

Ameloblastic neoplasia spectrum: a cross-sectional study of MMPS expression and proliferative activity



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Objective. To compare the proliferation and the expression of matrix metalloproteinases (MMPs; MMP-2 and MMP-9) in solid and unicystic ameloblastomas with ameloblastic carcinomas.

Study Design. Five cases of ameloblastic carcinoma (AC), 18 cases of solid ameloblastoma (SA), and seven of unicystic ameloblastoma (UA) were selected. The immunohistochemical expression of MMPs was assessed by the percentage of positive tumor cells and stained stroma. The mean argyrophilic nucleolar organizer region (AgNOR) and the percentage of cells with more than one AgNOR per nucleus were evaluated.

Results. Statistically significant higher mean AgNOR was observed in AC than in SA and UA. MMP-2 was expressed similarly in tumor and stroma among groups. MMP-9 was higher in the stroma of SA than that of UA ($P = .0484$).

Conclusions. The cell proliferation was related to the greater aggressiveness of AC. High expression of MMP-2 and MMP-9 in all lesions highlighted the importance of these enzymes in the biology of ameloblastic tumors. (Oral Surg Oral Med Oral Pathol Oral Radiol 2016;121:396-401)

Ameloblastoma is a benign odontogenic tumor with locally aggressive behavior, accounting for 1% of all jaw tumors.¹ According to the classification of the World Health Organization proposed in 2005, the variants of ameloblastoma may be classified as solid/multicystic, desmoplastic, unicystic, and extraosseous. There are significant differences among the biologic behaviors of these variants.^{2,3}

For solid ameloblastoma (SA), surgical resection with safe margins has been the treatment of choice to prevent recurrence and development of malignancy. In contrast to the aggressive growth and local invasion profile of its solid counterpart, unicystic ameloblastoma (UA) is considerably less destructive and more amenable to conservative treatment.^{4,5}

Ameloblastic carcinoma (AC) is a rare malignant odontogenic tumor, which combines the histologic characteristics of ameloblastoma with cytologically atypical features, regardless of the presence or absence of metastasis. Histologically, AC displays cellular pleomorphism with mitotic activity, focal necrosis, perineural invasion, and nuclear hyperchromatism, supporting the differential diagnosis with ameloblastoma.^{3,6,7} Several therapeutic approaches are available, including curettage, resection, and radiotherapy. Conservative treatment may result in multiple recurrences and metastases, demonstrating the importance of early diagnosis. Complete tumor removal seems to be strongly related to patient survival.^{6,8}

Enzymes related to cell invasion may be potentially useful for evaluation of the biologic behavior of different ameloblastic neoplasms.⁹ Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes, which are important in matrix degradation during tumor growth, invasion, and induction of angiogenesis.^{10,11} Several studies have demonstrated that MMP-2 and MMP-9 secreted by both stromal and tumor cells into the surrounding tissues

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Statement of Clinical Relevance

The results of this study highlight the clinical relevance of the expression of matrix metalloproteinases (MMP-2 and MMP-9) in ameloblastic tumors, suggesting a potential therapeutic approach to the inhibition of these molecules for future treatment of these tumors.

might contribute to the invasive capacity of ameloblastoma.¹²⁻¹⁵

Furthermore, the rate of cellular proliferation could contribute to the local aggressiveness of these neoplasms. The argyrophilic nucleolar organizer region (AgNOR) technique, which consists of silver impregnation of proteins associated with the active nucleolar organizer regions (NORs), can provide valuable information about cell proliferation velocity in these tumors. The faster the cell cycle occurs, the lower the possibility of aggregating the NORs together during interphase. Therefore, the AgNOR technique reflects the velocity of the cell cycle and not just the growth fraction.¹⁶⁻¹⁸

There are few comparative studies on the expression of invasiveness-related molecules and of proliferation markers among the spectrum of ameloblastic neoplasms. The present study assessed the proliferation rate and the expression of MMP-2 and MMP-9 in different clinical types of benign ameloblastoma and in ameloblastic carcinoma.

MATERIALS AND METHODS

This retrospective cross-sectional study included five cases of AC, 18 cases of SA (10 follicular and eight plexiform), and seven cases of UA (luminal type), which were retrieved from the files of the Department of Oral Pathology, School of Dentistry, Federal University of Rio Grande do Sul (Porto Alegre, Brazil) and from the Universidad del Uruguay (Montevideo, Uruguay), for the period 1984-2009. All samples were classified according with World Health Organization criteria.³ The diagnosis of ameloblastic carcinoma was based on the presence of cellular malignant features, such as atypical mitoses, nuclear pleomorphism, and nuclear hyperchromatism, in conjunction with typical areas of ameloblastoma.

This study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul. Demographic data as age, gender, race, and geographic location was recorded. The reason for some missing demographic data was the absence of records in the archives of the laboratories. Data on race was categorized as "Caucasian" and "non-Caucasian."

Serial sections from tissue samples, 3 μm in thickness, were obtained from paraffin-embedded samples, and the dewaxed sections were processed for antigen retrieval. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. After washing, the sections were incubated with primary antibodies for MMP-2 (clone HPA 001939, polyclonal, Sigma-Aldrich, Sweden) and MMP-9 (clone 15 W2, Novocastra Newcastle, UK). Peroxidase-linked secondary antibodies and diaminobenzidinetetrahydrochloride (Envision HRP Kit, Dako Corp., Carpinteria, CA) were

used to detect specific binding. The sections were counterstained with hematoxylin of Harris, dehydrated, and mounted. Microscopic images were captured with an Olympus binocular microscope equipped with an Olympus video camera (QColor 5, Olympus, Tokyo) and a computer. An oral squamous cell carcinoma sample, which was previously confirmed by MMPs expression, was used as positive control, and negative control was obtained by omitting the primary antibody. MMP-2 and MMP-9 expression was assessed by quantification of the first 1000 well-arranged tumor cells, and the percentage of positive-stained tumor cells was recorded. The percentage of area of stained stroma was also analyzed, supported by Image J 1.45 software (National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

Tissue samples were subjected to the AgNOR technique following the protocol described by Ploton et al.¹⁹ AgNORs were quantified according to the criteria established by Crocker et al.²⁰ AgNOR dots per nucleus were visually counted in the first 100 well-arranged, non-overlapping nucleated cells captured at $\times 1000$ magnification under immersion oil. The mean AgNOR (mAgNOR) numbers and the percentage of cells with more than one AgNOR per nucleus (pAgNOR >1) were calculated according to the methodology proposed by Xie et al.²¹

Quantification of MMP-2 and MMP-9 expression and AgNORs was performed by a single observer, who was blinded to avoid variability in the analysis and to maintain the same standard of evaluation. An interexaminer calibration was performed prior to data analysis. During the study, intraexaminer calibration was performed, considering an ICC ≥ 0.75 acceptable.

Statistical analysis was performed by using the SPSS 18 software (SPSS Inc., Chicago, IL). According to data distribution, differences between groups were assessed by the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison post-test or by using ANOVA followed by the Tukey post hoc test. Statistical significance was set at $P < .05$. The correlation analysis between variables was obtained with Spearman test correlation.

RESULTS

The clinical features for all studied patients samples are summarized in Table I. The mean age of the patients was 33.83 years (range 12-86 years). Most of the lesions were located on the mandible (see Table I).

MMP-2 was expressed in the cytoplasm of both peripheral columnar cells and stellate reticulum-like cells in all ameloblastic tumors (Figure 1). A similar expression of MMP-2 was seen in tumor cells and stromal cells of ameloblastic carcinoma and benign forms of ameloblastoma. The expression of MMP-2

Table I. Clinical and demographic features of patient sample

Type	Site	Gender	Mean age	Race
Unicyclic ameloblastoma	7 mandible	2 male	33.83 (±21.91)	2 Caucasian
		5 female		5 non-Caucasian
Solid ameloblastoma	12 mandible	9 male	39.33 (±20.87)	13 Caucasian
	6 maxilla	7 female		3 non-Caucasian
		2 unknown		2 unknown
Ameloblastic carcinoma	3 mandible	3 male	66.66 (±22.47)	5 unknown
	2 unknown	2 unknown		

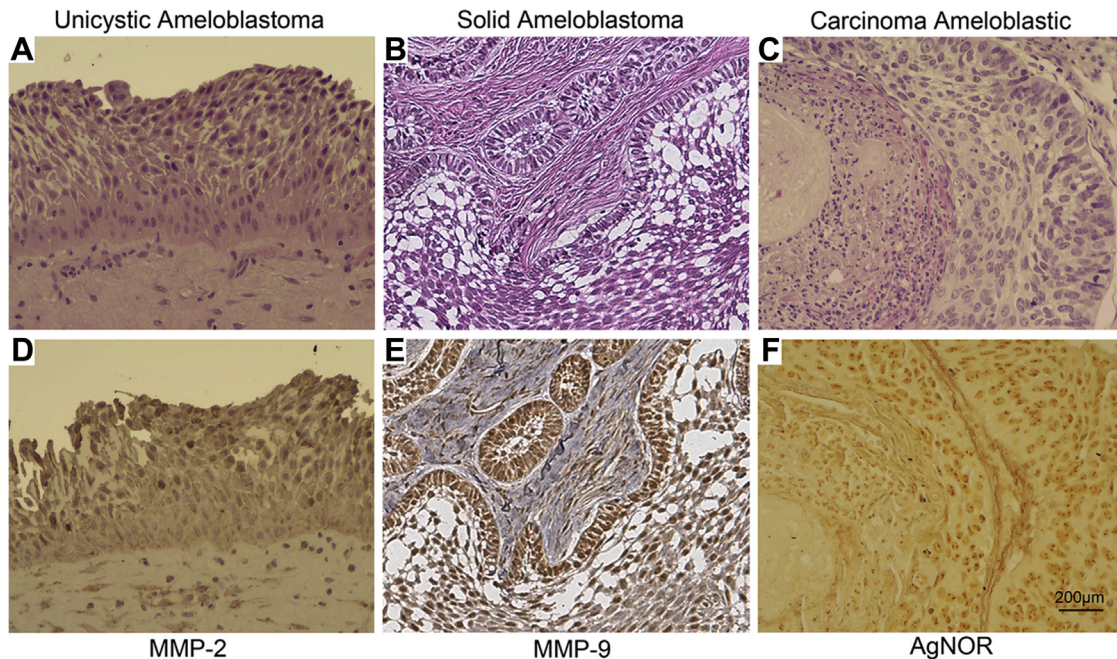


Fig. 1. Photomicrography of stained sections of ameloblastic tumors. **A**, unicyclic ameloblastoma. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02172. **B**, Solid ameloblastoma A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02173. **C**, Ameloblastic carcinoma. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02170. (original magnification ×400; bar scale = 200 µm). **D**, Matrix metalloproteinase 2 (MMP-2) staining in unicyclic ameloblastoma (tumor cells = 97.20%; tumor stroma = 73.20%). A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02174. **E**, MMP-9 staining in solid ameloblastoma (tumor cells = 99.50%, tumor stroma = 60.93%). A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02171. **F**, Argyrophilic nucleolar organizer region (AgNOR) staining in ameloblastic carcinoma (mAgNOR = 4.93; pAgNOR>1 = 94). A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02169. AgNOR, argyrophilic nucleolar organizer region; mAgNOR, mean argyrophilic nucleolar organizer region; pAgNOR<1, percentage of cells with more than one AgNOR per nucleus.

in stroma area decreased from 77.79% in AC, to 75.7% in SA, and to 69.62% in UA, although no statistical difference was observed between groups (Figure 2).

MMP-9 was also detected in both tumor cells and stroma cells in all samples (see Figure 1). However, there was a statistically significant higher expression of MMP-9 in the stroma of solid ameloblastoma (59.42 ± 13.07) than in that of unicyclic ameloblastoma (39.09 ± 20.99), and this difference was statistically significant (P = .0484). Furthermore, the expression of

MMP-9 was higher in the tumor cells of SA compared with those of AC (see Figure 2).

The mAgNOR of ameloblastic carcinoma was 3.22 (±1.64), whereas that of SA was 1.74 (±0.16) and that of UA was 1.77 (±0.18). There was a statistically significant increase in the mAgNOR and pAgNOR>1 when AC was compared with SA and UA (Table II). The analysis of correlation between variables revealed a positive association among MMP-2 stromal staining and pAgNOR>1 (r = 0.364; P = .04). It means that increased MMP-2 expression in tumor

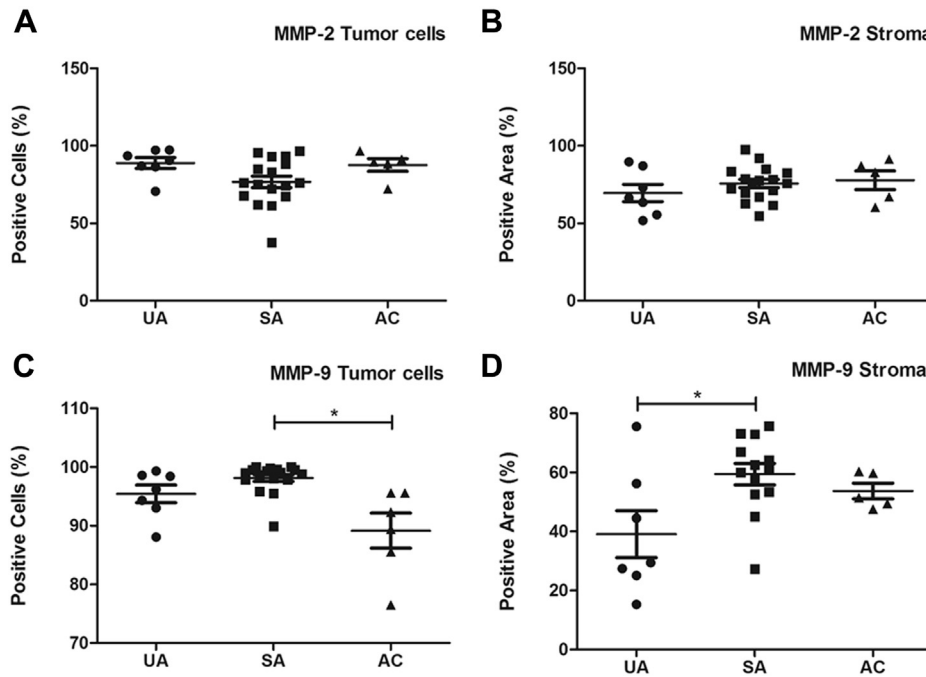


Fig. 2. Distribution of expression of matrix metalloproteinase 2 (MMP-2) (A and B) and MMP-9 (C and D) in tumor cells (A and C), and stroma (B and D).

Table II. AgNOR quantification (mAgNOR and pAgNOR) in the pathologic groups

	Unicystic ameloblastoma mean (SD)	Solid ameloblastoma mean (SD)	Ameloblastic carcinoma mean (SD)	P value
mAgNOR	1.55 (0.18)*	1.47 (0.16)*	3.22 (SD = 1.64)*	<.01
pAgNOR>1	40.86 (12.39)*	38.33 (11.28)*	68.40 (SD = 18.73)*	<.01

AgNOR, argyrophilic nucleolar organizer region; mAgNOR, mean argyrophilic nucleolar organizer region; pAgNOR>1, percentage of cells with more than one AgNOR per nucleus; SD, standard deviation.

*Statistically significant.

stroma was correlated with increased proliferative activity.

DISCUSSION

Odontogenic neoplasms with ameloblastic features present diverse clinical features from cyst-like lesions to very aggressive life-threatening tumors. It is important to understand the biologic mechanisms responsible for the clinical aspect to improve the therapeutic approach for these tumors. The behavior of neoplasms is determined partly by proliferation and extracellular matrix (ECM) degradation. This study evaluates, for the first time, a spectrum of ameloblastic tumors with very different clinical features, analyzing the expression of MMPs and proliferation to better comprehend the distinct biologic behavior of these lesions.

Comparing the malignant with benign forms of ameloblastoma, the combination of increased cell proliferation with an ability to degrade ECM appears to be the most significant factor to justify the aggressive behavior of

the ameloblastic carcinoma. When the benign forms of ameloblastoma are compared, a similar proliferation activity is observed; however, an increase in MMPs expression in the stroma of the solid ameloblastoma compared with unicystic luminal ameloblastoma was detected, which may explain why both solid and unicystic ameloblastomas are slow growing, although the solid ameloblastoma has greater infiltrative capacity. To better compare our results with previous reports, we have performed a systematic search of papers on this subject. The database PubMed was searched using the terms “MMP-2” and “ameloblastoma”; “MMP-9” and “ameloblastoma”; “MMP-2” and “ameloblastic carcinoma”; and “MMP-9” and “ameloblastic carcinoma,” and 21, 23, two, and one articles were obtained, respectively. From the 47 references obtained, 10 were duplicated. Next, items that were not from the English language literature (4 Chinese and 2 Russian) or articles that did not compare similar clinical groups (34) were excluded (UA vs SA vs CA; UA vs SA; UA vs CA; SA vs CA). A total of three articles that compared clinical types of ameloblastoma to MMP-2

and MMP-9 expression were included ([Supplemental Table I](#)). A literature review was also performed using the terms “AgNOR” and “ameloblastoma”, or “AgNOR” and “ameloblastic carcinoma,” and 11 and two articles were obtained, respectively. Two articles were duplicated. Next, reports that were not from the English language literature (1 Chinese) or articles that did not compare similar groups (6) were excluded. A total of four articles that compared clinical types of ameloblastoma to AgNOR quantification were included (see [Supplemental Table I](#)).

AC is a tumor with aggressive behavior, which, according to our results, may be associated with a higher proliferative rate compared with other benign ameloblastic tumors. This difference between malignant and benign odontogenic tumors are in accordance with Prasanna et al.,²² who concluded that proliferative markers, such as AgNORs, observed in these tumors could explain the variations in their growth pattern. In a comparison of the benign forms of ameloblastoma, we did not observe a statistically significant difference in proliferation by AgNOR quantification. Both solid and unicystic ameloblastomas presented similar mAgNORs, substantially lower than in ameloblastic carcinoma. This low proliferative rate correlates to the slow growth of benign ameloblastomas.²³ However, the finding of similar mAgNORs in SA and UA is in contrast to previous reports of a statistically higher mAgNOR in SA.²⁴⁻²⁶ A closer comparison of studies revealed differences in AgNOR quantification criteria, as well in sample size (see [Supplemental Table I](#)). One important factor that can explain the different results with regard to cells counted, is that both basal and parabasal cells were considered in our study, whereas Colemann et al.²⁴ quantified only basal cells, Ananthaneni et al.²⁶ did not specify which cells were counted (basal or parabasal), and Seifi et al.²⁵ used different criteria for quantification, considering also AgNORs outside the nucleus, which may explain why they observed much higher values than the other studies. The important information that was missing in all the cited articles was the type of unicystic ameloblastoma that was assessed. We selected only confirmed luminal unicystic ameloblastomas because these do not present infiltrative growth in contrast to the mural variant.

With regard to the expression of MMPs, all ameloblastic lesions studied presented positive expression of MMP-2 and MMP-9. The expression of MMP-9 was different among the ameloblastic lesions. To the best of our knowledge, this is the first article to compare MMP-9 in SA and UA and show a higher expression of MMP-9 in both the tumor cells and tumor stroma of SA in comparison with UA. This observation, along with similar mAgNORs, suggests that both are slow-growing tumors but with different ability to secrete

MMP-9 and consequently degrade the ECM. When comparing benign tumors versus malignant tumors, we observed a statistically significant increase in the expression of MMP-9 in the tumor cells of SA compared with those of AC and no difference in the MMP-9 expression in the stroma of these lesions. Our results are in contrast to those of Yoon et al.,¹⁵ who observed no differences in tumor cells and stronger expression in the stroma of AC. When comparing both studies, we identified differences in sample size and quantification. In this study, 1000 cells were analyzed, whereas Yoon et al. used a semiquantitative score analysis.

MMP-2 was expressed in both the tumor cells and stroma of all the tumors in this study. A higher expression was observed in AC compared with SA, although this difference was not statistically significant. Similar results were detected by Yoon et al.¹⁵ and Zhang et al.²⁷ When comparing benign ameloblastomas, we observed higher MMP-2 tumor stroma expression in SA but higher tumor cells expression in UA, again not statistically significant. The only other study that compared MMP-2 in solid and unicystic ameloblastomas, evaluated gene expression by using real-time polymerase chain reaction and showed lower expression in unicystic ameloblastoma; however, it is not clear if tumor cells and tumor stroma were assessed together or separately.²⁸

A correlation between MMP-2 tumor stroma expression and increased AgNORs per nucleus was observed. This suggests that MMP-2 activity could be releasing mitogenic factors present in the ECM. Pinheiro et al.²⁹ observed the expression of active forms of MMP-1, -2, and -9 in solid ameloblastomas and suggested that these enzymes could release mitogenic factors present in bone matrix, thus increasing cell proliferation and contributing to the local invasiveness of the tumor.

An important issue must, however, be considered: Although many studies have associated the MMPs with degradation of ECM in neoplasia, the role of these proteins is very complex. MMPs can also act in many pathways that involve cell migration, proliferation, angiogenesis induction, tissue remodeling, and inflammation. Therefore, their activities could also be important at other stages of tumor development when MMP-dependent signaling is more relevant biologically than ECM degradation.³⁰ Thus, one MMP can have opposing effects in different tumor types, and thus the use of MMPs has to be carefully considered and evaluated for each specific kind of neoplasia.³¹

CONCLUSIONS

Our results suggest that the cell proliferation activity is related to the greater aggressiveness of ameloblastic carcinoma in comparison with ameloblastoma. A high immunohistochemical expression of MMP-2 and

MMP-9 was observed in all the lesions studied. This result demonstrates the importance of these enzymes in the process of invasion and infiltrative growth of ameloblastic lesions. Therefore, inhibition of these molecules is a potential therapeutic approach for the treatment of ameloblastic tumors.

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Supplemental Table I. List of articles comparing MMPs expression and AgNOR quantification in the ameloblastic neoplasia spectrum

<i>Author (year), source</i>	<i>Markers</i>	<i>Methods</i>	<i>Sample</i>	<i>Main results</i>
Zhang et al. (2009), China	MMP-2	FFPE/IHC/ RT-PCR	7 UA 62 SA 6 AC	MMP-2 Immunoeexpression Ameloblastic carcinoma 100%/ameloblastoma (solid + unicystic) 84.06%, $P < .01$ MMP-2 mRNA expression (mean \pm SD) Ameloblastoma (1.425 \pm 0.174), primary ameloblastoma (1.577 \pm 0.249), recurrent ameloblastoma (1.561 \pm 0.208), $P = .959$
Zhang et al. (2010), China	MMP-2	RT-PCR	24 UA 18 SA	MMP-2 mRNA expression (mean \pm SD) Unicystic ameloblastoma (mean = 0.64; SD = 0.05)/solid/multicystic ameloblastoma (mean = 0.66; SD = 0.02), $P < .05$
Yoon et al. (2011), South Korea	MMP-2 MMP-9	FFPE/ IHC	10 SA 7 AC	MMP-2 expression <i>Tumoral cells:</i> Weak reaction in 70% of ameloblastoma/strong reactivity in tumor cells in AC, $P = .001$ <i>Stromal cells:</i> Weak to moderate expression without significant difference between the two tumors MMP-9 expression <i>Tumoral cells:</i> Moderate to strong expression without significant difference between the two tumors <i>Stromal cells:</i> Weak to moderate expression in ameloblastoma/stronger expression in AC, $P = .13$
Colemann et al. (1996), South Africa	AgNOR	- FFPE	15 UA 15 SA	mAgNOR (mean \pm SD) Unicystic ameloblastoma (mean = 1.68; SD = 0.10)/solid ameloblastoma (mean = 2.09; SD = 0.10), $P < .05$
Seifi et al. (2011), Iran	AgNOR	FFPE	15 UA 15 SA	mAgNOR (mean \pm SD) Unicystic ameloblastoma (Mean = 6.1; SD = 2.56)/multicystic ameloblastoma (mean = 7.4; SD = 2.72), $P = .001$
Ananthaneni et al. (2014), India	AgNOR	FFPE	7 UA 7 SA	mAgNOR (mean \pm SD) Multicystic ameloblastoma (mean = 1.97; SD = 0.41)/unicystic ameloblastoma (mean = 1.45; SD = 0.42) $P = .0021$ Few clusters of AgNOR in multicystic ameloblastoma and irregular clusters in unicystic ameloblastoma
Prasanna et al. (2014), India	AgNOR	FFPE	10 SA 2 AC	mAgNOR (mean \pm SD) - Solid ameloblastoma (mean = 2.14; SD = 0.262) Ameloblastic carcinoma (mean = 3.03)

AC, ameloblastic carcinoma; AgNOR, argyrophilic nucleolar organizer region; FFPE, formalin-fixed, paraffin-embedded; IHC, immunohistochemistry; mAgNOR, mean argyrophilic nucleolar organizer region; MMP, matrix metalloproteinase; RT-PCR, real-time polymerase chain reaction; SA, solid ameloblastoma; SD, standard deviation; UA, unicystic ameloblastoma.