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IMPERATIVE IMPORTANCE OF CONNECTIVE TISSUE STROMA IN EVALUATING BIOLOGICAL BEHAVIOUR OF ODONTOGENIC LESIONS BY EMPLOYING MYOFIBROBLAST COUNT: AN IMMUNOHISTOCHEMICAL STUDY.

Dr. Kanak Tiwari* M.D.S., Senior Lecturer, Department Of Oral And Maxillofacial Pathology And Oral Microbiology, Saraswati Dental College And Hospital, Tiwariganj, Lucknow. U.P. *Corresponding Author

Dr. Anil Singh (M.D.S.), Professor, Department of Dentistry, Government Medical College and Associated Hospital, Rajouri.

Dr. Kunal Sah (M.D.S., PhD.), Professor and Head, Department of Oral and Maxillofacial Pathology and Oral Microbiology, Saraswati Dental College and Hospital, Lucknow.

Dr. Shweta Singh (M.D.S.), Associate Professor, Department of Oral and Maxillofacial Pathology and Oral Microbiology, Saraswati Dental College and Hospital, Lucknow.

ABSTRACT **Context:** Myofibroblasts (MFs) were initially described by Giulio Gabbiani in 1971. They play a crucial role in organogenesis and morphogenesis and were considered to be predominant in the lesions with locally aggressive behaviour such as Keratocystic odontogenic tumor (KCOT) and Ameloblastoma. **Aims:** The present study explores the presence of MFs using alpha smooth muscle actin (α-SMA) and also to relate their density in odontogenic cysts (OCs) and odontogenic tumors (OTs), which can be of considerable importance in predicting their possible biological behaviour and growth potential. **Settings And Design:** An immunohistochemical analysis of cases of dentigerous cyst (DC), Keratocystic odontogenic tumor (KCOT) and Ameloblastoma. **Methods And Material:** For the present study, twenty paraffin embedded tissue blocks each one of DC, KCOT and Ameloblastoma were chosen. The diagnosis of tissues selected was confirmed through hematoxylin and eosin staining. Tissue sections were examined and interpretation for the number of myofibroblasts using α-SMA immunohistochemical staining was done. **Statistical Analysis:** The Kruskal-Wallis test followed by Tukey's post hoc test was used to compare the study parameters among the groups. **Results And Conclusion:** KCOT showed the highest number of myofibroblasts, whereas DC showed the lowest. Among the groups, there were significant differences between the myofibroblast counts among DC and KCOT and between DC and ameloblastoma, whereas the difference in counts was not statistically significant between KCOT and ameloblastoma. A positive correlation was observed between the myofibroblast count and the known biologic behavior of the lesions.

KEYWORDS : Ameloblastoma, dentigerous cyst, keratocystic odontogenic tumor, myofibroblasts, smooth muscle actin.

INTRODUCTION:

The most plentiful and key cells existing in the connective tissue are the fibroblast cells. Fibroblasts cells are solely responsible for formation, maintenance and destruction of the fibrous components and ground substance of connective tissue.[1]

The fibroblast cells are phenotypically heterogenous and may acquire smooth muscle cell like properties and are termed as Myofibroblasts. Giulio Gabbiani, eminent researcher and discoverer of myofibroblast cells defined these cells for the first time in 1971, as differentiated fibroblast cells in the granulation tissue.[2,3]

MFs are phenotypically altered, plump and spindle shaped fibroblast cells. These cells are characterized by presence of smooth muscle myofilaments and α- Smooth Muscle Actin (α-SMA) expressibility. MFs are crucial for the veracity of human body due to its predominant part in physiological tissue repair, but these cells also possess the capacity to promote tumour development. [4,5,6]

Several Odontogenic Cysts (OCs) and Tumours such as Keratocystic Odontogenic Tumour (KCOT) and Ameloblastoma have the potential for aggressive behaviour and local recurrence. Electron microscopic studies conducted on OCs and OTs have revealed that MFs were particularly more in lesions with locally destructive behaviour such as Keratocystic odontogenic tumour and Ameloblastoma. [7,8]

Therefore the present study has been undertaken to investigate the presence of MFs using α-SMA and also to compare their density in OCs and OTs, which can be of great value in predicting their possible biological behaviour and growth potential.

MATERIAL AND METHOD:

The present retrospective study was undertaken in the Department of Oral Pathology and Microbiology, Saraswati Dental College, Lucknow, after obtaining clearance from the Institutional Research and Development Committee (IRDC) and Institutional Human Ethics Committee (IHEC).

A total of 60 histopathologically confirmed cases of odontogenic cysts and odontogenic tumours were included in the study. Paraffin embedded tissue blocks of these confirmed cases were retrieved from the archives of the department.

The Study Group Comprises Of:

Group A: Keratocystic Odontogenic Tumour (n=20)
Group B: Dentigerous Cyst (n=20)
Group C: Ameloblastoma (n=20)

Inclusion Criteria

Group A: Histopathologically diagnosed cases of keratocystic odontogenic tumour without inflammation.

Group B: Histopathologically diagnosed cases of dentigerous cyst.
Group C: Histopathologically diagnosed cases of solid ameloblastoma (including all histopathological subtypes) and unicystic ameloblastoma.

Exclusion Criteria

Cases with presence of inflammatory infiltrate in the connective tissue were excluded from the study group.

The α-SMA positive cells in the blood vessel wall (served as control).

For all the groups (n=60) every slide was stained with H & E Stain and was also stained for SMA expression.

Four micrometer thick sections were then obtained from each of the selected cases for immunohistochemical staining with alpha-smooth muscle actin (α-SMA). The slides were deparaffinized by passing them through two changes of xylene for 5 min each. They were hydrated in two changes of 100% ethanol for 1 min each. The slides were then transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs. pressure for 15 min. After allowing cooling, they were washed in phosphate buffer solution. The slides were then treated with protein block reagent for 10 min. Immunohistochemical staining was then performed using Bio Genex primary antibody (mouse antihuman antibody α-SMA) as per the manufacturer's instructions. The slides were then mounted in DPX and observed under light microscope for the results.

- **SMA positive:** Cells with brown staining.

- **SMA negative:** Cells with no stain.

For each batch of slides positive and negative controls were run. Five representative fields were selected for counting in each of the SMA stained slides. SMA positive cells were counted just below the epithelial lining for cystic lesions. For solid tumours, it was done from the field immediately surrounding the epithelial tumour islands and strands. The SMA positive cells were tagged and were subjected for microscopic evaluation by two experienced oral pathologists. Counting was performed using "Olympus BX51" light microscope with a 10x eyepiece and 40x objective. The area encompassed by $1cm^2$ graticule was taken as one microscopic field. The SMA positive cells (MFs) were identified in each of the five fields chosen. Those cells immediately surrounding the blood vessels were not counted as they served as control.

The slides were examined under 10x objective magnification and then the counting of SMA positive cells was done under 40x objective magnifications using an OLYMPUS BX51 Microscope. Tagging of the SMA positive cells was done manually, using IMAGE PRO EXPRESS 6.0 for WINDOWS (media cybernetics).

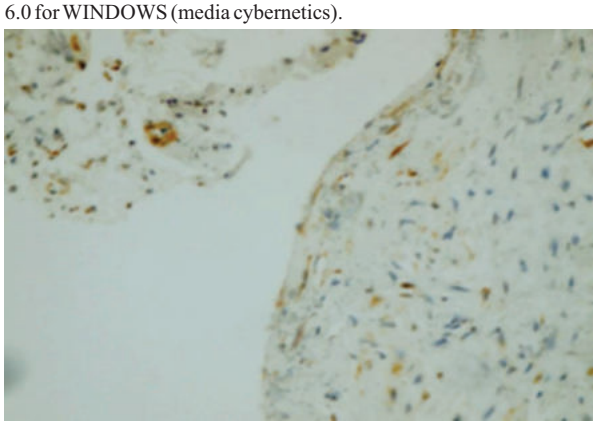

Fig. 1. Photograph showing α-SMA positive cells in fibrous wall in DC (IHC stain, X400).

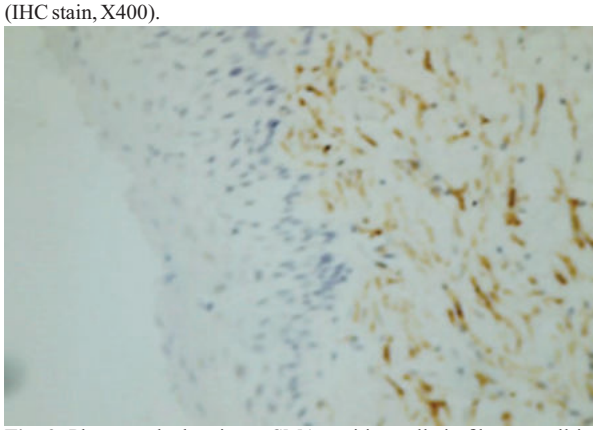

Fig. 2. Photograph showing α-SMA positive cells in fibrous wall in KCOT (IHC Stain, X 400).

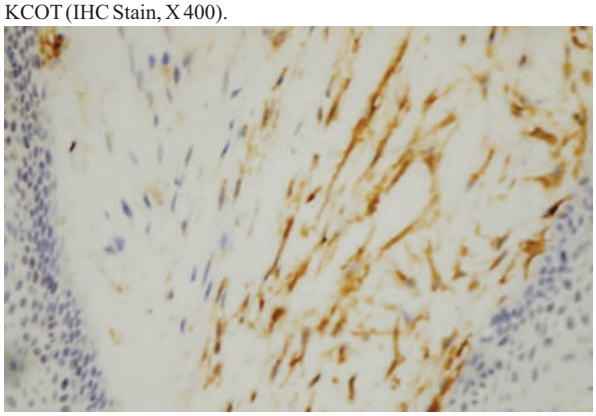

Fig.3. Photograph showing α-SMA positive cells in connective tissue stroma surrounding tumour islands in Ameloblastoma (IHC Stain, X 400).

Statistical Analysis

The data collected was entered into Microsoft excel spread sheet and all the analysis was carried out on SPSS 16.0 version (Chicago, Inc., USA).

Descriptive data was presented in the form of frequency, percentage for categorical variables and in the form of mean, median and standard deviation. The Kruskal-Wallis test followed by Tukey's post hoc test was used to compare the study parameters among the groups.

RESULTS:

A total number of sixty cases were taken for the study, twenty from each group and the mean count of α-SMA positive cells (MFs) was calculated and compared.

In **Group A (KCOT)**, the mean MF count was found to be 114.3550.98 with minimum of 18 and maximum of 180 MF cells.

In **Group B (DC)**, mean MF count was observed to be 61.00 48.02 with minimum of 0 and maximum of 163 cells.

In **Group C (Ameloblastoma)**, the mean MF count was found to be 112.6 073.42 with minimum of 2 and maximum of 248 MF cells.

Comparison of the total mean MF count of the three groups, as assessed by Kruskal-Wallis test was **significantly (p=0.007) different** among all the groups.

Table-1: Comparison Of Total MF Count Among The Groups

Groups	Total (Mean±SD)	Minimum	Maximum
Group A	114.35±50.98	18	180
Group B	61.00±48.02	0	163
Group C	112.60±73.42	2	248
p-value[1]	0.007*		

[1]Kruskal-Wallis test, *Significant

Further, comparing the difference in mean MF count between the groups, as assessed by, Tukey post hoc test was found to be **significantly (p=0.01) higher** in **Group A** than **Group B**. The total MF count was also **significantly (p=0.02) higher** in **Group C** than **Group B**, while the difference in mean MF count was found to be **non-significant (p=0.99)** between **Group A** and **Group C**.

Table-2: Post HOC Comparison Of Total Difference In Mean MF Count In All Five Fields Between The Groups

Groups	Groups	p-value[1]
Group A	Group B	0.01*
	Group C	0.99
Group B	Group C	0.02*

[1]Tukey's post hoc tests, *Significant

Table-2, shows the comparison of Total difference in mean MF count in all five fields among the groups. Kruskal-Wallis test showed that there was significant (p=0.007) difference in Total MF count among the groups. The post hoc tests showed that total MF count was significantly (p=0.01) higher in Group A (114.35±50.98) than Group B (61.00±48.02). Total MF count was also significantly (p=0.02) higher in Group C (112.60±73.42) than Group B (61.00±48.02).

The comparison of all the five fields among groups showed the highest MF count in KCOT (114.35±50.98) and lowest in DC (61.00±48.02). Ameloblastoma showed a value close to but slightly lesser than that of KCOT (112.60±73.42) (Table 6). Thus, the total number of MFs was found to be maximum in KCOT, which was marginally higher than that observed in Ameloblastoma, while the total MF count observed in DC was found to be much lower than that found in KCOT and Ameloblastoma.

On comparing the difference in mean MF count between the groups by Tukey post hoc test, a significant difference (p=0.01) was found in MF count among KCOT as compared to DC. The total mean MF count was also found to be significantly (p=0.02) higher in Ameloblastoma than in DC but, the total mean MF count between KCOT and Ameloblastoma was found to be non-significant (p=0.99).

DISCUSSION:

Odontogenic lesions are an imperative aspect of Oral and

Maxillofacial Pathology. Odontogenic apparatus gives rise to these lesions and they consist of two main categories: Odontogenic Cysts (OCs) and Odontogenic Tumours (Ots). [9]

Odontogenic Cysts and Tumours, particularly those having aggressive behaviours like, KCOT and Ameloblastoma are defined as benign lesions, but they demonstrate local infiltrative nature along with high growth and recurrence rate. It is now well-established that harmonized communications amongst the epithelial and stromal cells are crucial in controlling the growth and clinical behaviour of these lesions. [10,11]

Discrepancies acquired by epithelial component during the development of these pathologies is demonstrated by various proliferative markers, e.g., Ki-67, IPO-38, impaired expression of tumour suppressor genes and their protein products, e.g., PTCH, p53 and MDM2; and abnormal activity of cell-cycle-related pathways, e.g., HSP30 and telomerase. However, only few studies have been undertaken to investigate non-epithelial factors that could contribute to the variable biological behaviour of different OCs and OTs. [12,13,14]

The role of tumour stroma in tumour progression is an important area of current research and has become a potential target for therapeutic intervention. The presence of stromal MFs, which is an important component of tumour stroma, has been linked to the biological behaviour of both benign and malignant tumours. [15,16]

Vered et al. (2005) conducted an Immunohistochemical (IHC) study to assess the frequency of MFs in different OCs and OTs and correlated it to their aggressive biological behaviour. Zidar et al. (2002), described that invasion beyond the basement membrane is necessary to evoke a myofibroblastic stromal reaction. [17,18]

It is now known that MFs are differentiated fibroblasts that express α-SMA and have characteristics intermediate between the conventional fibroblasts and smooth muscle cells. Fibroblasts acquire the phenotype of MFs through TGF-β1, secreted by stromal cells which in turn upregulate their expression of α-SMA. [19]

Research over the past years has shown that presence of cancer cells is followed by some changes that occur within the epithelium due to which the normal stroma is transformed into a reactive one. The neoplastic cells secrete TGF-β1, which promotes the differentiation of fibroblasts into MFs. These in turn secrete cytokines and matrix metalloproteases (MMP) which contribute to the destruction of extra cellular matrix (ECM) facilitating tumour growth and invasion. [20,21]

The present study deals with evaluation of MFs immunohistochemically by using α-SMA in OKC, DC and Ameloblastoma and the evaluation of their biological role. The mean number of α-SMA positive cells (MFs) per HPF were calculated for each group and the mean MF count in field one (F1) was found to be higher in KCOT (26.40±14.29) than Ameloblastoma (24.75±15.41) and was found to be lowest in DC (16.65±10.99).

The comparison of all the five fields among groups showed the highest MF count in KCOT (114.35±50.98) and lowest in DC (61.00±48.02). Ameloblastoma showed a value close to but slightly lesser than that of KCOT (112.60±73.42) (Table 6). Thus, the total number of MFs was found to be maximum in KCOT, which was marginally higher than that observed in Ameloblastoma, while the total MF count observed in DC was found to be much lower than that found in KCOT and Ameloblastoma.

On comparing the difference in mean MF count between the groups by Tukey post hoc test, a significant difference (p=0.01) was found in MF count among KCOT as compared to DC. The total mean MF count was also found to be significantly (p=0.02) higher in Ameloblastoma than in DC but, the total mean MF count between KCOT and Ameloblastoma was found to be non-significant (p=0.99).

The present study was also found to be concomitant with a study done by Mashhadiabbas F et al. (2010), in which IHC phenotype, distribution and significance of proliferation of MFs (α-SMA positive cells) was analysed, in DC, KCOT and Ameloblastoma. The mean number of positive cells in KCOT was found to be higher than that in Ameloblastoma while, DC showed lowest number of MF count. Similar study was done by Vered et al. (2005), which showed the highest MF positivity in KCOT and least in DC. [8,17]

Study conducted by Joshi P et al. (2014) also reported that, MF positivity was marginally higher in KCOT than in Ameloblastoma but, without any statistically significant difference. [22]

While, studies conducted by Roy S. et al. (2016) and Syamala D. et al. (2016), found a higher mean MFs count in Ameloblastoma followed by OKC and DC again showed least number of α-SMA positive cells in its stroma. [20,23]

Similarly, a study conducted by Kouhsoltani M. (2016), demonstrated MF cells density in a significant number of odontogenic cysts and tumours and the outcome of the study showed, higher density of MFs in OTs in comparison to OCs. [24]

The comparison of all the research work done in context of MF cell density in OCs and OTs suggested that, there was only a marginal difference in MF positivity among KCOT and Ameloblastoma which was assessed to be statistically non-significant, and the variance in MF count between Ameloblastoma and DC, and KCOT and DC in all the studies were found to be statistically noteworthy.

The increased number of MFs in the stroma of KCOT is suggestive of its aggressive clinical course. Keeping in mind its high recurrence rate, aggressive clinical behaviour and its association with nevoid basal cell carcinoma syndrome, WHO (2005) reclassified this unique lesion as a neoplasm and renamed it as 'Keratocystic Odontogenic Tumour'. [25]

In 2017, KCOT was again termed OKC by WHO, but it is important and clinically relevant to separate KCOT from other odontogenic cysts as KCOT expresses features of cysts as well as benign tumours. Authors have reported that behaviour of some KCOTs is as aggressive as a benign neoplasm such as Ameloblastoma. [13,25]

The presence of MFs in the stroma of such aggressive lesions, like KCOT and Ameloblastoma, shows the involvement of MFs in the creation of a permissive microenvironment in the stroma for growth and progression of such lesions, when compared to other odontogenic lesions such as DC, which showed lesser number of MFs in this study and which goes in accordance with its reported lower growth potential. Researches over the past years suggest that the stromal MFs are the determinants of the aggressive nature of a lesion. Rothouse et al. (1980), first described the presence of MFs in the stromal component, while Smith (1986), first questioned the relationship between MFs and the aggressive behaviour of a neoplasm. [26,27,28]

Lombardi and Morgan in 1995 confirmed the presence of MFs in the wall of OCs and suggested that MFs might be part of a homeostatic response to the distension caused by cyst enlargement. Fregnani et al., Souza Freitas et al. and Kumamoto in 2006, reported abundant presence of MFs and high levels of MMPs in ameloblastoma and mentioned their role in degradation of ECM and the basement membrane components. [12,29,30,31]

This study confirms the role of stromal microenvironment in the growth and progression of aggressive lesions like KCOT and Ameloblastoma.

CONCLUSION:

Based on the above observations, we can conclude that, MFs play a significant role in determining the biological behaviour and growth potential of aggressive lesions. MFs also confirm the role of stromal microenvironment in the growth and progression of such lesions, thus it is essential to demonstrate the MF cells in such aggressive lesions to rule out their behaviour.

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