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IMMNUNOHISTOCHEMICAL EVALUATION OF THE ROLE OF MYOFIBROBLASTS IN KERATOCYSTIC ODONTOGENIC TUMOR

Andleeb Manhas[1], Amani Mahajan[2], Sadaf Antoo[3], Monika Negi[4], Muzaffar Parray[5],Swati Parhar[6]

[1,2,3,6]*Department of Oral Pathology and Microbiology, Swami Devi Dyal Hospital and Dental College, Golpura, Barwala, Panchkula, Haryana, India;*

[4]*Department of Oral Pathology and Microbiology, Himachal Institute of Dental SciencesPaonta Sahib, Himachal Pradesh, India;*

[5]*Resident Skims Srinagar, India*

E mail:dr.swatiparhar@gmail.com[6]

ABSTRACT:

Introduction: Odontogenic lesions are relatively common lesions and account for a substantial part of total oral biopsies by an oral pathologist. Some lesions like the keratocysticodontogenic tumor (KCOT) exhibit a locally aggressive behavior.Myofibroblasts (MFs) are specialized fibroblasts. These cells play a role in physiological processes like wound healing and pathologic conditions like reactive lesions, benign tumors, locally aggressive tumors and malignancies affecting oral cavity. Earlier, it was believed that the presence of Myofibroblasts (MFs) at the invasive front of the tumor was a part of the host defence mechanism against the tumor, but recent studies suggest that the presence of these cells at the tumor front promotes the growth and invasion of the tumor. The presence of Myofibroblasts has been reported in the stroma of many odontogenic lesions, including both cysts and tumors. Studies conducted both on odontogenic cysts and tumors revealed that the cells were particularly more in lesions with locally aggressive behavior like odontogenic keratocyst (OKC).

The aim of the study was to evaluate the role of Myofibroblasts in KeratocysticOdontogenic Tumor and its objectives were to compare the expression and distribution pattern of Myofibroblasts in KeratocysticOdontogenic Tumor (KCOT) as compared to their expression in Oral Squamous Cell Carcinoma and to derive a co-relation between the stromal Myofibroblasts and the known biologic behavior of KeratocysticOdontogenic Tumor (KCOT).

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Material and Methods: The study specimen comprised of 30 Specimens of Keratocysticodontogenictumor(KCOT and10 Specimens of Oral Squamous Cell Carcinoma.

Results: 7 out of 30 (23.30%) of the slides of keratocysticodontogenic tumor showed intensely positive staining to α-sma and 16 out of 30 (53.30%) of the slides of keratocysticodontogenic tumor showed moderately positive staining to α-sma. 8 out of 10 (80.00%) of the slides of oral squamous cell carcinoma showed intensely positive staining toα-sma and 2 out of 10 (20.00%) of the slides of oral squamous cell carcinoma showed moderately positive staining to α-sma.

Conclusion: According to the results of our study, quantitative evaluation and pattern of MFs in KCOT which is accepted as aggressive odontogenic lesion shows positive results, but at the same time differs from that of OSCC. This suggests that there is a positive association of MFs and aggressiveness of the lesion at the growth front. To conclude, we feel that at this point of time, we can safely suggest that increase in number of Myofibroblasts and change in their distribution pattern are of great value in predicting the possible biologic behavior of such lesions, and may hold prognostic significance in the near future.

KEY WORDS: α-sma, KCOT, MMPs, Ihc,alpha smooth muscle actin.

1. INTRODUCTION:

Odontogenic lesions form a significant part of total biopsies which are received by an oral pathologist. They range from small reactive lesions to large aggressive and destructive lesions.[1] one of the common odontogenic lesions is Keratocysticodontogenic Tumor (KCOT). This lesion has been surrounded by controversies ever since its discovery in 1876. Though it was originally described as a cyst, Toller was the first one who suggested that it is a tumor and not a cyst. It is defined as a cystic intraosseous tumor of odontogenic origin, with a characteristic lining of parakeratinized stratified squamous epithelium and potentially aggressive behavior. It is commonly seen in 2nd to 3rd decade of life and has a male predilection.[3] Odontogenickeratocyst, thoughinitially considered to be a cystic lesion, it was classified as a benign neoplasm by the WHO in 2005 and was renamed as keratocysticodontogenic tumor. This decision was taken due to its locally aggressive behavior and high recurrence potential and its association with genetic and chromosomal abnormalities.[1,4.] However, it was again reclassified as a cyst in cyst in the current 2017 classification of odontogenic lesions by the WHO. Never the less, this lesion has remained an enigma for the oral pathologists as several authors still consider it to be a tumor. Myofibroblasts are a type of specialized fibroblasts which have smooth muscle like features. Gibbani, first observed them in granulation tissue under an electron microscope.[5,6] These cells are large spindle shaped with long cytoplasmic processes, amphophilic cytoplasm and indented nuclei with conspicuous nucleoli.[7] Their role in physiological as well as pathological processes is well established. They are found in pathologies like reactive lesions, benign tumors and malignancies affecting the oral cavity. Some of the authors have also called them as Cancer associated fibroblasts.[10] Myofibroblasts are thought to secrete several enzymes and matrix metalloproteinase, (MMPs- 1,2,3,9,13, and 14) which lead to ECM

degradation[11] as well as they release different growth factors such as PDGF, Basic Fibroblast Growth Factor, Keratinocyte Growth Factor, Stem Cell Factor, Epidermal Growth Factor, Granulocyte – Macrophage colony stimulating factor and cytokines. Recent studies have proposed that they promote tumor progression due to their ability to secrete several growth factors and enzymes.[18]The presence of Myofibroblasts has been reported in the stroma of many odontogenic lesions, including both cysts and tumors. Numerous reports in the literature suggest that these cells were particularly more in lesions with locally aggressive behavior like odontogenic keratocyst. (OKC).[15]Many studies were carried out to investigate the role of MFs in malignant neoplasms including oral squamous cell carcinoma, only a few studies have investigated their presence and role in odontogenic lesions.[18] The odontogenic keratocyst is one of the most aggressive known odontogenic cysts. KCOT although defined as benign is known to demonstrate locally aggressive behavior. Hence, this study was undertaken to investigate the presence of Myofibroblats (MFs) in Odontogenic Keratocyst and to correlate the same with the known and reported biological behavior of this lesion.

2. MATERIAL AND METHODS:

The study was carried out in the department of Oral Pathology and Microbiology of Swami Devi Dyal dental hospital and College, Barwala. Paraffin-embedded tissue specimens of Keratocysticodontogenic tumor were retrieved from the archives of the department of Oral Pathology and Microbiology of Swami Devi Dyal Hospital and Dental College, Barwala.
The study specimens included were categorized as follows:
Group 1: 30 Specimens of Keratocysticodontogenic tumor (KCOT).
Group 2: 10 Specimens of Oral Squamous Cell Carcinoma.
Data on patient age, gender, and lesion site was obtained from the biopsy requisition forms submitted. The tissue sections will be stained using the following methods:

1. Standard hematoxylin and eosin (H & E) stain; and
2. IMMUNOHISTOCHEMICAL STAINING USING PRIMARY MONOCLONAL ANTIBODY (primary mouse monoclonal antibody clone 1A4 also k/as asm-1) AS PER MANUFACTURER'S INSTRUCTIONS.

3. EQUIPMENT FOR MICROSCOPY AND IMAGE CAPTURE

1. Olympus research microscope with CCD video camera
2. Q Capture pro 7 Image Analysis Software.

4.IMMUNOSTAINING EVALUATION

- Ten representative fields were selected for counting in each of the α_SMA stained slides using a grid.
- Counting was performed using **binocular microscope** with a 10x eye piece and 40x objective.
- **The α_SMA positive cells in theblood vessels were taken as internal control**.
- Those cells immediately surrounding the **blood vessel wall** were not counted. All other positively stained cells in each field were counted and their numbers were recorded**.**

- The total number of MFs in all the ten fields counted for a slide were calculated.
- The mean number of MFs were calculated.
- The percentage of α-sma positive cells in each group was calculated by noting the number of positive and negative cases separately for each group.

The mean number of alpha sma positive cells per field were calculated as follows:

0 (**NIL)** = NO positively stained cells in the high-power field.

1 **(WEAK**) = 1-25% of the area per high power field showing positively stained cells.

2 (**MODERATE**) = 25-50% of the area per high power field showing positively stained cells.

3 (**INTENSE**) = 50-100% of the area per high power field showing positively

The mean number of alpha sma positive cells per field were calculated using this above-mentioned criterion with the use of a grid. We also evaluated the varied arrangement of Myofibroblasts in KCOT and classified them according to the distribution and arrangement of Myofibroblasts as follows:

- FOCAL: If the myofibroblast had focal arrangement or had no special arrangement in the different areas of the stroma
- NETWORK: If they show vesicular nucleus and abundant cytoplasm arranged in multiple rows with an interwoven network of cytoplasmic extensions forming a network in the stroma.
- SPINDLE: If they are arranged in one or three rows in a regular order at the periphery of the neoplastic islands or in the connective tissue with distinctive cell margins around tumor islands and malignant tissue.

5. RESULTS:

More than half of cases (53.30%) of keratocysticodontogenic tumor stained immunohistochemically for antibody to α-SMA showed moderately positive staining, 23.30% showed intensely positive staining, 6.70% showed weakly positive staining and 16.70% showed negative staining to ALPHA SMOOTH MUSCLE ACTIN. This implies that few cases of KCOT may show locally aggressive and infiltrative behavior. Majority of cases (80%) of oral squamous cell carcinoma exhibited intensely positive staining and only a few cases 9 (20%) showed moderately positive staining to ALPHA SMOOTH MUSCLE ACTIN. that in a comparison of staining of Odontogenic Keratocyst and oral squamous cell carcinoma, more intensely positive staining was seen in oral squamous cell carcinoma (80%) as compared to that in the keratocysticOdontogenic tumor group (23.3%); whereas moderatelypositive staining was more common in the keratocysticOdontogenic tumor group (53.3%), as compared to the positive control group (20%) which is in accordance with the literature.

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TABLE 1
SHOWING COMPARISON OF POSITIVITY TO ALPHA SMOOTH MUSCLE ACTIN STAINING BETWEEN ODONTOGENIC KERATOCYST AND ORAL SQUAMOUS CELL CARCINOMA.

% OF POSITIVITY * Group Crosstabulation					
			Group		
			MYOFIBROBLAST COUNT IN KCOT	**MYOFIBROBLAST COUNT IN OSCC**	**Total**
% OF POSITIVITY	**Intense**	**Count**	7	8	15
		% within Group	23.30%	80.00%	37.50%
	Moderate	**Count**	16	2	18
		% within Group	53.30%	20.00%	45.00%
	Nil	**Count**	5	0	5
		% within Group	16.70%	0.00%	12.50%
	Weak	**Count**	2	0	2
			6.70%	0.00%	5.00%
Total		**Count**	30	10	40

	% within Group	100.00%	100.00%	100.00 %	

This table shows that shows that a comparison of staining of OdontogenicKeratocyst and oral squamous cell carcinoma that maximum cases of KCOT exhibited moderately positive staining to alpha smooth muscle actin where as majority of cases of OSCC exhibited intensely positive staining to alpha smooth muscle actin.

TABLE 2

SHOWING COMPARISON OF THE DISTRIBUTION PATTERN OF MYOFIBROBLASTS BETWEEN ODONTOGENIC KERATOCYST AND ORAL SQUAMOUS CELL CARCINOMA

% OF POSITIVITY * Group Crosstabulation

		Group		**Total**
		MYOFIBROBLAST PATTERN IN KCOT	**MYOFIBROBLAST PATTERN IN OSCC**	
FOCAL	**Count**	8	0	8
	% within Group	26.70%	0.00%	20.00%
NETWORK	**Count**	5	7	12
	% within Group	16.70%	70.00%	30.00%
SPINDLE	**Count**	12	3	15
	% within Group	40.00%	30.00%	37.50%
NONE	**Count**	5	0	5
		16.70%	0.00%	12.50%
Total	**Count**	30	10	40
	% within Group	100.00%	100.00%	100.00%

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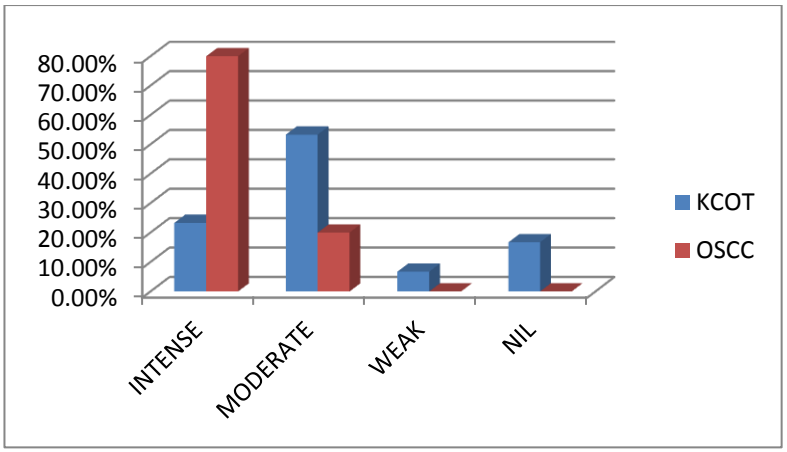
This table shows that a comparison of the distribution pattern of Myofibroblasts between OdontogenicKeratocyst and oral squamous cell carcinoma.maximum cases of KCOT exhibited spindle distribution pattern where as majority of cases of OSCC exhibited *network distribution* pattern of myofibroblasts.

GRAPH 1
COMPARISON OF POSITIVITY TO ALPHA SMOOTH MUSCLE ACTIN STAINING BETWEEN ODONTOGENIC KERATOCYST AND ORAL SQUAMOUS CELL CARCINOMA.



GRAPH 2
COMPARISON OF THE DISTRIBUTION PATTERN OF MYOFIBROBLASTS BETWEEN ODONTOGENIC KERATOCYST AND ORAL SQUAMOUS CELL CARCINOMA.

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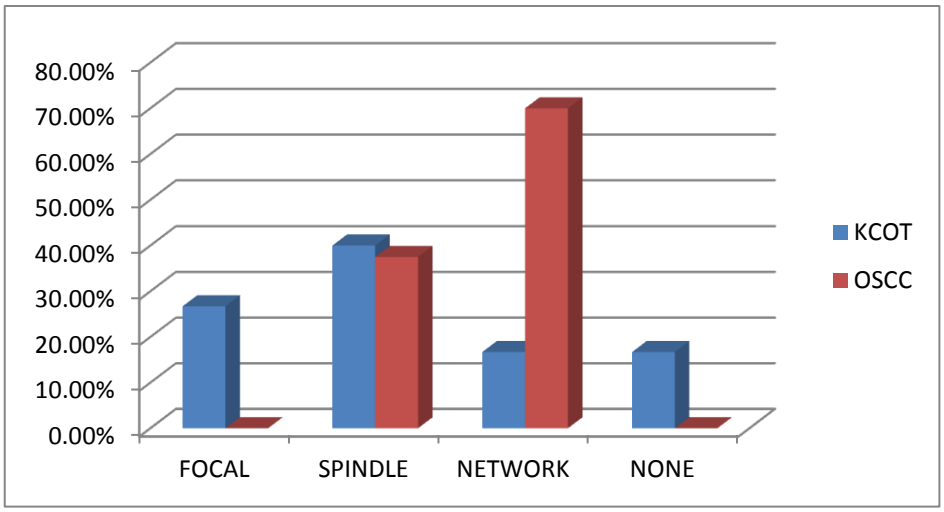

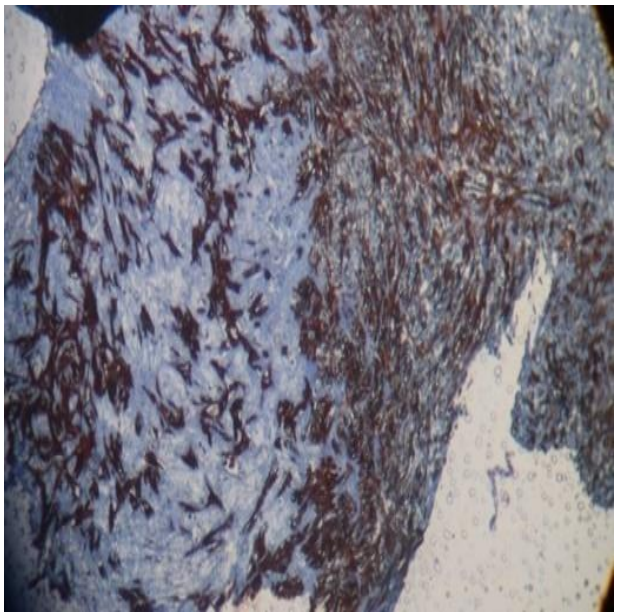

PICTURE 1:This picture shows an immunohistochemically stained section of keratocysticodontogenic tumor (40x) showing Myofibroblasts in a spindle distribution pattern.

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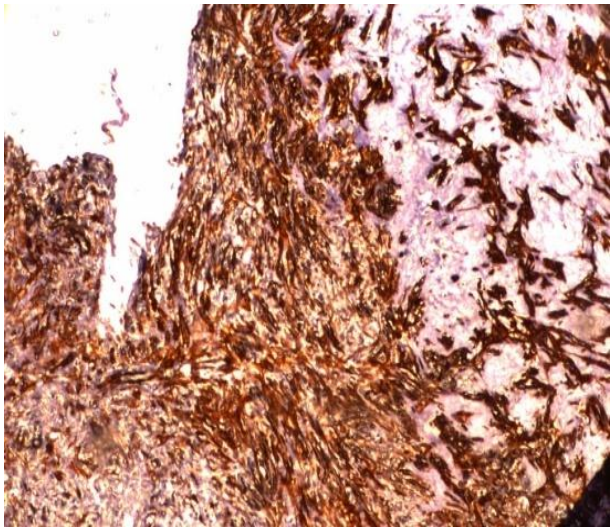
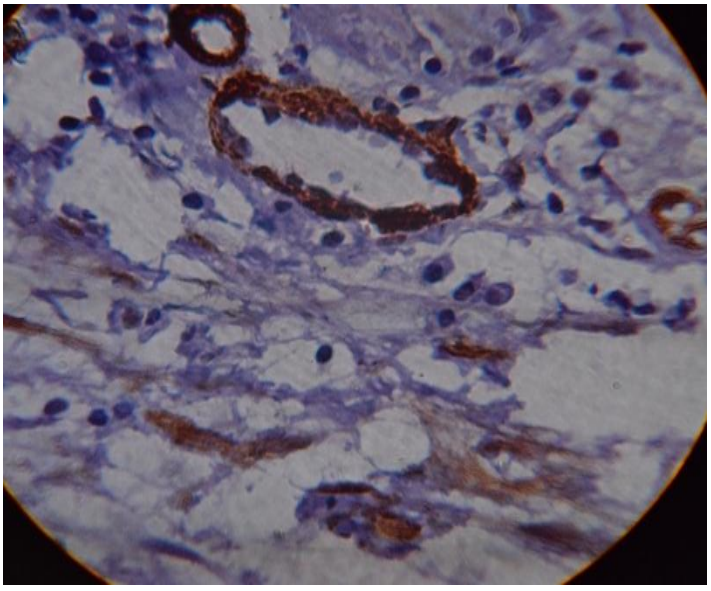


PICTURE 2: This picture shows an immunohistochemically stained section of keratocysticodontogenic tumor (40X)showing intensely positive staining of myofibroblasts in Network distribution pattern.



PICTURE 3: This picture shows immunohistochemically stained section of keratocysticodontogenic tumor (40x) showing α-sma positive blood vessel in the stroma.

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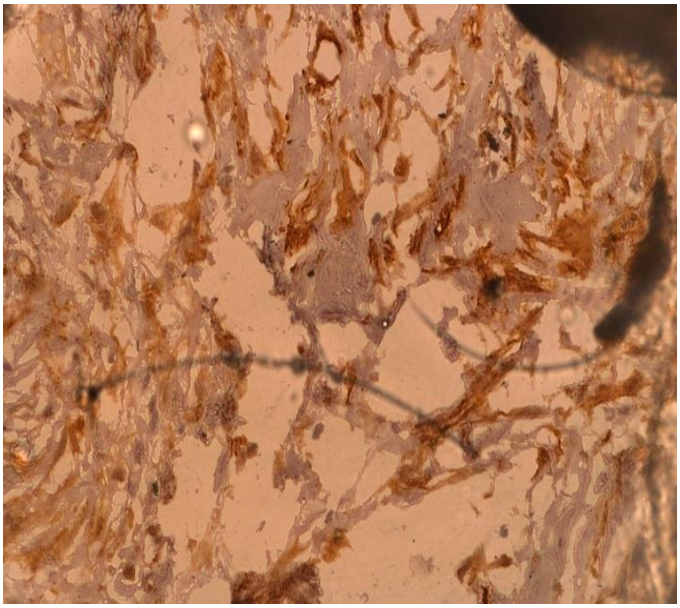

PICTURE 4:This picture shows an immunohistochemically stained section of keratocysticodontogenic tumor showing weakly positive staining for Myofibroblasts**.**(40x)

6. DISCUSSION:

Myofibroblasts (MFs), first observed by "Gibbani" in granulation tissue during wound healing are specialized fibroblasts that possess contractile functions, and are thought to develop from Fibroblasts under conditions of stress. It has been thoughtthese cells play a role in physiological processes like wound healing and pathologic conditions like reactive lesions, benign tumors, locally aggressive tumors and malignancies affecting oral cavity. Their prominent appearance in neoplastic lesions lead to the supposition that they probably have some role to play in the pathology of some lesions, Their association in neoplastic conditions has been so widely studied in a short span of time, that some investigators refer to these cells as **Cancer Associated Fibroblasts.**It is now evident that MFs arise from variety of sources, and do not have a common origin, as was previously thought. The various sources include leukocytes, fibroblasts, vascular smooth muscle cells, pericytes. However, the major source of MFs in whatever lesion they appear is the fibroblast. Alpha-smooth muscle actin (alpha-SMA) is the actin isoform that predominates within vascular smooth-muscle cells and plays an important role in fibrogenesis. Myofibroblasts are metabolically and morphologically distinctive fibroblasts expressing alpha-SMA, and their activation plays a key role in development of the fibrotic response. The expression of alpha-SMA correlates with the activation of Myofibroblasts. Alpha Smooth Muscle Actin has been described as the most relevant molecule in identifying differentiated fibroblasts. Myofibroblasts were identified by immunohistochemical detection of alpha smooth muscle actin through numerous studies in a variety of pathological conditions including Oral Squamous Cell Carcinoma, Dysplasia, Verrucous Carcinoma (VC) and Oral Submucous Fibrosis (OSMF).

The OdontogenicKeratocyst has been a lesion of interest to the Oral Pathologist, ever since its first report by**Philipsen in 1952** . From its initial description as a Primordial cyst to its more recent classification as a benign tumor by the WHO in 2005, it has presented as a lesion

whose nature has intrigued and confused us for decades. Over the years, the epithelial features of this lesion have been widely studied. However, it is intriguing to suppose that the epithelial morphology is, in many instances, a response to underlying meachanisms. Numerous studies have been undertaken in the recent past on the presence of altered fibroblasts (myofibroblasts) in the stroma of Oral Squamous Cell Carcinoma. In general, it is said that the stroma is necessary for the maintenance of the epithelium. This statement reflects, a supportive but passive role of the connective tissue. However recent studies show that alteration of stromal component resulting from neoplastic changes in the adjacent epithelium includes the appearance of Myofibroblasts. TGF- β1 and PDGF released by neoplastic cells, even at pro-invasive state are responsible for differentiation of Myofibroblasts. TGF- β1 is extremely chemotactic for fibroblasts. Cancer cells secrete TGF-β1andfibroblasts migrate towards them, leading to their transdifferentiation into Myofibroblasts. These cells in turn, produce several factors and proteineases that together promote invasion and growth of neoplastic cells.

Myofibroblasts also are thought to secrete matrix metalloproteases which contribute to the destruction of the ECM facilitating tumor growth .Due to their ability to modify ECM, MFs are thought to actively participate in both tumor invasion and metastasis.It has been suggested that these myofibroblasts secrete **Fibroblast activation proteins (FAP**).Fibroblast activation proteins are a member of serine protease family, selectively expressed in the stromal fibroblast associated with epithelial cancers. The proteolytic activity of FAP has been shown to support tumor growth and proliferation. The expression of FAP on cell surface has dramatic effects on the motility, matrix degradation and invasive behaviour of cells.[14]Thepresence of myofibroblasts has been co-related with aggressiveness of the oral squamous cell carcinomas but the role of Myofibroblasts in epithelial odontogenic cyst has not been thoroughly investigated till now. As mentioned previously that KeratocysticOdontogenic Tumor although benign are known to demonstrate locally aggressive behavior and as few studies that have shown that it KCOT shows certain molecular genetic alterations that are also present in some neoplasms. Therefore, we attempted to study their presence in the stroma of the Keratocysticodontogenic tumor in order to determine if this variant could be helpful in determining the course and prognosis of the lesion. Though, all slides would have staining of the blood vessel wall as a positive internal control, we also thought it best to have a separate positive control group (of Oral Squamous Cell carcinoma lesions).

Further more, **Priya J et. al** (2014)[2] have described varied arrangement of Myofibroblasts in pathologic lesions, and have classified them according to the distribution and arrangement of the Myofibroblasts into:

- FOCAL: If the myofibroblast had focal arrangement or had no special arrangement in the different areas of the stroma
- NETWORK: If they show vesicular nucleus and abundant cytoplasm arranged in multiple rows with an interwoven network of cytoplasmic extensions forming a network in the stroma.

- SPINDLE: If they are arranged in one or three rows in a regular order at the periphery of the neoplastic islands or in the connective tissue with distinctive cell margins around tumor islands and malignant tissue.

Those of the positive cases, 23.30% showed intense staining, 53.30% showed moderate staining and 6.70% showed weak staining to α-SMA. We saw ***focal, spindle*** as well as ***network*** patterns, where 30% of the slides were showing ***spindle and 26.70%*** **showed** ***focal patterns***, whereas few slides (16.70%) exhibited network pattern. From the control group (oral squamous cell carcinoma), 10 out of 10 samples showed positive staining. 80% of the cases demonstrated intense and 20% demonstrated moderate staining to α-SMA. The pattern which was seen in the control group was predominantly of ***network*** followed by ***spindl***e type. This finding co-related well with the fact that some KeratocysticOdontogenic tumors behave more aggressively than others. **Gabhane H.M.et al. (**2015)[19] stated that the stroma is not only responsible for the support and maintenance of the epithelial tissue but it also plays a role in the aggressive behavior of these lesions. **KCOT** contains numerous myofibroblasts in their stroma, as seen in our study which is similar with that found in cases of OSCC. **Roy and Swati** in another study evaluated stromal myofibroblasts expression in keratocysticodontogenic Tumor and orthokeratinizedOOC and found that the mean number of alpha SMA positive cells in the connective tissue 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. This increased number of Myofibroblasts in KCOT correlates with its aggressive biological behaviour, a finding that was corroborated by our study. Based on these findings, we find it reasonable to assume that a positive link can be established between the number of Myofibroblasts in the stroma and the aggressiveness of the odontogenic cyst.

7. CLINICAL SIGNIFICANCE:

Numerous studies in the literature have established the fact that the presence of Myofibroblasts in the stroma of various lesions correlate directly with the aggressive behavior of these lesions. KCOT contains numerous Myofibroblasts in the stroma, as seen in our study, thus this can be clinically implied to predict the biological behavior, growth and progression of this lesion and this observation can therefore can hold prognostic significance in the future.

8. LIMITATIONS:

The limitation of this study was its small sample size and further studies comprising of a larger sample size are required to further establish the role of Myofibroblasts in Kcot and its relation with the known aggressive biological behavior of this lesion.

9. CONCLUSION:

Myofibroblasts, discovered recently have received widespread interest as is is supposed that they may hold the key to predicting the nature of progress of a lesion, and thus may have prognostic significance. This is important, not only from an academic standpoint, but also

from a clinical viewpoint, and it has direct implications on treatment protocol. In our study, we found positive staining for Myofibroblasts in 83.3 %age of the lesions stained positively for alpha SMA. Also, we found a predominantly spindle shaped pattern in KCOT, as compared to a predominantly network pattern in OSCC cases. Therefore, according to the results of our study, quantitative evaluation and pattern of MFs in KCOT which is accepted as aggressive odontogenic lesion shows positive results, but at the same time differs from that of OSCC. This suggests that while there is a positive association of MFs and aggressiveness of the lesion at the growth front, but the aggressiveness is not comparable to cancer, which is a well established clinical fact. To conclude, we feel that at this point of time, we can safely suggest that increase in number of Myofibroblasts and change in their distribution pattern are of great value in predicting the possible biologic behavior of such lesions, and may hold prognostic significance in the near future.

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