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Immunohistochemical evaluation of myofibroblasts in odontogenic keratocyst, dentigerous cyst and different clinical variants of ameloblastoma: A comparative study

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ABSTRACT

Background: Odontogenic cysts and tumors exhibit varying degrees of aggressiveness in their biological behavior. Odontogenic keratocyst (OKC), dentigerous cyst (DC), ameloblastoma are most common odontogenic cysts and tumors to occur in the oral cavity. Myofibroblasts (MFs) in the connective tissue stroma participate in the matrix degradation process by secreting matrix metalloproteinase 2, transforming growth factor beta1 and may contribute to variation in their biological behavior. Its activity is identified by alpha-smooth muscle actin (α-SMA) marker. With this background, the present study aims to evaluate the frequency of MFs using α-SMA to determine the biological behavior of OKC, DC, and different clinical variants of ameloblastoma.

Materials and Methods: A retrospective study was carried out with total of 60 samples which include 10 cases each of OKC, DC, 30 cases of different clinical variants of ameloblastomas and 10 normal mucosa taken as controls. All are stained immunohistochemically using α-SMA and were analyzed for the same. Comparison between more than 2 groups done by one way analysis of variance test with the level of significance of $P \leq 0.0001$, i.e., <0.05.

Results: Statistically significant difference in the mean number of MFs observed between certain groups, with higher mean number in solid ameloblastoma (SA) (32.45) followed by OKC (28.79), unicystic ameloblastoma (24.53), desmoplastic ameloblastoma (7.44), and DC (1.72).

Conclusion: Higher frequency of MFs noticed in SA, OKC which are key cells for connective tissue remodeling by interacting with epithelial cells and other connective tissue cells to facilitate progression of cysts and tumors thereby contributing to their biological behavior.

Key Words: Actins, ameloblastoma, matrix metalloproteinase 2, myofibroblasts, odontogenic cysts, transforming growth factor beta1

INTRODUCTION

Oral cavity is a site for various pathologies among which odontogenic cysts and tumors are the most important entities.[1,2] Myofibroblasts (MFs) are stromal components in these cysts and tumors, which constantly interact with the tumor cells, cause matrix degradation, and create a favorable environment for their infiltrative growth.[3] These are difficult to identify in routine hematoxylin

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and eosin stained sections hence certain special stains and immunohistochemical markers used for locating MFs. One such marker is alpha-smooth muscle actin (α-SMA), an actin isoform which helps in identifying MFs in connective tissue stroma.[4] Mashhadiabbas *et al*., (2010) Roy *et al*., (2016), Syamala *et al*., (2016), Kouhsoltani *et al*., (2016), Annegowda *et al*. (2018) investigated the role of MFs for the biological behavior with comparison among cysts and tumors and no unified results have yet been obtained.[5] With this view, the present work directs to study the facts and then form an opinion about the frequency of MFs using α-SMA in order to know the behavior of odontogenic keratocyst (OKC), dentigerous cyst (DC) and an individual clinical variants of ameloblastoma. Sherlin *et al*., in 2013 studied few cases of desmoplastic ameloblastoma (DA).[6] We wish to include an equal number of clinical variants of ameloblastoma along with the cysts to clearly delineate biological variation between cysts and tumors.

MATERIALS AND METHODS

A retrospective study carried out with a sample size of 60 which includes 10 each of previously diagnosed cases of OKC, DC, solid ameloblastoma (SA), unicystic ameloblastoma (UA) and DA, 10 normal oral mucosa specimens that is the soft tissue overlying the impacted teeth taken as controls. The biopsy performed in patients who had undergone minor surgical procedures like extraction of impacted teeth devoid of inflammation. The source of sample was from hospital population and the sample collected from the dental records of Oral Pathology department retrospectively from 2009 to 2018. The number of sample population was minimal, as of which the sample size was 10 for each case. Ethical clearance taken for procedure carried out with ethical clearance number SVSIDS/OP/3/2016.

According to Masthan *et al*., 2015 DA is considered separately as it is characterized by an unusual histomorphology, including extensive stromal collagenization or desmoplasia, leading to the proposed term ameloblastoma with pronounced desmoplasia or DA. Radiographically it produces mixed radiolucent – radiopaque lesion with diffuse border that indicates that the tumor is more aggressive than other ameloblastic variants.[7]

Inclusion criteria

Previously diagnosed cases of different clinical variants of ameloblastoma, OKC and DC with no history of malignancy either orally or systemically included in the study group.

Exclusion criteria

Paraffin-embedded blocks of OKC, DC and different clinical variants of ameloblastoma associated with other odontogenic cysts, tumors and blocks with insufficient tissue were excluded from the study.

Staining procedure

It is an indirect method of immunohistochemical staining procedure which includes primary α-SMA mouse anti-human antibody and anti-mouse rabbit secondary antibody. 3 μm thick sections mounted on silane coated slides and dewaxing done in xylene. The slides rehydrated in graded solutions of ethanol, rinsed with distilled water, placed in 3% hydrogen peroxide for 10 min, and then rinsed with distilled water for 15 min. Antigen retrieval done by placing slides in citrate buffer solution (pH 6) in a microwave oven at 92°C for 10 min. After cooling to room temperature for 20 min, the slides exposed to primary α-SMA mouse anti-human antibody (DAKO, Glostrup, Denmark) dilution 1:100, for 60 min at room temperature and then rinsed with phosphate buffer saline (PBS) for 10 min. Sections were then incubated with a universal immune peroxidase polymer anti-mouse rabbit Histofine R kit (secondary antibody) for 30 min. The sections rinsed with PBS for 10 min and reacted with 3,3'-diaminobenzidine substrate-chromogen kit, rinsed with PBS for 2 min, counterstained in Harris hematoxylin and were then mounted using dibutyl phthalate xylene.[5]

Counting criteria

Brown colored end product present at the site of target antigen stains the cytoplasm of MF cells with immunohistochemical marker indicative of positive reactivity.[8] In each immunohistochemically stained section representative fields were randomly selected. Selection of random fields makes sure that every element gets equal chance for being part of the sample. Hot spot fields might be biased and makes difficult for every element for being part of the sample equally. MFs below the cystic epithelium in cysts and surrounding the tumor islands in ameloblastomas were counted.[9] From each tissue section 10 fields chosen and MFs (excluding those surrounding blood vessels) counted. Total number

of positive cells for all the 10 examined fields per case calculated and mean number derived for all the 10 cases in each lesion.[5] Total 3 observers (qualified Oral and Maxillofacial Pathologists) evaluated the slides. Intra class correlation coefficient for SA was 0.999; UA was 1.000; DA was 0.997; OKC was 0.999; DC was 0.914; NM was 0.844.[10] Counting done using Olympus compound microscope CH20i with a Labomed eyepiece ×10 magnification and objective ×40.[5]

Statistical analysis

Data analyzed by graph pad prism software version 6.0. Data summarized as mean ± standard deviation (SD) for continuous data. Comparison between more than two groups done by one way analysis of variance test and followed by Bonferroni's multiple comparison test for continuous normal data followed by Kruskal-Walli's test and Dunn's Multiple Comparison test for continuous non normal data. All the $P < 0.05$ considered as statistically significant.

RESULTS

All the samples subjected to immunohistochemical staining using α-SMA antibody for MFs detection. Comparison done based on the frequency of MFs among the groups presented in Table 1.

Table 2 presents intergroup comparison where n is number of cases, SD is Standard deviation.

On comparison between the groups SA (32.45) showed the highest mean number followed by OKC (28.79), UA (24.53), DA (7.44), DC (1.72) and control group (0.36) showed the least mean number. Statistically significant difference in the mean number of MFs seen between SA and DC, SA and control, UA and control, OKC and DC, OKC and control groups with a $P \leq 0.0001$. No statistical significant difference seen between SA and UA, SA and DA, SA and OKC, UA and DA, UA and OKC, UA and DC, DA and OKC, DA and DC, DA and control, DC and control. Out of 10 cases of DC, 7 cases (70%) showed α-SMA positivity.

Figures 1-6 depicts immunohistochemical expression of α-SMA positive MFs in SA, UA, DA, OKC, DC, normal mucosa respectively under ×10 magnification.

MFs counted immediately below cystic epithelial lining for OKC, DC, and area adjacent to tumor islands in different variants of ameloblastoma. Among the tumors SA and among the cysts OKC showed higher number of MFs indicating aggressive behavior of lesions. UA, DA ranked next indicating less aggressiveness compared to SA. DC ranked the least among all the lesions.

DISCUSSION

Odontogenic cysts and tumors with aggressive behavior cause a large amount of destruction in the orofacial region with complex pathological phenomena, different cell types i.e., epithelial and stromal cell interactions are critical in controlling the growth and clinical behavior of these lesions.[10]

MF is an important constituent of tumor stroma.[11] These are now recognized as main effectors of tumor needs, with regards to angiogenesis, MMPs production for collagen breakdown, further invasion and host immune response suppression. Local recurrence and overall survival are negatively influenced by stromal

Table 1: Mean number of alpha-smooth muscle actin positive myofibroblast cells among different groups (nos)

Case number	Group A (SA)	Group B (UA)	Group C (DA)	Group D (OKC)	Group E (DC)	Group F (normal mucosa)
1	73.1	119.2	0.7	61.8	3.1	0.5
2	65.1	22.8	41.3	50.5	1.6	0
3	15.7	3.1	4	34.2	2	0.9
4	43.6	3.2	4.9	32.3	0.7	0.2
5	9	0.2	2.1	1.9	1.7	0.2
6	68.8	0.1	0.4	31.6	3.4	0.5
7	24.5	25.5	3.7	31.5	0	0.9
8	3.1	33.7	12.5	17.1	0	0
9	15.9	9.5	0.8	24.3	4.7	0.2
10	5.7	28	4	2.7	0	0.2
Overall mean per group	32.45	24.53	7.44	28.79	1.72	0.36

SA: Solid ameloblastoma; UA: Unicystic ameloblastoma; DA: Desmoplastic ameloblastoma; OKC: Odontogenic keratocyst; DC: Dentigerous cyst; nos: numbers

Table 2: Intergroup comparison using alpha-smooth muscle actin for the evaluation of myofibroblast cells (nos)

Groups	n	Minimum	Maximum	Mean±SD	*P*
A	10	3.10	73.10	32.45±27.71	<0.0001
B	10	0.10	119.20	24.53±35.58	
C	10	0.40	41.30	7.44±12.40	
D	10	1.90	61.80	28.79±18.79	
E	10	0.00	4.70	1.72±1.62	
F	10	0.00	0.90	0.36±0.33	

Statistical tests include one-way analysis of variance test, Bonferroni's multiple comparison test, Kruskal-Walli's test and Dunn's multiple comparison test. *n*: Number of cases; SD: Standard deviation; nos: numbers

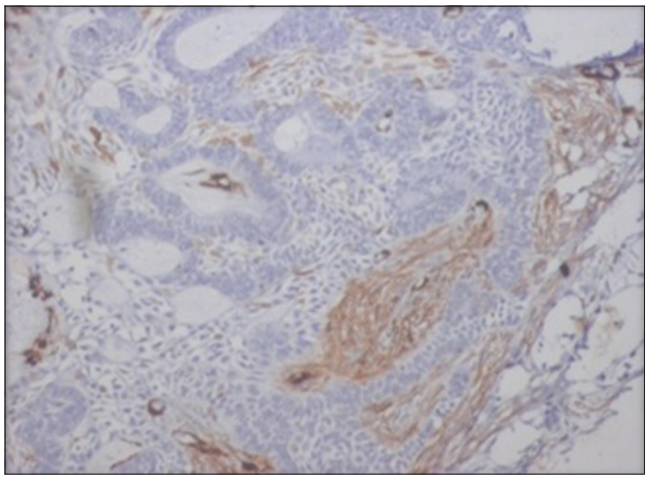

Figure 1: Immunohistochemical expression of α-SMA positive MFs in solid ameloblastoma under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

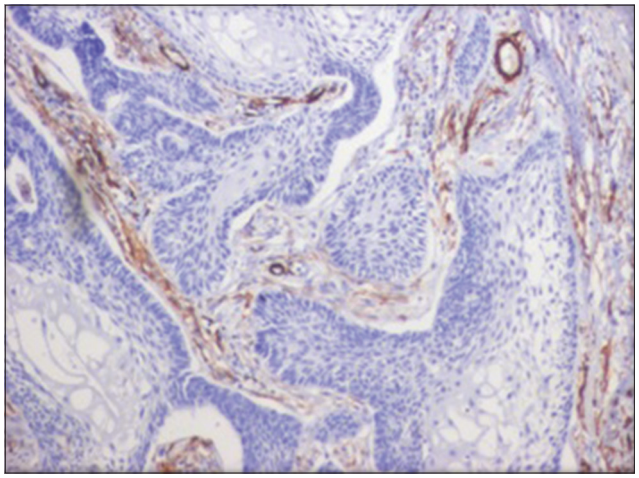

Figure 2: Immunohistochemical expression of α-SMA positive MFs in unicystic ameloblastoma under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

MFs.[5] α-SMA is a tumor marker and actin isoform predominantly seen within vascular smooth muscle cell with a role in fibrinogenesis along with fibroblast and MFs.[4]

The present study is an attempt to compare and evaluate the frequency of MFs in SA, UA, DA, OKC, DC and normal mucosa using α-SMA immunostaining to know the biological behavior of the lesions. A sample size of 60 cases of which 10 each of previously diagnosed cases of OKC, DC and 30 cases of different clinical variants of ameloblastoma such as SA, UA and DA taken as study group. Ten normal mucosa specimens taken as controls. Out of 10 cases of DC, 7 cases (70%) showed α-SMA positivity.

Statistically a significant difference for the mean number of MFs seen between SA and DC, SA and control, UA and control, OKC and DC, OKC and control group with a $P \leq 0.0001$.

Vered *et al*. assessed immunohistochemically the frequency of stromal MFs in different odontogenic cysts and tumors and found that among the cysts mean number of MFs in OKCs was significantly higher than in DC and among tumors SA showed highest mean number of MFs when compared to UA and ameloblastic fibroma. They suggested a positive link between number of MFs and aggressive biological behavior in odontogenic cysts and tumors.[12] The above study results are in accordance to present study where mean number of MFs is higher in SA followed by OKC, UA and DC.

Nadalin *et al*., Gabhane *et al*., analyzed the MFs presence in radicular cyst (RC), DC and OKC and observed higher mean number of α-SMA positive cells in OKC when compared to DC. They indicated that differentiation of MFs in the stroma produces collagen and synthesize enzymes such as MMP-2, which could be associated with tumor growth and progression.[3,13] These results are in accordance to the present study.

Shruthi *et al*., Santos *et al*., (2014, 2017) conducted an immunohistochemical study to assess the frequency of stromal MFs in OKC, ameloblastoma and correlated the same with the behavior of these lesions and observed a higher number of MFs in SA in comparison to OKC. They demonstrated that presence of higher frequency of MFs in the stroma is responsible for aggressive behavior of the odontogenic cysts and tumors.[14,15] These results are similar to the present study where higher frequency of MFs observed in SA when compared to OKC.

Kouhsoltani *et al*. performed a study to investigate MFs density in UA, SA, OKC, DC, RC, odontogenic myxoma (OM), adenomatoid odontogenic tumor and calcifying odontogenic cyst (COC) and correlated

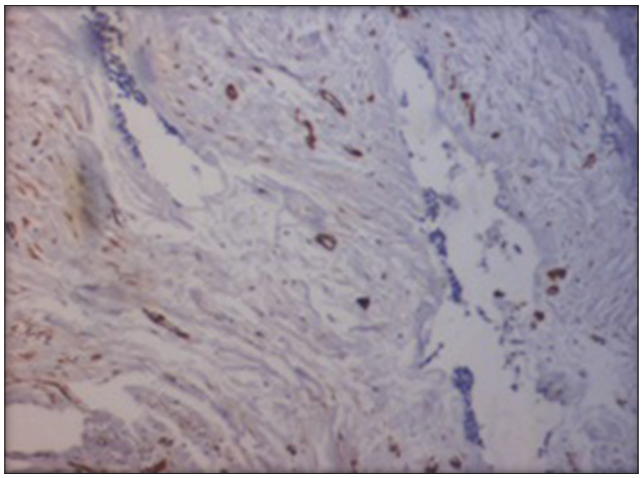

Figure 3: Immunohistochemical expression of α-SMA positive MFs in desmoplastic ameloblastoma under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

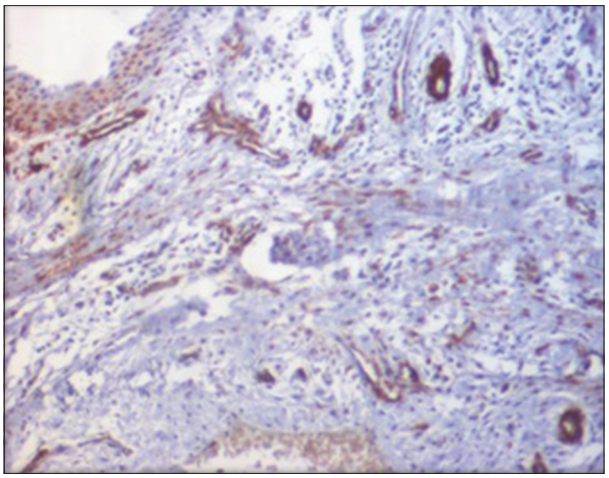

Figure 5: Immunohistochemical expression of α-SMA positive MFs in dentigerous cyst under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

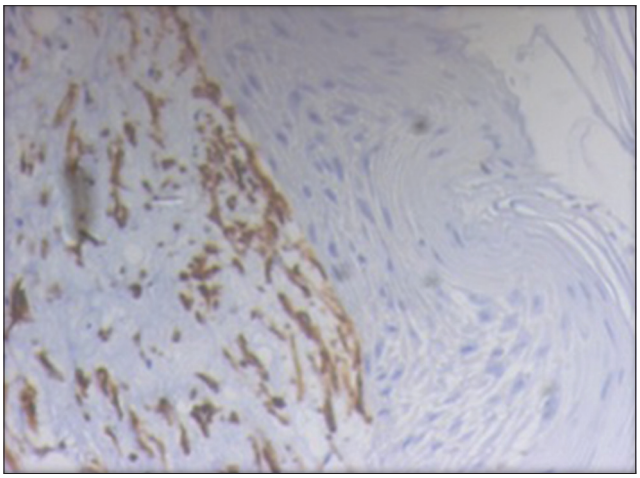

Figure 4: Immunohistochemical expression of α-SMA positive MFs in odontogenic keratocyst under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

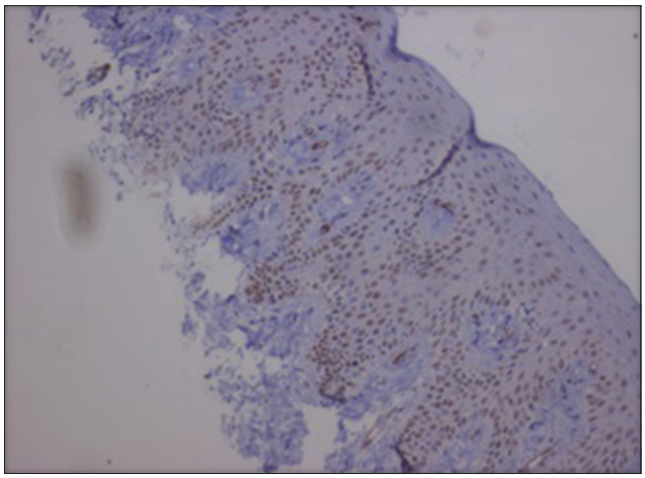

Figure 6: Immunohistochemical expression of α-SMA positive MFs in normal mucosa under ×10. α-SMA: Alpha-smooth muscle actin; MFs: Myofibroblasts.

the same with its clinical behavior. The number of MFs were significantly higher in odontogenic tumors compared to odontogenic cysts demonstrating the role of MFs in the aggressive behavior of these lesions.[10] The results are in accordance to the present study where higher mean number of stromal MFs observed in SA followed by OKC, UA, DA and DC.

Similar to the present study Roy *et al*., Syamala *et al*., Annegowda *et al*., observed higher number of MFs in ameloblastoma and OKC followed by DC. They arrived at a judgment that MFs may have a role in predicting the biological behavior and growth potential of these lesions.[5,16,17]

Mashhadiabbas *et al*., analyzed the distribution and proliferation of MFs in DC, OKC and ameloblastoma and observed that the mean number of MFs are more in OKC followed by DC and ameloblastoma and concluded that the higher frequency of stromal MF in the OKC implies that MFs can contribute to aggressive nature of this cyst but between odontogenic cysts and ameloblastoma, the stromal MFs presence has no correlation with invasiveness.[9] These findings are in contrary to the present study results where ameloblastoma showed a higher mean number of MFs followed by OKC, DC.

Smitha *et al*. performed an immunohistochemical study to detect the MFs presence in SA and UA and observed higher number of MFs in UA compared to SA. They suggested that the minimal stromal component in plexiform type contributed to the decrease in mean

number of MFs in SA.[11] The results of the study are in contrary to the present study where SA showed higher mean number of MFs compared to UA.

Several reasons may account for similarities and differences in results such as size of sample, minor methodological differences. Similar results are because the variables might be the same and as the cases are limited to 10 or so on, there is a chance that the results may be similar or little difference may arise. Results are contrary basing on the number of cases and the kind of research, study methods and way of approach is concerned. α-SMA positive MFs emergence in the supportive connective tissue helps in predicting the possible biological behavior and growth potential of these lesions. α-SMA positive MFs have also reaffirmed the role of stromal microenvironment in the growth and progression of the aggressive lesions by constantly interacting with tumor cells. The results of present study showed a significant role of MFs in the aggressive behavior of odontogenic lesions.

CONCLUSION

This study showed higher frequency of MFs in SA and OKC, suggesting the role of MFs in the aggressive behavior of these lesions. Therefore, we must evaluate stromal component of these lesions and further therapies should target stromal constituents.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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