Cell proliferation and apoptosis immunolocalisation suggest an association between aggressiveness and the preferred location of mural unicystic ameloblastomas: A multicentric Study

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Research Article

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Abstract

Objectives

This study aimed to assess whether the different biological behaviour between subtypes of unicystic ameloblastoma (UA) are related to the expression of proteins that modulate cell proliferation and apoptosis.

Materials and methods

Immunohistochemical study with a sample of 32 cases of UA, 11 cases of conventional ameloblastoma (CAM) and 10 dental follicles (DF) cases was performed. Cell proliferation was assessed using Ki-67 status and apoptosis by expression of Caspase-3. Positive cells were quantified in each sample and the difference among groups was compared.

Results

Mural UA (MUA) showed a higher immunostaining of Ki-67 (p < 0.05) and a lower immunostaining of Caspase-3 (p < 0.05) compared to luminal and intraluminal subtypes of UA (LIUA) and CAM. For both proteins, the LIUA and CAM groups showed no statistical difference. The neoplastic cells of the cystic capsule of the MUA showed a higher expression of Ki-67 protein (p < 0.0001) and a lower expression of Caspase-3 (p < 0.0001) compared to the lumen. DF showed lower Ki-67 and Caspase-3 immunostaining (p < 0.05) than neoplasms, except when comparing Caspase-3 expression between DF and MUA, as there was no statistical difference.

Conclusions

The higher immunoexpression of Ki-67 and lower of Caspase-3 in MUA, in the parenchyma cells inside the cystic capsule, suggests an association between the biological behaviour and the location of neoplastic cells in the tumour.

Clinical Relevance

Uncovering the mechanisms related to the difference in the biological behaviour of each UA subtype can contribute to directing more effective treatments.

Background

Ameloblastoma is the most prevalent benign odontogenic tumour [1], which has an ectodermal origin and is characterised by the proliferation of the odontogenic epithelium, without ectomesenchyme. In addition,
in line with the guidelines of the World Health Organisation (WHO, 2022) [2], although it presents with a slow growth pattern, it is considered an aggressive tumour, due to its capacity for local tissue invasion and destruction. Among the variations of this tumour, CAM is the most common, followed by UA [3].

During the clinical and radiographic examination, the UA presents as a cyst, however, at the histological examination, a typical ameloblastic epithelium is observed [3] and the proliferation site in the cystic cavity denominates three other histopathological subtypes. These include the luminal unicystic ameloblastoma (LUA), in which the tumour is confined to the luminal surface of the cyst and the lesion has a fibrous cystic wall, with a lining consisting entirely or partially of the ameloblastic epithelium; intraluminal unicystic ameloblastoma (IUA), which has one or more nodules projecting from the cystic lining towards the lumen; and mural unicystic ameloblastoma, which demonstrates greater invasiveness by infiltrating the fibrous wall of the cyst, and therefore, exhibits a more aggressive behaviour compared to the other subtypes [4]. In the 2017 classification [5], the possibility of changing the labelling of the category of MUA subtype from unicystic ameloblastoma to conventional ameloblastoma was raised, based on the need for aggressive surgical treatment for both lesions. In the 2022 classification [2], the MUA was kept under the UAs. Interestingly, CAM and UA mutations of BRAFp.V600E were found in several cases. However, only CAM showed additional mutations [6], which potentially explains CAM’s clinical behaviour, but not MUA’s.

There are significant differences in the biological behaviour between these variants, which implies the choice of either conservative or aggressive treatments, such as surgical resection of the lesion with safety margins, as well as the prognosis of the lesions. The variations in their biological behaviour may be attributable to the expression of proteins related to tumour invasion, infiltration and destruction of healthy tissue. Furthermore, these proteins are found to adhere to and destroy the extracellular matrix [7]. Among the various mechanisms of the typical biological behaviour of tumours with cystic areas are cell proliferation and apoptosis. There is a report that in CAM there are more apoptosis-inhibiting proteins than apoptosis-promoting proteins [8].

The process of cell proliferation impacts tissue homeostasis and also several biological and pathological events, such as the development of tumours. The identification of cell proliferation markers can be an effective diagnostic tool to understand and predict the biological behaviour of several lesions. A marker of cell proliferation that stands out in studies is the Ki-67 protein, whose expression was observed in several phases of the cell cycle and disappears after mitosis [9].

In contrast to cell proliferation, apoptosis occurs by the activation of members of the family of cysteine aspartic proteases (caspases), such as Caspase-3, which in its active form is considered a marker of apoptosis, due to the effector function in this biological phenomenon [10]. It is believed that the formation of ameloblastomas may be the result of an imbalance between cell proliferation and cell death, but little is known about this etiopathogenesis of their formation [10].

In previous studies [11, 12], we hypothesised that neoplastic epithelial cells of the CAM that are close to the bone would present a higher rate of proliferation and be stimulated by growth factors released by
bone resorption to synthesise and secrete metalloproteinases. Therefore, in this sense, this study aims to evaluate the immunoexpression rates of Ki-67 and Caspase-3 proteins in UA subtypes, in CAM and DF, and also to verify the differences in the immunostaining of these proteins between the cells that are located in the lumen and cystic capsule regions of the MUA since the neoplastic epithelial cells of the MUA are closer to the bone being reabsorbed by the neoplasm. Therefore, there is an urgent need to gain a deeper insight into the mechanisms related to the difference in the biological behaviour of each subtype, which is essential to direct appropriate treatment approaches for each UA subtype.

**Material And Methods**

*Study design and ethical approval*

This study was carried out as per the criteria established by the Ethics Committee in Research with Human Beings of the Institute of Health Sciences of the Federal University of Pará – ICS/UFPA and approved under protocol number 4,570,860, following the Declaration of Helsinki [13]. Immunohistochemical reactions were performed on 53 human-derived samples: 32 UA samples, 11 primary CAM samples and 10 DF samples.

The samples and clinical data of the patients were collected from the archives of the Centre for the Diagnosis of Mouth Diseases (CCDB) of the Faculty of Dentistry of the Federal University of Pelotas, the São Leopoldo Mandic Research Centre and Institute, and the Laboratory of Pathological and Immune Anatomy -histochemistry at the Faculty of Dentistry of the Federal University of Pará. All samples were diagnosed based on imaging analysis and trans-surgical exams, combined with the histological analysis of the entire lesion to rule out an invasion of tumour epithelium into the cystic capsule, in the cases of UA.

*Sample*

The total sample was divided into four groups, according to the WHO classification (2022) for head and neck tumours: the MUA group with eighteen samples of the mural subtype, the LIUA group with eight samples of the luminal subtype and six of the intraluminal. The CAM group consisted of 11 samples microscopically diagnosed as conventional ameloblastoma, which served as a positive control, owing to the established protein expression in this tumour [14–16]. Additionally, the DF group was included, consisting of 10 samples of dental follicles which are normal dental tissue that does not characterise cystic or neoplastic alterations [2]. Following the same diagnostic pattern, all cases of UA and CAM were diagnosed based on the clinical, radiographic, surgical and anatomopathological aspects.

*Histopathological data*

For immunohistochemical evaluation, the loaded histological slides were deparaffinised and washed with xylene dehydrating ethanol solution. Subsequently, the samples were immersed in 3% hydrogen peroxide and methanol (1:1) to block endogenous peroxidase activity. Antigen retrieval was performed using citrate buffer (pH 6.0) in a Pascal pressure chamber (Dako Cytomation, Carpinteria, CA, USA) for 30s
at 125ºC. After treatment with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline solution for 1 h, the sections were incubated for 1 h in a humid chamber at room temperature with the primary antibodies, i.e., Monoclonal antibody Rabbit Anti-Human Ki-67, Clone SP6 (Spring Bioscience, Pleasanton, CA, USA), diluted 1:25, overnight and Activated Rabbit Anti Caspase-3 Polyclonal Antibody, CPP32 (Diagnostic BioSystem, Pleasanton, CA, USA), diluted at 1:600, for 1 hour and incubated separately. Slides were incubated and treated at room temperature with a dextran polymer-based complex (Reveal; Spring Bioscience, Pleasanton, CA), and diaminobenzidine (DAB) was used as a chromogenic agent (Liquid DAB + Substrate, Spring Bioscience®). Finally, slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich) and were mounted with mounting medium (Permount, Fisher Scientific, Fair Lawn, NJ, USA). CAM samples were used as a positive control and a negative control was performed with the omission of the primary antibody, replaced by non-immune serum.

From each sample/quantitation area, a total of 5 images were acquired randomly by a microscope (AxioScope, Carl Zeiss, Oberkochen, Germany) equipped with a colour camera (AxioCam HRC, Carl Zeis) at 400x magnification. The DAB-immunomarked nuclei in the tumour parenchyma were counted using a specific plugin of imaging software (Cell Counter, ImageJ, NIMH, Bethesda, MD, USA). The tumour parenchyma cell count was performed to verify the percentage of nuclear immunostaining (labelling index) according to Siqueira et al. (2010) [17]. In the case of MUA, both the cells that invaded the cystic capsule and the cells present in the lumen were counted for comparison between these areas.

**Data analysis**

The data obtained were analysed by the GraphPadPrism 8 software (GraphPad Software Inc., San Diego, CA, USA). A non-parametric distribution was evidenced by the Shapiro-Wilk test, thus, the differences between the groups were evaluated by the Kruskal-Wallis ANOVA test with Dunn’s post-test. The difference between the MUA capsule and lumen areas was evaluated using the Mann-Whitney test. The 95% confidence interval was assumed ($\alpha = 0.05$).

**Results**

The distribution of samples of the different types of ameloblastoma into groups was performed after the collection of clinical and anatomopathological data, which can be seen in Table 1.
Table 1
Clinical and demographic characteristics of the ameloblastoma samples: histological subtype, sex, age and anatomical region of the lesion.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unicystic type</th>
<th>Conventional type</th>
<th>Dental follicle</th>
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<tr>
<td></td>
<td>Luminal</td>
<td>Intraluminal</td>
<td>Mural</td>
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<tr>
<td>Sex (n = 53)</td>
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<tr>
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<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
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<td>NR*</td>
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<td>Age (n = 53)</td>
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<td>10 – 19</td>
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<td>20 – 29</td>
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<td>30 – 39</td>
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<td>80 – 89</td>
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All samples from CAM, MUA, LIUA and DF groups showed expression of Ki-67 and Caspase-3 proteins in neoplastic parenchyma cells.

MUA had a relatively higher Ki-67 immunostaining rate (p < 0.05) and a lower rate of Caspase-3 (p < 0.05) compared to LIUA and CAM. For both proteins, the LIUA and CAM groups showed no statistical
difference. The neoplastic cells of the cystic capsule of the MUA showed a higher percentage of expression of Ki-67 protein ($p < 0.0001$) and a lower percentage of Caspase-3 ($p < 0.0001$) compared to the lumen. DF showed lower rates of Ki-67 and Caspase-3 immunostaining ($p < 0.05$) than neoplasms, except when comparing Caspase-3 expression between DF and MUA, as there was no statistical difference.

The immunohistochemical staining of the proteins studied was located in the cords and islands of the odontogenic tumour epithelium. Ki-67 labelling was predominantly nuclear. The histological images and the representative graph of the comparisons for the Ki-67 protein between the UA, CAM and DF groups are shown in Figure 1, the result of the comparison between the location (capsule/lumen) of the neoplastic cells of the MUA can be visualised in figure 2.

Caspase-3 labelling was verified in the cell nucleus and was found to be diffused in the cytoplasm of tumour parenchyma cells. The histological images and the analysis of the results of the comparisons for the Caspase-3 protein between the UA, CAM and DF groups are shown in Fig. 3, and the comparison of the immunostaining rate of this protein, between the regions of neoplastic cells present in the capsule and lumen of the MUA, can be verified by Fig. 4.

Discussion

The clinical data of the studied UA samples displayed a higher prevalence of the mural subtype, with most patients aged 25 years or less and mostly females. The most prevalent site was in the mandible region. In the CAM samples, patients were predominantly over 25 years of age and the predominant location was the mandible.

Ledesma-Montes et al. and Reichart et al. [17, 18] observed a higher prevalence of UAs in young patients, with a mean age in the third decade of life, a higher incidence in the posterior region of the mandible, and most of them were classified as the mural variant. Eom et al. [19] found that CAM is more frequent in patients who are in their third to seventh decade of life, and the mandible is the most affected location. The clinical data of the present study corroborate the findings in the literature.

In the immunohistochemical evaluation of the expression of Ki-67 and Caspase-3 proteins, in the neoplastic parenchyma of the UAs and CAMs variants, the labelling of the proteins was studied in all samples.
When evaluating the predominance of nuclear labelling of the Ki-67 protein, it was found that the mural subtype showed higher immunoexpression of this protein than the other subtypes and CAM. In contrast, the Caspase-3 protein was less expressed in the parenchyma of the MUA, when compared to the other subtypes, showing no difference when compared to the CAM.

Ahlem et al. [16] suggested that the Ki-67 immunostaining index in cells is related to proliferation, to local invasion, and that it constitutes a prognostic factor. In the research by Carreón-Burciaga et al. [20], in which they used immunohistochemistry, the authors report that there is no difference in the rates of nuclear staining of Ki-67 in UA when compared to those of CAM, as well as Bologna-Molina et al. [21]. However, Carreón-Burciaga et al. did not perform a comparative study on Ki-67 immunoexpression between UA subtypes. Bologna-Molina et al, however, when comparing unicystic variants, greater expression of proteins was found in the luminal subtype. They justify this finding with the possibility of a relationship between the fact that in this subtype, there are fewer stellate reticulum-like cells, and consequently, most of the cells counted corresponded to those from the basal or suprabasal layers, which are more likely to be positive.

Sah et al. [4] also analysed the expression of Ki-67 in UA subtypes and have shown higher values in the mural and intraluminal variants, than luminal. In the present study, a higher nuclear expression of Ki-67 was observed in the MUA when compared to the LIUA group and the CAM. The higher expression of this protein typically implies a greater potential for cell proliferation, which would explain the fact that MUA has a greater tendency to local invasiveness when compared to other UA variants. Furthermore, in our research, it was observed that MUA has a higher immunoexpression of Ki-67 than CAM. Perhaps this can be explained by the fact that the neoplastic epithelial cells in the UA that invade the capsule are closer than the surrounding bone, while in the CAM the neoplastic epithelial cells would be both close and far from the bone, when they are located in more central areas. However, these cells are equidistant from the surrounding bone matrix so they would not receive as much stimulus resulting from bone resorption. Additionally, some studies have shown that in tumour microenvironments with hypoxia, there is an increase in apoptosis and the formation of cystic areas in the central portions of the CAM [10, 22, 23].

The area with the most expressive markings in MUA was the neoplastic cells of the cystic capsule. Since the parenchymal cells of the MUA are closer to the bone surrounding the cyst, these results can be justified by studies that state that both neoplastic and stromal cells secrete metalloproteinases, which degrade the bone matrix and cause an additional release of growth factors. These mitogens, in turn, are released randomly and are likely to increase the rate of cell proliferation of ameloblastoma parenchyma cells [11, 12] (Fig. 5).

The DF samples showed lower Ki-67 immunostaining rates when compared to the other groups. It is understood that the fact that DF epithelial nests do not show a neoplastic growth pattern led to this result.

In this study, the immunoexpression rate of the pro-apoptotic protein, Caspase-3, in different types of ameloblastoma was evaluated. The statistical difference was observed when comparing the expression
of this protein in the mural subtype, with the lowest rates, to the other UA subtypes. The lower expression of Caspase-3 in MUA may result in longer survival of tumour parenchyma cells, which possibly influences their biological behaviour. From what could be observed, so far, this is an unprecedented finding in the literature. Sandra et al. [8], when studying tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) reported that ameloblastoma has an anti-apoptotic proliferation site in the periphery and a pro-apoptotic differentiation site in the centre of the parenchyma tumour.

These results can possibly explain the difference between Caspase-3 expression in MUA and CAM found in the present study. Probably, the lower expression in the parenchyma cells present in the cystic capsule of the MUA would contradict the higher expression in the centre of the CAM. In addition, tumour cells typically demonstrate high demand for oxygen during proliferation, and access to oxygen in the central area of the tumour becomes restricted, which leads to the creation of hypoxic microenvironments [10]. This process occurs in most solid tumours and can result in an increase in aggressiveness at the front of tumour invasion (tumour periphery) [24] or lead to tumour death by apoptosis in the centre of the tumour [22]. This validates the result of quantification in different regions of the MUA, in which the highest rates of immunostaining for the apoptosis-promoting protein were found in the cystic lumen region (tumour centre).

Some studies have also concluded that there are more apoptosis-inhibiting than activating proteins, suggesting that conventional ameloblastoma parenchyma cells may have a high survival activity, and thus their high recurrence rate [8, 22, 25, 26]. This can be attributable to a greater proliferation of CAM at the bone-neoplasm interface than at the centre of this tumour [22, 24].

By silencing an anti-apoptotic protein (Bcl-2) in an assay with ameloblastoma cells, Kim et al. [25], observed that there was inhibition of tumour formation in vivo and suggest that studying apoptosis modulators may favour the prediction of recurrences and the development of effective chemotherapeutic agents for the treatment of these lesions.

González-González et al. [26] reiterated that the behaviour of ameloblastoma is closely related to the potential of tumour cells to inhibit apoptosis and claim that it is also associated with the ability to initiate a proliferative phase of lesions.

Still, in this context, so far the literature has pointed out that growth factors such as FGF, KGF, EGF and TGF-β, among others, participate and are released during bone remodelling/resorption [27]. A major proportion of the literature points out that these molecules may have an anti-apoptotic effect [28–31]. Thus, possibly the growth factors would also act by inhibiting apoptosis on the invasion front of MUA parenchyma cells. In addition to growth factors acting against apoptosis, there are reports that mutations in the SMO and BRAF genes also participate in the FGF pathway, which is associated with this phenomenon [32]. However, UA does not have additional mutations to BRAFp.V600E [6]. In this sense, only the BRAFp.V600E mutation, in cases of UA, would not justify the clinical behaviour of MUA.
The low expression of Caspase-3 in DF, which is a non-neoplastic tissue, as well as in UA, indicates that in this tumour, anti-apoptotic events overlap with apoptotic ones. The high expression in LIUA and CAM can be explained by not being so influenced by growth factors and genetic mutations, related to the inhibition of apoptosis.

The mentioned studies indicate the probable role of anti-apoptotic modulators in the local invasiveness of ameloblastoma. There were no studies in the literature that verified the expression of Caspase-3 in UA and its subtypes.

Since the method used for the development of this study (IHC) is limited, the results need confirmation with studies that investigate the biological mechanisms involved with the findings. In addition, studies with more expressive numbers of UA should be carried out to confirm the results.

In 2022, Wright and Vered [2] updated the classification of head and neck tumours, based on the publication made by the WHO (2022). Based on the studies carried out by these authors, they recommend that ameloblastoma subtypes should be treated conservatively when they are intraluminal and luminal, but the mural subtype should be treated with the same approach used for CAM, as it is associated with recurrences when treated by conservative methods.

In summary, we can extrapolate that the lower Caspase-3 labelling and the higher Ki-67 expression observed in the cystic MUA capsule could justify the more aggressive biological behaviour of this histological subtype.

**Conclusions**

The immunoexpression rates of Ki-67 and Caspase-3 proteins can contribute to a better understanding of the biological behaviour of UA. MUAs exhibit high immunohistochemical expression of Ki-67 and low expression of Caspase-3 when compared to LIUA variants, and therefore explain the more aggressive biological behaviour of MUA.

**Declarations**

**Author Contribution**

Rebeca Vieira Costa, João de Jesus Viana Pinheiro and Ruy Gastaldoni Jaeger carried out the conception and design of the project;

Rebeca Vieira Costa, Karolyny Martins Balbinot, João de Jesus Viana Pinheiro performed data acquisition;

João de Jesus Viana Pinheiro, Ruy Gastaldoni Jaeger, Ana Carolina Uchoa Vasconcelos, Vanessa Morais Freitas and Sérgio de Melo Alves Júnior analysed the validation of the results;
João de Jesus Viana Pinheiro, Ruy Gastaldoni Jaeger Fabricio Passador Santos and Adriana Etges performed the analysis and interpretation of the data;

Rebeca Vieira Costa, Karolyny Martins Balbinot, Gabriela Cristina Avertano Rocha da Silveira and João de Jesus Viana Pinheiro performed the research;

João de Jesus Viana Pinheiro, Sérgio de Melo Alves Júnior, Maria Sueli da Silva Kataoka were responsible for the resources;

Rebeca Vieira Costa, João de Jesus Viana Pinheiro, Maria Sueli da Silva Kataoka and Adriana Etges performed the writing of the manuscript;

João de Jesus Viana Pinheiro, Maria Sueli da Silva Kataoka, Sérgio de Melo Alves Júnior, Fabricio Passador Santos and Silvio Augusto Fernandes de Menezes performed the critical review and editing.

João de Jesus Viana Pinheiro was responsible for overseeing and administering the project and procurement funding.

All authors read and accepted the published version of the manuscript.

**Ethics Approval and Consent to Participate**

This study was carried out as per the criteria established by the Ethics Committee in Research with Human Beings of the Institute of Health Sciences of the Federal University of Pará – ICS/UFPA.

The Ethics Committee in Research with Human Beings of the Institute of Health Sciences of the Federal University of Pará – ICS/UFPA waived the Consent to Participate form and approved this research under protocol number 4,570,860.

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No funding was obtained for this study.

**Conflict of Interests**

The authors declare that they have no conflict of interest.

**References**

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apoptosis and proliferation in ameloblastomas. Dis Markers 2015: 301781. DOI: 10.1155/2015/301781


Figures
Figure 1

Proliferating marker Ki-67 is higher on MUA (A) when compared to (B) LIUA, (C) CAM and (D) DF samples. The markings were predominantly nuclear in the neoplastic cell parenchyma. Scale: 20µm. (E) Comparison of the immunoexpression of Ki-67 between the samples of MUA, LIUA, CAM and DF, *** p < 0.0001, ** p < 0.001, * p < 0.05
Figure 2

Proliferating marker Ki-67 is higher on (A) Neoplastic MUA cells in the cystic capsule region when compared to (B) Neoplastic MUA cells in the lumen region. Scale: 50µm. (C) Comparison of the immunooexpression of Ki-67 between the capsule and lumen areas of MUA, *** p < 0.0001
Figure 3

Apoptosis marker Caspase-3 is lower on (A) MUA, when compared to (B) LIUA, (C) CAM except for (D) DF samples. The markings were weakly diffused into the cytoplasm in the parenchyma of neoplastic cells. Scale: 20µm. (E) Comparison of the immunoexpression of Caspase-3 between the samples of MUA, LIUA, CAM and DF, *** p < 0.0001, ** p < 0.001, * p < 0.05
Figure 4

Apoptosis marker Caspase-3 is lower on (A) Neoplastic AUM cells in the cystic capsule region when compared to (B) Neoplastic AUM cells in the lumen region. Scale: 20 µm (C) Comparison of the immunoexpression of Caspase-3 between the capsule and lumen areas of AUM, *** p < 0.0001.
Figure 5

Illustration summarising our hypothesis about the difference between the location of neoplastic cells in Mural Unicystic Ameloblastoma. Neoplastic AUM cells are present both in the lumen and in the cystic capsule, with those in the capsule being closer to the area of bone degradation than those in the lumen. The formation of the neoplasm causes local bone resorption through the secretion of proteolytic enzymes that solubilise the bone matrix and thus release growth factors. Therefore, cells that are closer to the bone resorption area will be influenced by the mitogens released at that location, whereas cells present in the lumen are more likely to undergo apoptosis since cells present in the central area of solid tumours suffer from the hypoxia microenvironments present in this region with little access to oxygen.

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