100 cells were counted. These cells were taken from representative areas of the three odontogenic lesions. In the MUA cases, 100 cells from mural islands or nodules and 100 cells from luminal areas were assessed.

In the cystic epithelia of DC and in the luminal proliferation area of MUA, the graticule was positioned in order to count always the same proportion between the basal and parabasal cells. In the areas of mural proliferative islands of MUA and in the CA, the same proportion of peripheral and central cells was counted. Two calibrated examiners performed the AgNORs quantification, and the mean of NORs/nucleus of the two measurements was obtained.

The comparison among the means of NORs/nucleus among the three lesions, CA, CD and MUA (mural proliferation and luminal proliferation areas), was done by one-way analysis of variance (ANOVA) followed by Holm-Sidak test as post-hoc comparison test (α=0.05).

Results

In all cases of CA, MUA and DC, AgNORs were visualized within the cellular nucleus as distinct and homogeneous dark dots with rounded morphology and regular boundary.

The mean number of NORs/nucleus of luminal proliferation areas for MUA (1.68±0.13) was significantly lower (P<0.05) than for the mural proliferation areas of MUA (2.02±0.22) and CA (2.06±0.22), whereas it was not statistically different from DC (1.83±0.32). Data are summarized in Table 1 and expressed as mean ± standard deviation values.

Table 1. Comparison among the mean number of NORs/nucleus of CA, UA, and CD by ANOVA test.

Lesion	NORs/nucleus (Mean±SD)
CA	2.06±0.22
Mural areas of MUA	2.02±0.22
Luminal areas of MUA*	1.68±0.13
DC	1.83±0.32

* P<0.05 when compared to CA and mural area of MUA.

Discussion

Previous studies have related the cellular proliferation index with the biological behavior of ameloblastomas (8-10,16), and compared the proliferative activity of ameloblastomas with those of odontogenic cysts (8-13). Variations in AgNOR size and number might be dependent on the stage of the cell cycle, the transcriptional and metabolic cell activity or the number of NOR-bearing chromosomes in the karyotype (17). Audaciously, some authors have related the occurrence of smaller and more numerous AgNORs with the malignant potential of the lesions (18,19). According to these authors, AgNORs features can be used to assess the evolution of the degree of cellular proliferation as a prognostic measure for the tumor.

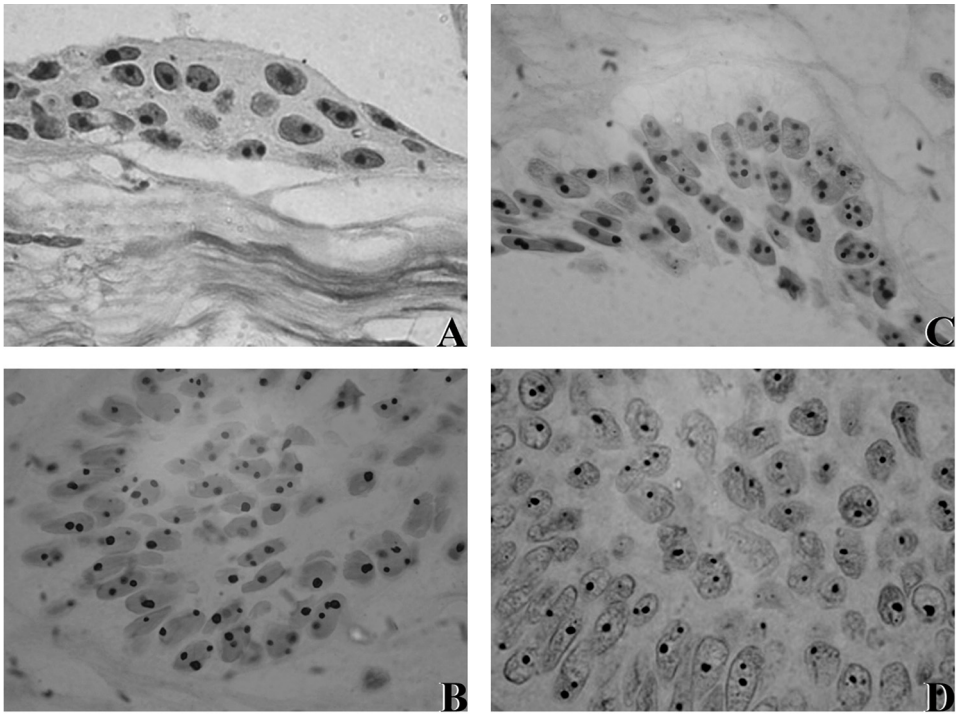

Fig. 1. **(A)** AgNOR staining in epithelium of the dentigerous cyst; **(B)** mural proliferation areas of unicystic ameloblastoma; **(C)** luminal proliferation areas of unicystic ameloblastoma; **(D)** and conventional ameloblastoma. The AgNORs are shown as black dots within the cellular nucleus.

The results of the present study showed a larger number of NOR per nucleus in CA than in luminal proliferation areas of MUA. These data could explain the less aggressive biological behavior of unicystic ameloblastomas presenting only luminal type of proliferation (LUA). On the other hand, it is in accordance with the clinically observed aggressive behavior of solid lesions of ameloblastomas (2,20). Moreover, these results combined with previous reports suggest a positive correlation between the number of AgNORs and the biological behavior of tumors (21).

In the present study all 10 UA cases were MUA, and both luminal and mural areas were analyzed. When CA was compared to mural proliferation areas of MUA, no statistically significant difference was found. This result reinforces the already known aggressive behavior of MUA, which is similar to CA, considering recurrence ratio and prognosis (5,22,23). Besides, it seems that MUA is a lesion with higher proliferative capacity than LUA and IUA (24).

In contrast to the present results, Eslami et al. (10) did not find any statistically significant difference of AgNORs number per nucleus between UA and CA in odontogenic keratocysts. Some important methodological differences could explain such conflicting results as Eslami et al. (10) did not distinguish UA subtypes, grouping all MUA, LUA, and IUA areas in their AgNOR counting. Therefore, the higher proliferation activity of mural proliferation areas could have been masked by the low proliferation index of luminal and intraluminal sites, as these last ones would be more frequent.

In the present study, caution was taken to have proportional number of basal and parabasal cells in lesions of cystic features (luminal areas of MUA and DC) for AgNOR counting. Also, the peripheral and central cells in epithelial odontogenic islands of mural areas of MUA and CA were counted in the same proportion. Eslami et al. (10) reported similar AgNOR counting only for OC, but this entity was not included in the present analysis. Curiously, although those authors have justified such counting procedure for OC based on the differences related to proliferation between the distinct zones of cystic epithelia (25), they did not use the same method to quantify AgNOR for other lesions. It is important to highlight that there is a lack of studies which used the AgNORs technique to analyze UA lesions distinguishing mural and luminal proliferation areas as adopted here. Coleman et al. (9) counted only the basal cells in unicystic ameloblastomas and in dentigerous cysts. Because they did not include other parabasal areas in their AgNOR counting, these tumors may have shown fewer AgNORs in these lesions than the real situation.

Finally, when the DC proliferation index values were compared to the values observed in the studied variants of ameloblastoma, no statistically significant difference was found. The DC was included in this study especially because its epithelium lining can originate the ameloblastic epithelium (1). Although the DC can become a large lesion and expand the cortical bone of the jaws, the mechanism of expansion of the DC is considered rather passive, occurring by accumulation of fluid in the lumen instead of by the proliferation of its epithelium (13). A previous study showed different results when DC was compared with ameloblastomas, being the AgNOR counts of DC higher than AU (9). Coleman et al. (9) attributed these differences to the variations in metabolic, proliferation or transcriptional activity observed in these entities. However, Eslami et al. (10) described a lower number in DC when compared with UA and CA. Again, the lack of standardization of AgNOR counting may have interfered in their data analysis.

Other methods such as immunohistochemistry have been used to investigate cell proliferation antigens to compare cellular proliferation among CA, UA, and DC (11-13). Li et al. (24), based on the differences among the biological behavior of intraosseous ameloblastomas, compared the proliferative activity of these lesions by evaluating Proliferating Cell Nuclear Antigen (PCNA) and Ki-67. These authors showed that mural islands invading the fibrous capsule had higher number of these antigens than intraluminal nodes and luminal epithelia in UA lesions. They also showed that CA exhibited higher PCNA and Ki-67 than all UA variants. Moreover, the precise localization of PCNA positive cells in CA and UA, defined by Piatelli et al. (11), was similar to the one found by Li et al. (24), *i.e.*, peripheral cells of CA tumor islands and of the mural proliferation areas in MUA had similar PCNA indexes. These authors observed equal numbers of PCNA positive cells when they considered the basal cells of DC and of UA cystic epithelia. Besides, Sandra et al. (25) observed two distinct patterns of apoptosis in ameloblastomas: an anti-apoptotic proliferating site in the outer layer (periphery) of the islands and a pro-apoptotic differentiating site in the inner layer (centre). Although these proliferation markers are distinct from the one used in the present study (AgNORs), these findings somehow reinforce the method of AgNOR counting, proportionally evaluating peripheral and central cells of CA and MUA islands, and basal and parabasal cells of cystic epithelia of MUA luminal areas and DC epithelium lining.

Based on the results exposed, it can be concluded that the luminal proliferation areas of MUA exhibits lower proliferation activity than the mural proliferation areas. Besides, the same significant differences were observed when the luminal proliferation areas of MUA and CA were compared. Since the amount of AgNOR proteins is strictly proportional to the proliferative status of the cells, these results strongly reinforce the differences in the biological behavior of these ameloblastoma variants. Moreover, AgNORs technique may be considered a good indicator of cell proliferation, but should not be regarded as a definitive or unique method.

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