Comparison of immunoexpression of α-SMA in inflamed and non-inflamed odontogenic keratocyst and ameloblastoma

Joshi Priya, Bhosale Satish, Hongal Bhagyalaxmi, Chougule Madhuri, Dudanakar Mahesh

Abstract
Odontogenic cyst and tumors constitute a group of heterogeneous lesions derived from the tooth forming apparatus and odontogenic epithelial remnants. Odontogenic keratocyst and ameloblastoma though benign exhibit a locally aggressive behaviour. The coordination between the parenchymal and stromal cells plays an important role in pathologic phenomenon like tumor invasion and progression. Myofibroblast is one of the non-epithelial stromal factor which has acquired the capacitance of α-smooth muscle actin expression and synthesis of extracellular matrix components and collagen. However the role of myofibroblast is very sparse in these odontogenic lesions. Hence we designed a study to evaluate the role of α-SMA in Odontogenic keratocyst & Ameloblastoma with an aim to assess the frequency and significance of immunohistochemical expression of α-SMA in inflammed & non-inflammed Odontogenic keratocysts & Ameloblastoma. We conclude that more MF’s in the stroma, are associated with more aggressive behaviour of the odontogenic cyst or tumor.

Keywords: Ameloblastoma, α-SMA, inflammation, Odontogenic keratocyst.

1. Introduction
Odontogenic tumors constitute a group of heterogeneous lesions derived from the tooth forming apparatus and odontogenic epithelial remnants. Originated from the common origin, they range from hamartomatous or non-neoplastic tissue proliferation to malignant neoplasms and are most common in mandibular molar and canine area. [1] Majority of these lesions are benign, but some lesions like keratocystic odontogenic tumor (KCOT) and ameloblastoma exhibit a locally aggressive behavior [2]. Ameloblastomas are the second most frequent type of odontogenic tumors. Based on their clinical behaviour and prognosis, solid ameloblastomas show a locally invasive and infiltrative behaviour with frequent recurrence. [3] Many researchers have tried to uncover the cause of local invasiveness of ameloblastomas, but it is still an enigma and has not yet been clearly identified. A wide range of epithelial associated factors are implicated in the relative aggressive biological behaviour of the odontogenic epithelium but very few studies have investigated non-epithelial factors that could contribute to the variable biological behaviour of odontogenic tumors [4].

As far as odontogenic cysts are concerned, it was earlier considered that cyst wall is a passive membrane, but interest has centered on its potential for aggressive behaviour and propensity for recurrence or as a source of chemical mediators for bone resorption. The aggressive clinical behaviour and frequent recurrences following curettage have been the focus of several studies with an indication that the epithelial lining of OKC (Odontogenic Keratocyst) may have some intrinsic growth potential and thus leading to its reclassification as a tumor by the WHO (World Health Organization) [5]. The stroma is essential for maintenance of epithelial tissue. The coordination between the parenchymal and stromal cells plays an important role in pathologic phenomenon like tumor invasion and progression [6]. It is well established fact that many epithelial tumors are characterized by local accumulation of connective tissue cells which is called as stromal reaction. One of the cellular components of stromal reaction is myofibroblast (MF), a modulated fibroblast which has acquired the capacitance of α-smooth muscle actin (α-SMA) immuno-expression and synthesis of important amount of extracellular...
matrix components and collagen. Normally these cells are found in a variety of tissues ranging from normal (lymph nodes and blood vessels) to pathological conditions (reactive lesions, benign tumor, sarcomas). It is now a well-accepted fact that myofibroblasts play a key role in the connective tissue remodelling which takes place during wound healing and fibrosis [7].

Odontogenic lesions show variable biological behaviour in terms of invasiveness, growth, and recurrence rate. The presence of MFs has been demonstrated in stroma of various benign odontogenic lesions with local aggressive behaviour like OKC and Ameloblastomas [6, 8]. It has been hypothesized that the presence of these cells could be correlated with the aggressiveness of these lesions [8]. MFs have also been detected in the fibrous capsule of primary non syndromic OKCs (NSOKCs), recurrent NSOKCs, and syndromic OKC (SOKCs). These studies indicate that MFs are an important component of the fibrous capsule of OKCs [8]. The presence of stromal MF and enzymes synthesized (MMP-2) by them could also be related to their behaviour and development [10].

Various factors influencing the behaviour of odontogenic lesions include histological subtypes, proliferation capacity, apoptotic, vascular, genetic factors and changes within lesional or stromal tissue. The role of myofibroblast is limited in these odontogenic lesions and not many studies have been conducted to investigate the association between the MFs and their aggressive behaviour and inflammatory reaction. Hence we designed a study to assess the immunohistochemical expression of α-SMA in inflammed and non-inflammed odontogenic keratocyst and ameloblastoma and to correlate the frequency of myofibroblasts with the aggressive biological behaviour of lesions.

2. Materials and Methods
Histopathologically diagnosed 25 cases of odontogenic keratocyst & 25 cases of ameloblastoma with or without inflammation from the archives of the Department of Oral Pathology & Microbiology constituted the study group. Sections were obtained & stained with H & E to confirm the histopathological diagnosis and they were immunostained for α-SMA antibody. Sections were obtained & stained with H & E to confirm the histopathological diagnosis and they were immunostained for α-SMA antibody.

2.1 Principle of Immunostaining
The Super Sensitive Polymer DAB kit is based on the principle of antigen/antibody reaction in tissues. Primary α-SMA antibody combines with its corresponding antigen in tissues. Secondary antibodies which have a dextran polymer backbone conjugate with primary antibody. DAB (3’3’- diaminobenzidine) chromogen combines with antigen-antibody complex and a colored reaction product is formed [11]. The slides were evaluated by two independent oral pathologists, when there was a difference of opinion it was settled with consensus. Representative field with immunopositive cells were selected in 10X objective lens and subsequently observed in 40X objective lens. Tissue sections of each lesion were thoroughly examined. For cystic lesions, the objective lens was placed immediately beneath the cystic epithelial lining and for solid tumors (Odontogenic tumors) it was placed immediately adjacent to the periphery of the tumoral islands/nests/cords. Expression of α-SMA in context with inflammation in odontogenic keratocyst and ameloblastoma was also assessed.

Counts were performed using counting grid containing 100 squares that determined the perimeter of the chosen field. Five fields were chosen for each section. Each α-SMA-positive cell, excluding those surrounding blood vessels, was counted and the total number of positive cells for all five examined fields per case was calculated. This allowed calculation of the mean number of α-SMA positive cells per field. Results were presented as the mean number of α-SMA positive cells per field for each type of lesion as 0 (nil) = no positive cells, 1(weak) = 1–25% cells, 2(moderate) = 25–50% cells and 3(intense) = 50–100% positive cells. The intensity of inflammatory cells was estimated on the basis of the H&E stain and graded as 1-without inflammation or weaknesses; 2-moderate; 3-severe.

Considering the distribution pattern of myofibroblasts, the arrangement of positive-stained cells were classified into 3 groups [13].

1. Focal: If myofibroblasts had a focal arrangement or had no special arrangement in different areas of connective tissue and stroma.
2. Network: Myofibroblasts with vesicular nucleus and abundant cytoplasm arranged in multiple rows with an interwoven network of cytoplasmic extensions forming a network in the stroma of the connective tissue.
3. Spindle: Myofibroblasts arranged in one to three rows in a regular order in the periphery of the neoplastic islands or in the connective tissue with distinctive cell margins around tumor islands and malignant tissue.

4. Statistical analysis: The data was collected and subjected to statistical analysis and was assessed using chi-square test and SPSS (Statistical Package for Social Science) software (version 20.0). P value < 0.05 was considered to be statistically significant.

3. Results
3.1 Clinical data analysis
In total 50 cases of OKC and ameloblastomas there was a significant male preponderance as compared to females. In 25 cases of OKC, the age range was 12 to 80 years with mean age of 28.16 years and in 25 cases of ameloblastoma the age range was 13 to 65 years with mean age of 28.96 years [Table 1].

The site wise distribution of OKC showed 52% lesions in posterior mandible and 40% lesions in the posterior maxilla. One case was present in the anterior mandible and one case was present bilaterally crossing the midline involving the whole body of mandible. Ameloblastomas showed 60% lesions in the posterior mandible, 32% lesions in the anterior mandible and only 8% lesions in the posterior maxilla. The data of both the study group was statistically significant with p value of 0.0081 [Table 2].
### Table 2: Site wise distribution of cases of Odontogenic keratocyst and Ameloblastoma.

<table>
<thead>
<tr>
<th>Site involved</th>
<th>Odontogenic keratocyst</th>
<th>%</th>
<th>Ameloblastoma</th>
<th>%</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior mandible</td>
<td>1</td>
<td>4.00</td>
<td>8</td>
<td>32.00</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Posterior mandible</td>
<td>13</td>
<td>52.00</td>
<td>15</td>
<td>60.00</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Posterior maxilla</td>
<td>10</td>
<td>40.00</td>
<td>2</td>
<td>8.00</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Bilateral mandible</td>
<td>1</td>
<td>4.00</td>
<td>0</td>
<td>0.00</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>100.00</td>
<td>25</td>
<td>100.00</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square = 11.9213  P = 0.0081*

*P<0.05

### 3.2 IHC analysis

Out of 25 cases of OKC, 23 cases (92%) showed α-SMA positivity and only 2 cases (8%) showed negative staining. In ameloblastomas, 22 cases (88%) showed positivity and only 3 cases (12%) were negative for α-SMA. There was significant α-SMA expression in both the groups with p value of 0.037. [Table 3].

#### Table 3: Percentage (%) distribution of α-SMA positivity in Odontogenic keratocyst and Ameloblastoma.

<table>
<thead>
<tr>
<th>% Of Positivity</th>
<th>Odontogenic keratocyst</th>
<th>Ameloblastoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>23 (92%)</td>
<td>22 (88%)</td>
</tr>
<tr>
<td>Negative</td>
<td>2 (8%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

*p<0.05, p=0.037

In OKC, the α-SMA positivity was mainly observed in juxtaepithelial/subepithelial layer and mainly in lower 2/3rd of the cystic wall. Among 25 cases of OKC’s, 52% of cases showed moderate (n=13), 28% cases showed intense (n=7), 12% cases showed weak (n=3), and 8% (n=2) cases showed no expression for α-SMA. In ameloblastomas, immunopositive cells were mainly present in the extracellular matrix adjacent to tumor cells. 22 cases (88%) showed positive expression, 12 cases (48%) showed weak positivity (score=1), 7 cases (28%) showed moderate positivity (score=2), 3 cases (12%) showed intense positivity (score=3) and 3 cases (12%) were observed with negative expression. These values were statistically significant [p value of 0.0290 (p<0.05)] for OKC and Ameloblastomas.

In OKC the predominant pattern observed was subepithelial and network (52%) [figure1], subepithelial (36%) and focal (4%) [Figure 2A & 2B]. In ameloblastomas, spindle pattern was predominant (52%) [Figure 3A & 3B], followed by network (36%) [Figure 4A&4B]. The data for both the study group was statistically significant with p value of 0.00001. [Table 4].

#### Table 4: Distribution of α-SMA patterns in Odontogenic keratocyst and Ameloblastoma groups

<table>
<thead>
<tr>
<th>α-SMA pattern</th>
<th>Odontogenic keratocyst</th>
<th>%</th>
<th>Ameloblastoma</th>
<th>%</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepithelial</td>
<td>9</td>
<td>36.00</td>
<td>0</td>
<td>0.00</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Subepithelial and network</td>
<td>13</td>
<td>52.00</td>
<td>0</td>
<td>0.00</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Network</td>
<td>0</td>
<td>0.00</td>
<td>9</td>
<td>36.00</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Spindle</td>
<td>0</td>
<td>0.00</td>
<td>13</td>
<td>52.00</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Focal</td>
<td>1</td>
<td>4.00</td>
<td>0</td>
<td>0.00</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>8.00</td>
<td>3</td>
<td>12.00</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>100.00</td>
<td>25</td>
<td>100.00</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square = 45.000  P = 0.00001*

*P<0.05

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**Fig 1:** H&E stained section of OKC (10 X), Inset- IHC staining showing α-SMA positivity in subepithelial and network pattern (10X) in OKC.

**Fig 2A:** H&E stained section of OKC (10 X), Inset- IHC staining showing α-SMA positivity in subepithelial pattern (10X) in OKC.
Out of 25 cases of OKC, 11 were non-inflamed and 14 were inflamed. Among 14 inflamed cases, 4 were mild, 6 were moderate and 4 were severe [According to the criteria given by Mashadiabbas [8]. Out of 25 cases of ameloblastomas, 12 were non-inflamed and 13 were inflamed. Among 13 inflamed cases 6 were mild, 4 were moderate and 3 were severe. Regardless of the presence or absence of inflammation there was significant $\alpha$-SMA expression in OKC and ameloblastomas. The values were statistically non-significant ($p=0.399$) suggesting that inflammation did not affect the results.

4. Discussion

Ever since the Myofibroblasts (MF) were discovered by Gabbiani et al [13], in the early 1970’s and shown to actively promote dermal wound contraction, this cell has been on the rise and its importance shown for different physiological, practical and pathological processes. Gabbiani G, Ryan GB and Majne G et al [13] in 1971 performed experiments on granulation tissue with various pharmacological agents and concluded that granulating wound contained contractile cells. They coined the term ‘MYOFIBROBLASTS’ for these contractile cells. Myofibroblasts are differentiated fibroblasts that express alpha smooth muscle actin and have intermediate characteristics between classic fibroblasts and smooth muscle.
cells. Recent studies have shown that smooth muscle fibroblasts may originate from fibroblasts within the tumor stroma or even from carcinoma cells by the process of epithelial-mesenchymal transition. Several cytokines, including TGF-β, PDGF, IL-4 and IGF-II have been reported to induce myofibroblastic differentiation. Cancer cell derived transforming growth factor (TGF-β1) stimulation is the key cytokine. In recent years, the tumor microenvironment has become the focus of intense research, with the understanding that the alterations that occur in the tumor stroma might prove useful in prognosis and generate new therapeutic targets. Fibroblasts are among the most abundant cell type in the microenvironment of solid tumors, being particularly prominent in carcinomas of the breast, ovary, pancreas, colon and prostate.

There is abundant evidence that Carcinoma Associated Fibroblast (CAFs/MFs) can contribute to tumor growth and spread, mediated through the release of growth factors, such as, TGF-β and HGF. Differentiation of MF and induction of an inflammatory infiltrate leads to release of pro-angiogenic factors. Development of a desmoplastic stroma, partly in response to hypoxia, leads to tumor-specific interactions with tumor cell-surface receptors that enhance the invasion. A wide range of epithelial-associated factors are implicated in the relative aggressive biological behavior of the odontogenic epithelium that includes increased proliferative potential. Only a few studies have investigated non-epithelial factors that could contribute to the variable biological behavior of different types of odontogenic cysts and tumors.

In a study of 24 cases of ameloblastomas by Sherlin et al., smooth muscle actin positivity was noted at the tumor front of the lesion which clearly indicates the pivotal role of smooth muscle actin positive myofibroblasts in tumor progression. Similarly Vered et al. have observed α-SMA immunopositivity mainly in extracellular matrix adjacent to tumor cells. The present study also observed similar expression and significant α-SMA positivity (88%) in tumor stroma. We observed weak α-SMA positivity (48%) in 12 cases suggesting that the behaviour of the tumor may not be dependent entirely on stromal elements but also on other factors. Our results are in contrast to the results observed by Vered et al. but are in accordance with the study done by Mashhadiabas F and Moghadam Atarbash S.

In the present study out of 25 cases of ameloblastomas, 13 were inflammed [mild/moderate/severe]. Regardless of the presence of inflammation, α-SMA expression was seen and this result is in accordance with the study done by Mashhadiabas suggesting that inflammation does not affect the results. The immunohistochemical expression profile of α-SMA has been described in three patterns; namely spindle, network, focal. In the present study, we observed that the majority of the lesions showed the spindle pattern which were interconnected in a network like manner which is in accordance with the observations reported by Vered et al. There was significant α-SMA positivity (92%) in OKC’s. Mashhadiabas have shown distribution patterns of MF in subepithelial zone and throughout cyst wall. In the present study also we observed α-SMA predominantly in the subepithelial zone and lower 1/3rd of cystic wall. Out of 25 cases of OKC, 11 were non-inflammed and 14 were inflammed [mild/moderate/severe]. Regardless of inflammation significant α-SMA expression was reported, which is in accordance with a study done by Mashhadiabas.

Lombardi et al. demonstrated strong expression of myofibroblast mainly in subepithelial, midzone and periphery of OKC, Dentigerous cyst, Radicular cyst and epidermoid cyst. We also observed strong expression of myofibroblasts subepithelially (36%) and in a network pattern in 52% of cases and in only one case, focal pattern was seen (4%). The present study provides persuasive evidence that stroma of these lesions harbor MFs as reflected by α-SMA immunopositive cells. Our study also reveals that the MFs are distinctly heterogeneous in distribution and pattern of arrangement. It has been clearly shown in the literature that the mean number of MF in well recognized aggressive odontogenic lesions (e.g. SMA and OKC) are high and did not differ significantly from that in OSCC. Roy Swati and Garg Vipul evaluated stromal myofibroblasts expression in Keratocystic odontogenic tumor and Orthokeratinized odontogenic cysts (OOC) and found the mean number of α-SMA positive cells in the connective tissue wall of KCOT was significantly higher than that in OOC and stated that the two cysts not only differ in the epithelial characteristics, but also in the stromal wall component. The increased number of stromal MFs in KCOT in comparison to OOC correlates with its aggressive behavior and increased tendency towards recurrence. Thus a positive link can be suggested that more MFs in the stroma, a more aggressive behaviour of the odontogenic cyst or tumor. Odontogenic epithelium, mainly in SMA and OKC can act and modulate stromal MF in a manner similar to OSCC.

The curative surgery, particularly of aggressive odontogenic lesions could occasionally result in significant functional, aesthetic and psychological damage. Thus, in these cases, it is suggested to evaluate the frequency of MF in their stroma and a long term follow-up of the patients is required. Studies correlating the expression of myofibroblasts and biological behavior of these odontogenic lesions with special emphasis on recurrence and local invasion are essential in understanding the true nature of these lesions. Along with α-SMA, staining with Desmin and Vimentin helps to identify MF’s with greater specificity. The absence of MF in approximately 10% cases of OKC and Ameloblastoma suggests the role of factors other than myofibroblasts like epithelial proliferation and luminal contents. Since immunohistochemistry is a technique sensitive procedure, false positive results can be encountered. So proper quality control, handling of the reagents and staining protocol has to be followed.

5. Conclusion

The immunoexpression profile of α-SMA demonstrates that myofibroblasts are located at the interface of tumor cells and stroma in ameloblastoma while heterogeneous distribution juxtaepithelially and in deeper and peripheral part of cystic wall in OKC. Regardless of the presence or absence of inflammation there was significant α-SMA expression in both OKCs and ameloblastoma. When more MF are present in the stroma, a more aggressive behavior of the odontogenic cyst or tumor can be anticipated.

6. References

3. Andrade ESS, Miguel MCD, Pinto LP, Souza LBD. Ameloblastoma and adenomatoid odontogenic tumor: the role of α2β1, α3β1 and α5β1 integrins in local invasiveness and architectural characteristics. Annals of diagnostic Pathology 2007; 11:199-207.


