Original Article

Immunoexperssion of cancer stem cell marker (CD44) in ameloblastoma

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Abstract

Background: Ameloblastoma is the most frequently encountered benign, locally invasive tumor. Attempts to surgically resect the tumor often leave small islands of tumor, which later result in recurrence in 50%–90% of cases. This has raised questions regarding the tumor cell populations that are responsible for tumor growth and recurrence. In ameloblastoma, whether or not cancer stem-like cells are present remains undetermined. However, if cancer stem-like cells are present in ameloblastoma, it is important to identify which type of cell possesses the stem-like characteristics and is responsible for ameloblastoma progression and recurrence.

Aim: Our study aims at analyzing immunohistochemical staining to detect the expression of cancer stem cell (CSC) marker CD44 in relation to proliferative activity of tumor cells in histopathologically diagnosed cases of ameloblastoma variants and to derive a correlation between the CD44 expression and biologic behavior of the lesion.

Materials and Methods: A retrospective study, was conducted on total 25 cases ameloblastoma and were immunostained for CD44 expression. Results obtained were statistically analyzed.

Results: A positive correlation was observed between staining intensity of CD44 marker and the known biological behavior of the lesion. Intense staining reaction was found to be only in 8% cases, whereas 76% cases demonstrated moderate intensity and remaining 16% displayed mild immunoreactivity to CD44 marker. Staining location was more to be in stellate reticulum-like (SR-like) cells when compared to ameloblast-like (AB-like) cells. Intense immunostaining was localized in the small tumor follicles, especially in SR-like cells situated in close vicinity of peripheral AB-like cells whereas mild intensity of staining was observed in keratinizing areas.

Conclusion: CSCs marker positive expression in benign tumor like ameloblastoma may be responsible for its aggressiveness and recurrence. CD44 marker may be of great value in predicting the biological behavior and growth potential of ameloblastoma.

Keywords: Ameloblastoma, cancer stem cell, CD44, histological patterns, recurrence

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INTRODUCTION

Ameloblastoma is the most frequently encountered benign, locally invasive tumor.[1] It is second most common epithelial tumor of odontogenic origin.[2] According to WHO, ameloblastoma is classified into different types depending on the origin of tumourgenesis; solid/multicystic, extraosseous/peripheral, desmoplastic and unicystic. Based on their histological features, they are classified into follicular, plexiform, acanthomatous and granular types.[3]

Although many histological variants of ameloblastoma exists, but two cell types i.e., peripheral ameloblast-like (AB-like) cells and central stellate reticulum-like (SR-like) cells are present in all the variants.

Since decades there is an ongoing debate on to which variant of the tumor is considered more aggressive or which is most likely to recur. Moreover each type of ameloblastoma requires different forms of treatment as these tumor types differ in biological behavior and rate of recurrence.[1]

Recurrence may be attributed to improper surgical removal of tumor islands, which later results in 50%–90% of recurrent cases.[4] This raised a question regarding the nature of tumor cell population that are responsible for recurrence and tumor growth.

Recently, it has been hypothesized that functional heterogeneity of these tumors may account for the fact that not the entire tumor cells in solid tumor have similar ability to drive tumor formation. This observation led to the so-called "Cancer stem cell hypothesis." According to American Association for Cancer Research, "a cell within a tumor that possesses the capacity to self-renew and to cause heterogeneous lineages of cancer cells that comprise the tumor is known as cancer stem cell (CSC)."[4]

Within a given tumor, there exists a small population of cells with the capacity to behave like stem cells. Conventional treatment modalities target the bulk of tumor cells leaving CSCs unaffected, thus making eradication of tumor tissue difficult. One of the challenging problems is identifying CSCs, which may be an effective treatment modality.[5]

In past few years, several CD markers have been identified as solid CSC markers.[4] CD44 is family of cell surface glycoproteins that play a role in cell-cell and cell-extracellular matrix adhesion and interactions. Changes in CD44 expression are associated with tumor cell differentiation, progression and metastasis.[6]

The presence of CSCs like cells in ameloblastoma remains undetermined. However, if CSC-like cells are present in ameloblastoma, it is important to identify which type of cell possesses the CSC-like characteristics and is responsible for ameloblastoma progression and recurrence.

Therefore, in this study we analyzed the protein expression of most putative candidate stem cell marker, i.e., CD44 in two different cell types in histological variants of ameloblastoma. Study also compared the immunostaining expression among the different histological patterns of ameloblastoma.

MATERIALS AND METHODS

The retrospective study was conducted on paraffin-embedded blocks retrieved from the archived files of department of oral and maxillofacial pathology, Sri Sai College of dental surgery, Vikarabad. A total of 25 cases which were clinically and histopathologically diagnosed as ameloblastoma (n = 25; follicular variant = 9, plexiform variant = 5, unicystic variant = 6, acanthomatous variant = 3 and granular variant = 2) were stained for CD44 marker.

Immunohistochemistry

Tissue sections (3-μm) were cut and transferred on 3-amino-propyl-triethoxy silane coated slides for immunohistochemistry (IHC) staining. Briefly, sections were deparaffinized in series of xylene for 15 min and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in methanol for 8 min. Antigen retrieval was achieved by heat treatment using Tris-EDTA buffer solution (pH 9). The sections were incubated with Rabbit Monoclonal CD44 (Ready-to-use vial, PathnSitu Biotechnologies Pvt. Ltd.,) primary antibody for 45 min at room temperature followed by incubation with biotinylated secondary antibody for 15 min. Visualization of the IHC reaction was performed by developing the enzyme complex with DAB/H_2O_2 solution (PathnSitu Biotechnologies Pvt. Ltd.). Then, the sections were counterstained with hematoxylin and mounted.

Quality control for immunohistochemistry

Lymphocytes served as positive control for CD44 marker located in the stroma [Figure 1].

Interpretation

Assessment of CD44 positive cells was performed using a binocular light microscope at ×10 and ×40. Two observers (Observer A and B) performed the evaluation of distribution, staining intensity and percentage positive cells by selecting most reactive areas on the respective slide at ×10 and then counting at ×40 magnification.

Evaluation of immunohistochemical staining

The most representative areas were selected for scoring the immunostaining pattern. The criteria used to define CD44 positive cells were brown staining of the cells in the areas of peripheral AB-like cells and SR-like cells. The presence or absence of immunoexpression for CD44 was also considered. It was done as follows:

Each field was analyzed and classified according to its histological type and was scored as described below [Table 1]. Then each slide was again scored for 2 distinct cell types present in that particular variant [Table 2]:

i. AB-like cells
ii. SR-like cells.

Particular cell type was focused under lower magnifications to analyze the pattern of distribution.

Qualitative assessment was done by evaluating the staining intensity which was classified into four groups: 0 = no staining, 1 = mild staining, 2 = moderate and 3 = intense staining.

Quantitative analysis included the extent of staining and was classified as follows: 0 = no staining, 1 = 1%–10% positive cells, 2 = 11%–50% positive cells and 3 = >50% positive cells.

A final immunoreactive score was arrived at by adding both the above indices. Final score given was 0 = negative, 1–2 = mild, 3–4 = moderate and >4 = intense.

Evaluation of percentage positive cells

Similarly, in each slide, the most representative areas were focused under ×40 magnifications, and cell counting was done for each cell types. The percentage of positive cells were then calculated and graded. The slide was moved in single direction (right to left) to avoid repetition of already examined fields.

Five areas were randomly selected in each slide for particular cell type (AB-like and SR-like), and labeling index was calculated as follows:

Number of positive cells divided by total number of cells counted, multiplied by 100.

Statistical analysis

Statistical analysis was done using SPSS 20.0 (Chicago, IL, USA). Differences in the mean scores of AB-like cells and SR-like cells between various types of ameloblastoma was done using paired samples *t*-test followed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Of the 25 ameloblastoma cases, retrieved from the archives during the year 2010–2016, mandible was the most common jaw involved (88%). Ameloblastoma occurred in the age range of 13–62 years with mean age of 31.32 ± 10.3. Ameloblastoma showed almost equal distribution in males and females with male to female ratio of 1:1.27 [Table 3].

Table 1: CD44 staining intensity in variants of ameloblastoma

Ameloblastoma variants	Staining intensity			
	Intense	Moderate	Mild	Total
Follicular	1	8	0	9
Plexiform	1	4	0	5
Unicystic	0	5	1	6
Acanthomatous	0	0	3	3
Granular	0	2	0	2

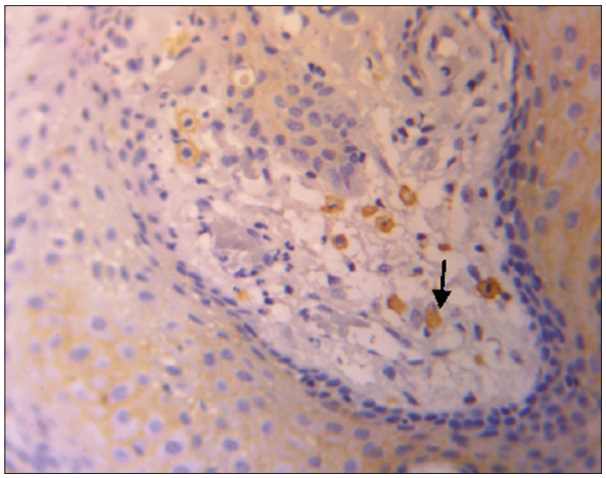


Figure 1: Photomicrograph showing positively stained CD44 cells in lymphocytes in the stroma which served as the control (IHC, ×40)

Table 2: CD44 staining intensity in two distinct cell types in variants of ameloblastoma

Ameloblastoma variants	Staining location (AB-like cells)			*P*	Staining location (SR-like cells)			*P*
	Intense	Moderate	Mild		Intense	Moderate	Mild	
Follicular (*n*=9)	0	2	7	0.005*	1	5	3	0.841
Plexiform (*n*=5)	0	2	3		1	2	2	
Unicystic (*n*=6)	0	0	6		0	3	3	
Acanthomatous (*n*=3)	0	0	3		0	3	0	
Granular (*n*=2)	0	2	0		0	1	1	

SR: Stellate reticulum, AB: Ameloblast (*P value >0.005) is considered stastically significant

Nearly, 36% of ameloblastoma cases were follicular type, 24% were unicystic type while plexiform, acanthomatous and granular formed 20%, 12% and 8%, respectively [Table 4]. All the cases demonstrated CD44 positively stained cells. Staining was cytoplasmic. Both peripheral and central cells stained positive with varying intensities.

The percentage of CD44 positive cases in each variant was almost 100% with intense staining reaction was found to be only in 8% cases [Figure 2], whereas 76% cases demonstrated moderate intensity [Figure 3] and remaining 16% displayed mild immunoreactivity [Figure 4 and Table 1].

The distribution pattern of CD44 positive cells was also observed in the stained sections in each variant and was found more to be in SR-like cells when compared to AB-like cells. Intense immunostaining was localized in the small tumor follicles, especially in SR-like cells situated in close vicinity of peripheral AB-like cells [Figure 2] whereas mild intensity of staining was observed in keratinizing areas of acanthomatous variant.

For AB-like cells, among the five variants statistically significant difference was found in staining intensity ($P = 0.005$) whereas for SR-like cells, there was no statistically significant difference observed among these variants ($P = 0.841$) [Table 2].

CD44 expression in AB-like cells was moderately positive in 6 cases (score 2) and remaining cases were mildly positive (score 1). No case displayed intense reaction [Table 2].

CD44 expression in SR-like cells was strongly positive in 2 cases (1 follicular variant and other was plexiform type), whereas 14 cases were moderately positive (score 2) and 9 cases scored 1 with mild staining [Table 2 and Graph 1].

The percentage value of AB-like and SR-like cells positive for CD44 staining was calculated out of total cells from each area. The final value of positive cells was considered for analysis. The mean values of percentage of positive cells from each group were statistically analyzed for comparison. The percentage of positivity value of SR-like cells was higher when compared to AB-like cells in all the variants.

Mean value of AB-like cells was found to be highest in granular variant (26.3 ± 1.8) followed by follicular (10.4 ± 8.7), plexiform variant (9.8 ± 5.4), acanthomatous (5.8 ± 1.11) and unicystic variant (4 ± 2.5).

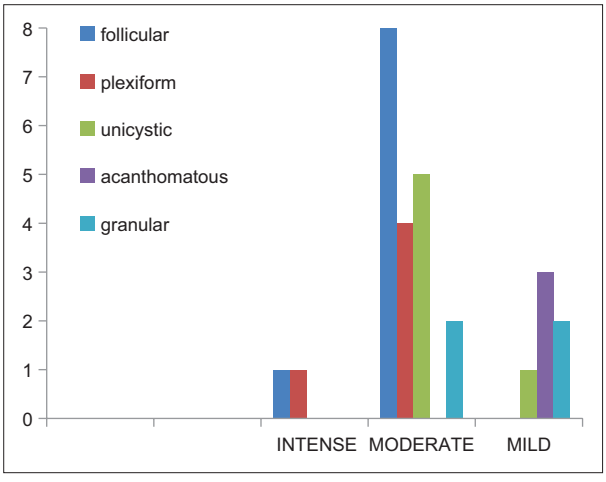
In SR-like cells, the mean value was highest for plexiform variant (24.5 ± 19.4) followed by follicular (21.2 ± 16.6), granular (20.3 ± 18.8), acanthomatous (17.2 ± 1.90) and unicystic variant (14.2 ± 10.7).

Difference between both the cell types was found to be statistically significant in follicular (0.03), unicystic (0.04) and acanthomatous variant (0.01) [Table 5].

DISCUSSION

Ameloblastoma as stated by Robinson, is usually unicentric, nonfunctional, intermittent in growth, anatomically benign and clinically persistent. The tumor is relatively uncommon and accounts for approximately 1% of all oral tumors. It occurs in all age groups but is most commonly diagnosed in the third and fourth decades. The tumor shows a marked predilection for the mandible with a preponderance that could be as high as 99.1%.[7] Even though patients undergo surgical excision of the tumor with free safety margins, the recurrence rate of ameloblastoma is very high.[8]

It has been hypothesized that tumors are likely to be initiated in normal stem cells or their immediate descendants and then are perpetuated by minority of these cells known as CSCs.[9] Treatment should focus on elimination of



Graph 1: CD44 staining intensity in SR-like cells of ameloblastoma variants

Table 3: Distribution according to location, gender and age

Group	Location		Gender		Male:female ratio	Age range (years)	Mean age±SD (years)
	Maxilla, *n* (%)	Mandible, *n* (%)	Male, *n* (%)	Female, *n* (%)			
Ameloblastoma	3 (12)	22 (88)	11 (44)	14 (56)	1:1.27	13-62	31.32±10.3

SD: Standard deviation

Table 4: Distribution of histological diagnosis among group

Histological diagnosis	Number of cases (%)
Follicular ameloblastoma	9 (36)
Plexiform ameloblastoma	5 (20)
Unicystic ameloblastoma	6 (24)
Acanthomatous ameloblastoma	3 (12)
Granular type ameloblastoma	2 (8)
Total	25 (100)

Table 5: Comparison of percentage of positive cells in two distinct cell types in variants of ameloblastoma

Variant	Mean±SD		*P*
	AB-like cells	SR-like cells	
Follicular	10.4±8.7	21.2±16.6	0.03*
Plexiform	9.8±5.4	24.5±19.4	0.1
Unicystic	4±2.5	14.2±10.7	0.04*
Acanthomatous	5.8±1.11	17.2±1.90	0.01*
Granular	26.3±1.8	20.3±18.8	0.758

*$P > 0.005$ is considered stastically significant. SR: Stellate reticulum, AB: Ameloblast, SD: Standard deviation

these CSCs for improving overall survival of the patient. Cancerous stem-like cells have been implicated as cancer initiating cells in range of malignant tumors. Xu *et al*. for the first time isolated these cancerous stem like-cells in pituitary adenomas and concluded that stem-like cells are also present in benign tumors.[10]

The present study was carried out to elucidate whether or not such cancerous stem-like cells are present in different histological variants of ameloblastoma to assess the aggressiveness with the help of most well-known CSC marker, i.e., CD44.

CD44 is one of the cell surface markers currently used to identify CSCs in various solid tumors.[4] This molecule functions as a principal receptor for hyaluronic acid, a major glycosaminoglycan of extra cellular matrix and is involved in adhesion, movement and activation of normal and transformed cells. CD44 is expressed widely in variety of cell types, including hematopoietic cells, macrophages, fibroblasts, epithelial cells, muscle cells and glial cells. Altered expression of CD44 molecule has been detected in various neoplasms and are associated with tumor growth, progression and metastasis.[11]

Even though many types of histological variants are seen in ameloblastoma, two distinct cell types are observed in all the subtypes; peripheral columnar cells or AB-like cells and central SR-like cells. Peripheral cells are situated at invasive front and no morphological variation can be observed whereas central SR-like cells show cellular differentiation and morphological change.

Literature revealed no study highlighting the expression of CD44 marker in both cell types (AB-like cells and SR-like cells)

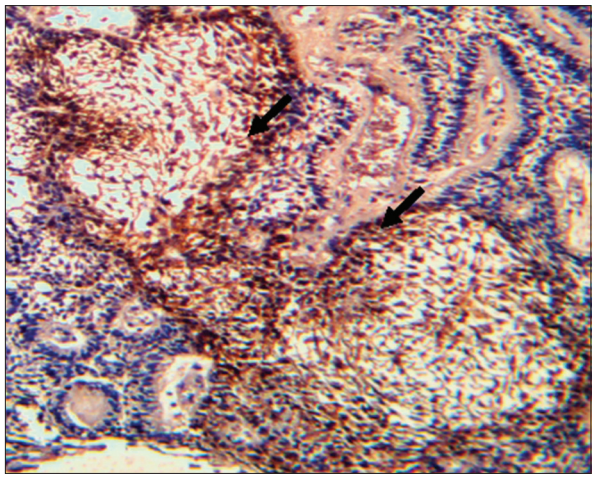


Figure 2: Photomicrograph showing intensely stained CD44-positive cells in stellate reticulum-like cells situated adjacent to peripheral ameloblast like cells of mixed variant of ameloblastoma (IHC, ×10)

of ameloblastoma in all its histological variants. The present study analyzed the immunoexpression of CD44 in ameloblastoma variants. Results demonstrated varying positive expression in all the cases suggesting presence of CSC-like cells which may be responsible for their proliferation and recurrence.

The present study comprised of 5 histological variants of ameloblastoma which were further classified according to cell types present in each of them (AB-like and SR-like cells). It was observed that, CD44 expression both quantitatively and qualitatively was more in SR-like cells when compared to AB-like cells (statistically significant in most cases).

Intense staining reaction was observed in central SR-like cells especially those cells which are situated adjacent to peripheral AB-like cells [Figure 2]. The finding was in accordance to Sathi *et al*. study. They analyzed CD44 expression along with Ki67, a proliferative marker expression. Ki67 was expressed in nuclei of peripheral AB-like cells and only a few positive cells were observed in nuclei of SR-like cells.[4]

Overall the findings suggest AB-like cells which have higher degree of differentiation and minimal expression for CD44 marker are less likely to have CSC-like cells. Thus, maintaining tumor cell proliferation.

The SR-like cells show intense CD44 marker expression and are devoid of Ki67 expression. Thus, these cells harbor the potential CSC like cells which may be responsible for tumor recurrence. In accordance with this hypothesis, these SR-like cells may change their morphology and differentiate into different types of cellular patterns, for example, granular, squamous and acanthomatous.

Study also demonstrates decreased CD44 immunoexpression for highly differentiated acanthomatous areas. The above finding was in accordance to Kumamoto *et al*.'s study, suggesting CD44 useful marker in detecting cellular differentiation in epithelial odontogenic tumors.[11]

Srinath *et al*.'s study suggested that these cell adhesion molecules are strongly expressed in active cells than in differentiated cells of odontogenic lesions and is therefore useful to predict the tumor progression.[6]

The interesting findings of the study results taking the histological variants of ameloblastoma into consideration are as follows:

i. Overall immnuoreactive scores for AB-like cells among the variants were found to be highest in granular variant (26.3 ± 1.8) suggesting it to be most aggressive or proliferative one [Figure 5]
ii. The scores of immnuoreactivity for SR-like cells were more in cases of follicular and plexiform type (24.5 ± 19.4) indicating them to be recurrent ones. Intense expression for CD44 was found in tumor follicles [Figure 6]
iii. Statistically significant difference in both the cell types was observed in follicular, unicystic and acanthomatous variants.

CONCLUSION

Understanding the biological function of the expression of CSC markers in ameloblastoma may aid in elucidating their role in tumor pathogenesis, and continued research may lead to the development of more effective therapeutic approaches. CD44 may be useful in detecting the active cells in ameloblastoma and to predict the tumor progression and recurrence rate.

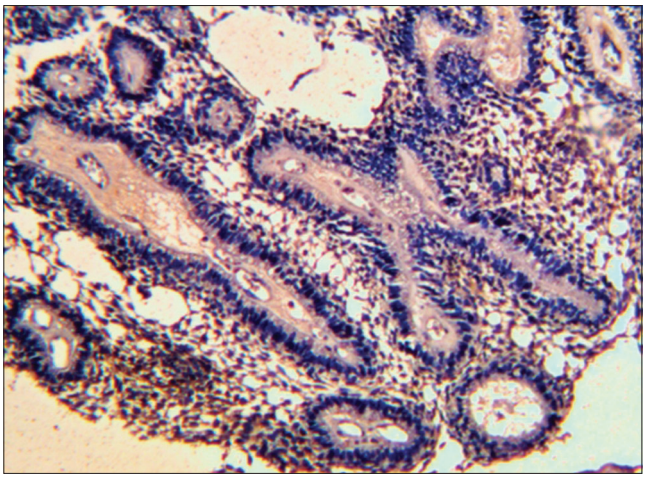


Figure 3: Photomicrograph showing moderately stained CD44-positive cells in stellate reticulum-like cells in plexiform variant of ameloblastoma (IHC, ×10)

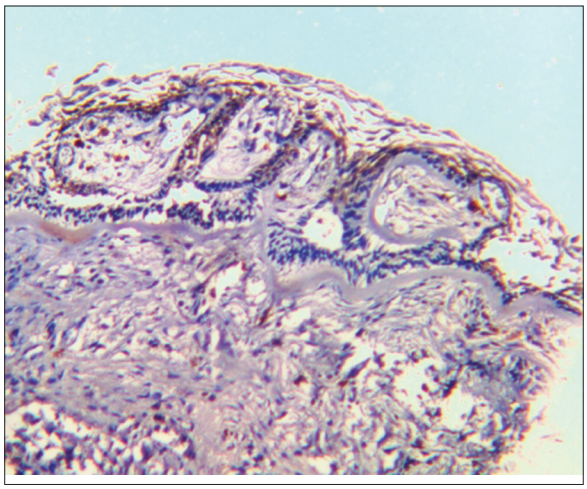


Figure 4: Photomicrograph showing mildly stained CD44-positive cells in stellate reticulum like cells in unicystic variant of ameloblastoma (IHC, ×10)

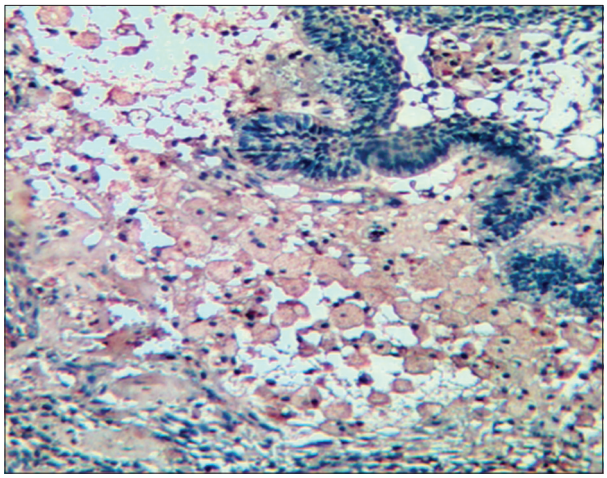


Figure 5: Photomicrograph showing moderately stained CD44-positive cells in stellate reticulum like cells of granular ameloblastoma (IHC, ×10)

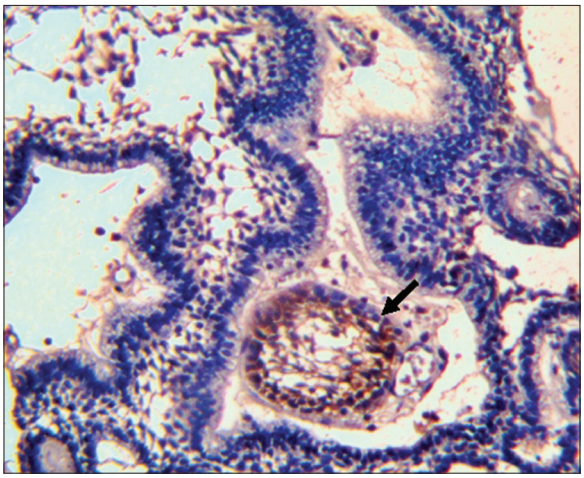


Figure 6: Photomicrograph showing intensely stained CD44-positive cells in stellate reticulum like cells of tumor follicle (IHC, ×10)

Routine IHC procedure should be performed in ameloblastoma cases to determine its recurrence and progression rate and keep the patient under follow-ups accordingly. Clinical surveys with large study cohorts will be needed to verify our findings.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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