BMC Oral Health

RESEARCH

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Comparative evaluation of calretinin immunohistochemical expression in calcifying odontogenic cyst and ameloblastoma

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Abstract

Background Calretinin is a 29 kilodalton (KDa) calcium-binding protein that is expressed in normal and tumoral tissues. The expression of calretinin has been shown in the dental epithelium during odontogenesis and in different odontogenic cysts and tumors such as ameloblastoma. Since the epithelium of calcifying odontogenic cyst (COC) is similar to ameloblastoma and in both lesions, an arrangement of loose cells similar to stellate reticulum is seen, we aimed to investigate the comparative expression of calretinin in COC and ameloblastoma.

Methods In this observational-analytical study, 11 paraffin blocks of each lesion were examined. The immunohistochemical expression of calretinin and its severity and distribution were evaluated. Data were evaluated using SPSS version 26 and $P<0.05$ was considered the level of significance.

Results Out of 11 ameloblastoma samples, calretinin staining was positive in 6 samples in the stellate reticulum-like cells and also in the lining of microcysts and macrocysts in some cases. Ameloblast like cells did not show staining. In COC samples, epithelial cells were not stained, but in 8 samples, staining was seen in the area of ghost cells. There was no significant difference in staining between ameloblastoma and COC ($P=0.33$), but the difference in staining in the stellate reticulum of ameloblastoma and COC was significant. ($P=0.012$).

Conclusions it is concluded that calretinin does not play a role in the pathogenesis and histogenesis of COC epithelium, but since ghost cells are caused by a degenerative process, the possibility of involving calretinin as an apoptotic factor in the formation of these cells is supported.

Keywords Calbindin 2, Ameloblastoma, Odontogenic cyst, Calcifying

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Introduction

Calcifying odontogenic cysts (COC) were first discovered by Gorlin in 1962 and in most cases present as a cystic lesion lined by odontogenic epithelium including 4–10 cell layers [1]. Palisaded and hyperchromatic columnar ameloblast-like cells are present in basal layer. Beyond the basal layer, epithelial cells are loosely arranged, resembling the stellate reticulum of the enamel organ [2]. Ghost cells are characteristic, rounded or stacked flattened pale eosinophilic cells within the epithelium with distinct outlines and karyolysis resulting in a ghost like appearance. These are variable in shape and number and often are calcified. Sometimes ghost cells are present in the cyst wall eliciting foreign body reaction and inducing dentin like matrix [3].

Ameloblastoma is a benign, slow-growing, locally invasive tumor and may arise from rests of dental lamina, a developing enamel organ, the epithelial lining of an odontogenic cyst, or from the basal cells of the oral mucosa [1, 3, 4]. It can be intraosseous solid multicystic or conventional, unicystic and peripheral. All subtypes of solid tumor commonly have columnar ameloblast like cells with reverse polarity surrounding epithelial islands, and the cells in the central region have loose attachments mimicking the enamel stellate reticulum [1, 2].

Calretinin is a 29 kDa calcium-binding protein acting as an intermediate for calcium intracellular signaling and as an important secondary messenger involved in multiple cellular functions such as proliferation and differentiation [5]. This protein also shares roles as a calcium buffer or its receptor, as well as an apoptosis regulator [6, 7]. Calretinin is also expressed in developing tooth buds during odontogenesis at several stages [8, 9]. Although several studies have suggested this protein as a specific marker for ameloblastoma [10–12]; some others do not confirm this issue and there is a controversy in this field [13, 14]. Since epithelial lining in COC is similar to unicystic ameloblastoma to some extent except the ghost cells; the aim of this study was to investigate the expression of calretinin in COC in comparison with ameloblastoma, and the possible role of calretinin in the pathogenesis and tumorigenicity of this type of cyst.

Materials and methods

In this observational-analytical study, 11 intra osseous conventional ameloblastoma samples and 11 samples of intra osseous COCs were analyzed. Six of the 11 ameloblastoma samples were obtained from the archives of the Dentistry Faculty of Babol University of Medical Sciences. The remaining five ameloblastoma samples and all COC samples were from the archives of the Dentistry Faculty of Tehran University of Medical Sciences. Patients' files were reviewed to extract demographic and clinical data, including age, sex, and the location of the lesion. Tissue sections were prepared from paraffin blocks with 4-µm thickness and stained with hematoxylin and eosin. The slides were examined by an oral and maxillofacial pathologist to confirm the diagnosis. Histological criteria to confirm the diagnosis of COC and conventional ameloblastoma were according to the WHO classification of head and neck tumors [3].

Tissue sections with 4-µm thickness were prepared on a charged slide and kept at 60 ℃ for one hour. The tissue sections were then deparaffinized and rehydrated. Antigen retrieval was performed using a citrate buffer. After the slides cooled, they were washed with the phosphate buffer saline (PBS). Endogenous tissue peroxides were neutralized by incubating the tissue sections with H2O2 3% for five minutes, followed by rewashing them with PBS for five minutes. The tissue sections were then incubated with the primary antibody (Rabbit anti-Calretinin monoclonal antibody, clone BSR235) for one hour. The tissue sections were again rinsed with PBS buffer for five minutes, and then two drops of the detection solution No. 1 were poured on the tissue slides, and incubation was continued for 15 min. Next, the tissue sections were washed with PBS buffer and then incubated with the polymer HRP solution (Master Diagnostica) for 30 min, followed by another round of PBS washing. One drop of the DAB solution (Master Diagnostica) with 1 mL of dilution buffer was subsequently added to the tissue sections for five minutes. The slides were finally rinsed with distilled water, and hematoxylin was poured on the slides for background staining. The slides were washed with distilled water, and after dehydration, they were placed in xylene, covered by a coverslip, and finally visualized under a light microscope. As the positive control for calretinin antibodies, the human cerebral cortex was used as recommended by the manufacturer (Master Diagnostica). For the negative control, primary specific calretinin antibodies were replaced with non-immune serum.

The distribution and intensity of immunohistochemical staining were measured in the samples. In COC samples, scoring was based on the number of stained ghost cells. Immunostaining was nuclear and cytoplasmic. Regarding the distribution of positive cells, the samples were divided into two groups: Focal (stained cells in each slide were < 50%) and Diffuse (equal or more than 50% of cells in each slide were stained) [14]. Staining intensity was graded between 0 and 3 (0 = no staining, 1 = mild staining (i.e., light brown cells under light microscopy), 2 = moderate (i.e., brown cells), and 3 = severe (i.e., dark brown)) [15].

Clinical and demographic data, including age, sex, and the location of the lesion, were extracted from patients' files.

Data analysis was conducted in SPSS software version 26 under Windows using descriptive statistics, as well as

Table 1 The intensity of calretinin immunohistochemical staining in each group

Group	Non-stained *N* %	Mild *N* %	Moderate *N* %	Severe *N* %	*P*- value
Ameloblastoma	5 45.5%	1 9.1%	1 9.1%	4 36.4%	0/330 = p*
Calcifying Odontogenic Cyst	3 27.3%	0 0%	8 72.7%	0 0%	
Total	8 36.4%	1 4.5%	9 40.9%	4 18.2%	

*fisher's exact test

the chi-square test, Fisher's exact test, and Mann-Whitney U test.

Results

The mean age of patients was 40.4 years in the ameloblastoma group and 31.7 years in the COC group. Most ameloblastoma samples were obtained from mandible and most COCs were located in maxilla.

Table 1 shows the staining intensity of the samples in each group. Among 11 ameloblastoma samples, five samples (45.5%) were negative for calretinin, and the other six samples (54.5%) were positive (one sample with mild positivity, one with moderate, and four with severe positivity). Regarding the site of staining, stellate reticulum-like cells and, in some cases, the lining between spaces of macro-cysts and micro-cysts were reported to be positive, but ameloblastoma-like cells showed negative reaction (Fig. 1). Among 11 samples of COCs, three samples (27.3%) showed negative staining, and eight samples showed positive staining (72.7%), all of which showed moderate intensity. Positive reaction in COC samples was limited to ghost cells, and epithelial cells in COCs including basal layer (ameloblast-like cells) and stellate-reticulum-like layer remained unstained in all cases (Fig. 2).

There was no significant difference in the intensity of staining between different groups ($P = 0.944$, Mann-Whitney U test), including between COC and ameloblastoma samples ($P = 0.330$, Fisher's exact test).

There was a significant difference in the number of samples showing positive staining in the stellate reticulum between ameloblastoma and COC samples (Table 2). Among six ameloblastoma samples showing positive calretinin staining, two samples showed focal and four samples showed diffuse pattern, respectively, while all eight COC samples showing positivity for calretinin staining revealed a diffuse pattern among ghost cells. The difference in staining pattern (dispersion) was not statistically different between the two groups (Table 3) ($P = 0.161$, Mann-Whitney U test).

In COC samples, epithelial cells showed completely negative staining for calretinin, while in ameloblastoma samples, negative staining was observed in ameloblast-like cells surrounding islands. In all samples with positive staining in ameloblastoma, positivity for calretinin was related to stellate reticulum-like cells and, in some cases, the cells lining the cystic space. Also, moderate to severe

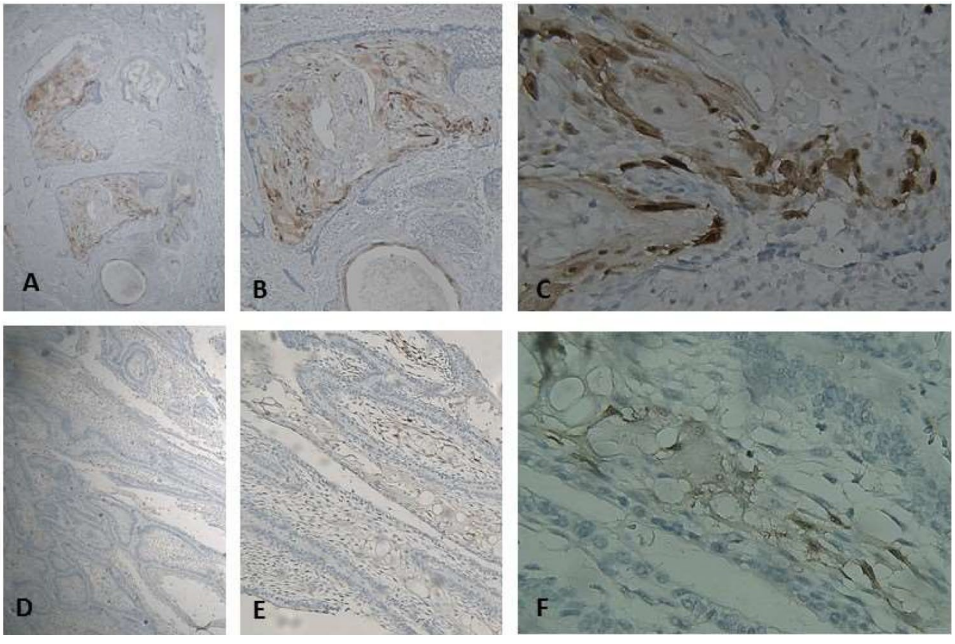

Fig. 1 Diffuse immunohistochemical staining in the center of the tumoral islands and lining of microcyst in Ameloblastoma at ×40 magnification (**A**), ×100 magnification (**B**), ×400 magnification (**C**), Diffuse immunohistochemical staining with moderate intensity in the stellate reticulum-like epithelium of Ameloblastoma at ×40 magnification (**D**), ×100 magnification (**E**), ×400 magnification (**F**)

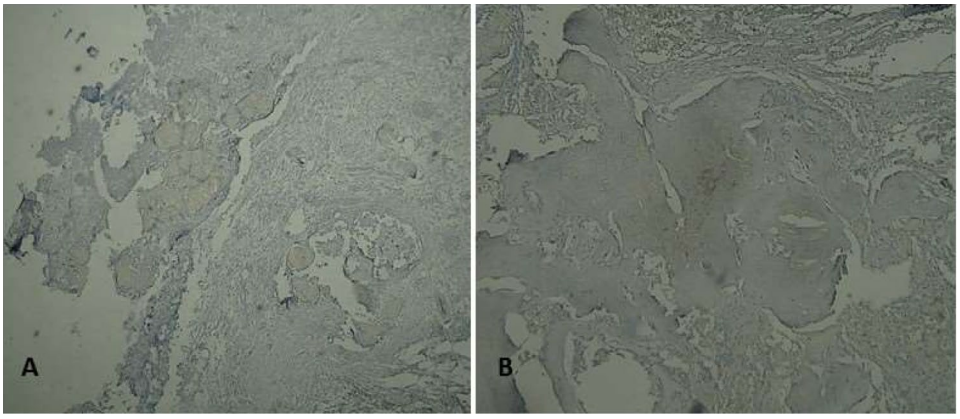

Fig. 2 Moderate immunohistochemical staining with diffuse dispersion in the areas of ghost cells of a Calcifying Odontogenic Cyst with ×40 magnification (**A**), Moderate immunohistochemical staining with diffuse dispersion in the ghost cells of a Calcifying Odontogenic Cyst at ×100 magnification (**B**)

Table 2 The number of positive and negative samples for the staining of the Stellate Reticulum

	Negative ***N*** **%**	**Positive** ***N*** **%**	***P*-value**
Ameloblastoma	5 45.5%	6 54.5%	P*=0/012
Calcifying Odontogenic Cyst	11 100%	0 0%	

*fisher's exact test

Table 3 Staining pattern

	Dispersion			
	Negative **N** **%**	**Focal** **N** **%**	**Diffuse** **N** **%**	**p-value**
Ameloblastoma	5 45.5%	2 18.2%	4 36.4%	0/161 = p*
Calcifying Odontogenic Cyst	3 27.3%	0 0%	8 72.8%	
Total	8 36.4%	2 9.1%	12 54.5%	

*Mann-Whitney U test

staining for calretinin was observed in isolated cells in the connective tissue and extravascular hemorrhage areas.

Discussion

In this study, 72.7% of COC samples showed moderate calretinin staining in ghost cells, and 54.5% of ameloblastoma samples had mild to severe staining in stellate reticulum-like cells similar to Pabbaraju's study in 2024 [12] and, in some cases, in the lining of macrocyst and microcyst spaces. In most previous studies, the rate of positivity for calretinin in ameloblastoma samples has been reported above 80% and, sometimes, up to 100% [10, 12–19], in contrast to our findings which almost half of ameloblastoma samples showed a positive reaction to this protein. Because previous studies showed a higher percentage of staining in ameloblastoma, negative samples were retested in another lab to minimize the possibility of laboratory error. For the third time, the experiment was repeated on one of the ameloblastoma samples using a different staining kit, and the results were the same as the first time. These results rejected the possibility of a false negative answer and confirmed the reliability of tests.

In order to intensity and distribution of staining, Altini et al. (2000) reported that more than 80% of uni-cystic ameloblastoma samples showed a positive reaction to calretinin, and the rate of positivity for solid ameloblastoma samples was 93.5%. These values were considerably higher than the rate observed in our study. But regarding the location of stained cells, as in other recent studies, stellate reticulum cells showed the most positive reaction in ameloblastoma [10].

In a study by Devilliers et al. (2008), all solid and unicystic ameloblastoma samples showed strong positivity for calretinin in the ameloblastic epithelium, but this staining was restricted to stellate reticulum-like cells, and only weak positivity was seen in basal layer cells in a few samples. However, scattered positivity was reported in the stroma, confirming the presence of mast cells in the connective tissue. Our results agreed with that of Devilliers et al. regarding the fact that only stellate reticulum-like cells in ameloblastoma samples showed positivity for calretinin [20].

In the study of Alaedini et al. in 2008, all ameloblastoma samples showed intense and diffuse calretinin expression in stellate reticulum-like cells, while marginal ameloblast-like cells showed no staining in none of the samples, showing a pattern similar to that observed in the present study. In a recent study, calretinin expression was also reported in the cells lining the cystic space [18]. Sundaragiri et al. (2010) reported that all ameloblastoma specimens showed strong positivity for calretinin, which was limited to stellate reticulum-like cells. In our study, we also observed positive calretinin staining for these cells; however, the number of positive samples

and the intensity of positivity were lower in our experiment [17]. In another study by D'silva et al. in (2014) all ameloblastoma samples showed strong positivity for calretinin in basal and granular squamous cells, as well as stellate reticulum-like cells. On the other hand, stellate reticulum-like areas showed positivity in about half of the samples with variable intensity from moderate to strong, while other cells revealed negative staining [13]. Also, in the study of Anandani et al. (2014), all four specimens of solid ameloblastoma were weakly positive for calretinin in the stellate reticulum region, and only in one sample a few basal cells were stained. Out of these four solid ameloblastoma samples, one case showed a diffuse staining pattern; however, half of 16 cases of uni-cystic ameloblastoma revealed focal staining only in the stellate reticulum-like region, and staining was intense in one case only [19]. The results of our study somehow agreed with those of the recent report. Koneru et al. (2014) observed that 90% of ameloblastoma samples expressed calretinin, and the staining pattern was diffuse in 78%, showing consistency with our study in terms of the staining pattern; however, the ratio of positive samples was lower in our study. Moreover, in the recent study, no positive reaction was observed in ameloblast-like marginal cells, and positive reaction was reported only in stellate reticulum-like cells, squamous metaplasia areas, and the lining of the cystic space, which was in line with our study regarding the negative reaction of ameloblast-like cells [14]. In another study by Imran et al. in 2016, all ameloblastoma samples showed positive calretinin staining and half of the samples revealed weak positivity [15]. In our study, on the other hand, more than half of the cases showed no reactivity for calretinin. Rudraraju et al. (2019) studied the expression of calretinin in various types of odontogenic cysts and tumors and observed that all uni-cystic ameloblastoma samples had a positive reaction to calretinin in columnar cells and basal cells, as well as in stellate-like reticulum cells. Staining was intense and diffuse in around 70% of the samples. Also, all solid ameloblastoma samples showed a positive reaction only in stellate reticulum-like cells, which was in agreement with our observation. In addition, in the areas of macrocyst and microcyst formation, the cells lining the cyst also showed positive staining for calretinin [16]. Cyst formation in ameloblastoma results from stromal degeneration [1], and consistently, we observed positive staining in ghost cells in COC samples, suggesting that calretinin may be possibly involved in the degeneration process. In the study of Varshney et al. (2020), all solid ameloblastoma and 85% of uni-cystic ameloblastoma samples were shown to express calretinin, with higher intensity and extent of staining in solid ameloblastoma; however, there was no mentioning of the type of positive cells. In solid ameloblastoma, 70% of the sample revealed a diffuse pattern, and 30% had focal dispersion, which was nearly similar to the pattern observed in our samples. Focal staining was noticed in two out of six positive samples in our study, and the rest of the samples showed a diffused pattern. Regarding the intensity of staining, unlike our study, none of the samples in the report of Varshney et al. were strong (grade 3), and most of them showed intermediate staining (grade 2) [5]. In the present study, 4 out of 6 positive samples had grade 3 intensity. In our literature review, we encountered no study investigating calretinin expression in COC samples. Regarding the fact that the cells in the upper epithelial layer in these types of cysts resemble stellate reticulum-like cells, and ghost cells are seen in the interspaced region, it can be assumed that the cells of this epithelial layer in COC samples can show positivity for this protein similar to solid and uni-cystic ameloblastoma. Nevertheless, these cells remained unreactive in all of our COC samples, but ghost cells showed diffused positivity in 72% of the samples. Since ghost cells result from a degenerative process, this observation suggests that calretinin, as an anti-apoptotic factor [7], can possibly be involved in the formation of these cells. Although previous reports indicate that the cells in the upper epithelial layer of uni-cystic ameloblastomas show positivity for calretinin, and despite similarities between cystic cell layers in COC and uni-cystic ameloblastoma [1], we observed no positive reaction in the cells of the basal layer, ameloblast-like cells, and stellate-like reticulum in these samples, suggesting that the development of these cells follow distinct and independent pathways in COC and ameloblastoma. It has been suggested that there may be a link between normal cells and their neoplastic counterparts [21], warranting more studies on molecular and immunohistochemical markers, such as calretinin, in normal and neoplastic tissues to better understand the biological behavior, associated factors, and tumorigenesis of odontogenic lesions [18, 19]. As mentioned earlier, calretinin acts as an anti-apoptotic factor [6]. Consistently, Imran et al. argued that the cellular distribution of calretinin indicates that this protein can be an anti-apoptotic and proliferation mediator in the outer layer and a differentiation apoptotic marker in the inner layer (i.e., the stellate reticulum-like region) [15]. Accordingly, Koneru et al. suggested that the variability observed in calretinin expression in marginal and central cells might be due to the dual anti-apoptotic/pro-apoptotic role of this protein [14, 22]. Another study showed that in ameloblastoma, the expression of pro-apoptotic proteins is dominantly observed in squamous metaplasia foci and in central tumoral islands with cystic degeneration. This expressional pattern suggests the involvement of apoptosis in cystic changes in ameloblastoma tumoral islands [15]. Therefore, calretinin may play a role in cell cycle regulation and apoptosis depending

on the ameloblastoma stage and interaction with other mediators [14].

Conclusions

Calretinin seems to play no role in the pathogenesis and histogenesis of the epithelial layer in COC. However, regarding the expression of this protein in ghost cells in COC samples, and since ghost cells are formed in a degenerative process, calretinin, an apoptotic mediator, may contribute to the development of ghost cells. An important fact is the positive immunostaining in stellate reticulum-like cells in ameloblastomas versus the negative immunostaining in stellate reticulum-like cells in calcifying odontogenic cysts. In this context, the suprabasal strata in the luminal epithelial lining of unicystic ameloblastomas should present positive immunostaining. The expression of the calretinin protein could be useful in the differential histological diagnosis of unicystic ameloblastomas versus developmental odontogenic cysts (including calcifying odontogenic cyst).

Abbreviations

DAB	Diaminobenzidine
IHC	Immunohistochemistry
PBS	Phosphate-buffered saline
COC	Calcifying Odontogenic Cyst
KDa	Kilo Dalton

Acknowledgements

Hereby, the Deputy of Research and Technology of Babol University of Medical Sciences for supporting this research and colleagues of oral and maxillofacial pathology department of faculty of dentistry of Tehran and Babol universities of medical sciences are gratefully acknowledged.

Author contributions

Mahdizadeh M and Bijani F: project conception and design; Mahdavi N and Mahdizadeh M: data acquisition; Bijani A and Bijani F: analysis and validation of the results; Bijani A: data analysis and interpretation; Bijani F and Mahdavi N: conducting the survey; Mahdizadeh M: writing the original draft preparation, writing revision, and editing; Bijani F, Bijani A, and Mahdavi N: substantive review; All authors were involved in writing the manuscript and approved the submitted and published versions.

Funding

This research received no external funding.

Data availability

Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate

The provisions of the Declaration of Helsinki were adhered to, given that the study is retrospective and paraffin blocks of patients' tissues were used from laboratory archive, there was no access to individual patients for informed consent, but written consent in Persian had been obtained from all patients who delivered their tissues to university centers for diagnosis for future research. Considering these points, the code of ethics was obtained from the Health Research Institute of Babol University of Medical Sciences under the code IR.MUBABOL.HRI.REC.1401.077. https://ethics.research.ac.ir/form/448pyrcs6nob9oa.pdf.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 9 October 2024 / Accepted: 20 January 2025
Published online: 29 January 2025

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outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (Centre). Histopathology. 2001;39(1):93–98.

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