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ORIGINAL ARTICLE

EVALUATING THE PRESENCE AND DISTRIBUTION OF MYOFIBROBLASTS IN ORAL SUBMUCOUS FIBROSIS, ORAL SQUAMOUS CELL CARCINOMA AND FIBROMA: AN IMMUNOHISTOCHEMICAL STUDY

Evaluación de la presencia y distribución de miofibroblastos en la fibrosis submucosa oral, el carcinoma escamocelular oral y el fibroma: un estudio inmunohistoquímico

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ABSTRACT

Background: Myofibroblasts are differentiated fibroblasts that carry out the function of physiological repair upon injury within the tissue and also are considered to be responsible for inducing fibrosis causing pathological remodelling of tissue. These cells take up alpha-smooth muscle actin (α-SMA) stain which helps to determine their biological activity in physiological as well as pathological conditions. **Aim:** To evaluate the presence and distribution of myofibroblasts in Oral Submucous Fibrosis (OSMF), Oral Squamous Cell Carcinoma (OSCC), and fibroma using α-SMA immunohistochemical (IHC) marker.

Materials and Methods: An the present study evaluation of the expression of myofibroblasts in OSMF (Group-1; n= 20), OSCC (Group-2; n=35) and fibroma (Group-3; n=20) using IHC using α-SMA in formalin-fixed, paraffin-embedded tissue specimens was carried out. The staining intensity, percentage of cells and staining index between the three groups was analysed. **Statistical analysis:** Spearman correlation test was used to analyse cell distribution, stain type, and percentage of stained cells across the three research groups.

Results: Staining Index was highest in group II i.e. OSCC (70%) compared to group I *i.e.* OSMF (20%). In OSCC cases, moderately differentiated oral squamous cell carcinoma exhibited intense staining intensity whereas OSMF showed mild to moderate intensity and fibroma did not show any intense staining intensity.

Conclusion: We conclude that fibroblast myofibroblast staining increases in increasing grades of OSCC. When compared to OSCC, OSMF (Oral Potentially Malignant Disorder) displayed a nearly identical ratio of mild to moderate staining intensity. When correlated with aetiology, fibroma, a benign tumour, only exhibits mild to moderate severity and is mostly associated with a traumatic background.

Keywords: Actins; Fibroma; Myofibroblasts; Oral Submucous Fibrosis; Oral Squamous Cell Carcinoma; Immunohistochemistry.

RESUMEN

Antecedentes: Los miofibroblastos son fibroblastos diferenciados que llevan a cabo la función de reparación fisiológica tras una lesión en el tejido y también se consideran responsables de inducir la fibrosis, lo que provoca la remodelación patológica del tejido. Estas células absorben la tinción de actina de músculo liso alfa (α-SMA), que ayuda a determinar su actividad biológica en condiciones fisiológicas y patológicas. **Objetivo:** Evaluar la presencia y distribución de miofibroblastos en fibrosis submucosa oral (OSMF), carcinoma de células escamosas oral (OSCC) y fibroma utilizando el marcador inmunohistoquímico (IHC) α-SMA.

Materiales y métodos: En el presente estudio se llevó a cabo la evaluación de la expresión de miofibroblastos en OSMF (Grupo 1; n = 20), OSCC (Grupo 2; n = 35) y fibroma (Grupo 3; n = 20) utilizando IHC utilizando α-SMA en muestras de tejido fijadas con formalina e incluidas en parafina. Se analizó la intensidad de tinción, el porcentaje de células y el índice de tinción entre los tres grupos. Se utilizó la prueba de correlación de Spearman para analizar la distribución celular, el tipo de tinción y el porcentaje de células teñidas en los tres grupos de investigación.

Resultado: El índice de tinción fue más alto en el grupo II, es decir, CCE (70%) en comparación con el grupo I, es decir, CME (20%). En los casos de CCE, el carcinoma de células escamosas oral moderadamente diferenciado exhibió una intensidad de tinción intensa, mientras que el CME mostró una intensidad de leve a moderada y el fibroma no mostró ninguna intensidad de tinción intensa.

Conclusión: Concluimos que la tinción de fibroblastos y miofibroblastos aumenta en grados crecientes de CCE. En comparación con el CCE, el CME (trastorno oral potencialmente maligno) mostró una proporción casi idéntica de intensidad de tinción leve a moderada. Cuando se correlaciona con la etiología, el fibroma, un tumor benigno, solo exhibe una gravedad de leve a moderada y se asocia principalmente con un trasfondo traumático.

Palabras Clave: Actinas; Fibroma; Miofibroblastos; Fibrosis de la submucosa bucal; Carcinoma de células escamosas de cabeza y cuello; Inmunohistoquímica.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC), one of the most prevalent malignancies, still presents a constant number of new cases and associated mortality. The longterm survival rate of patients with oral cancer has not changed significantly, despite recent advances in primary prevention and therapy. Patients with oral cancer continue to have high rates of morbidity and mortality, which have increased in developing countries.[1-3] Among the many intrinsic variables influencing tumour biology, interplay between tumour cells and their surrounding microenvironment are critical for cancer formation and progression.[4]

Cancer-associated fibroblasts (CAFs) are a subset of cells in the tumour stroma that have a myofibroblast like phenotype and have been found in the stroma of several carcinomas such as OSCC[5] and breast[6] colorectal[7] and hepatocellular carcinoma.[8,9]

As in oral cancer, clinical and histologic criteria are at times insufficient to determine tumour aggressiveness, the components of tumor microenvironment determine the aggressive nature of the disease. In many cases, fibroblast proliferation precedes the development of malignancy, and fibroblasts are commonly detected around early or pre-malignant tumours.[10,11]

The progressive nature of the alterations seen has led to the supposition that the bulk of stromal fibroblasts are produced from local fibroblasts that have suffered tissue malfunction.[12] Observations and experiments of the study related to early lesions showed fibroblasts surrounding these lesions increasing the possibility of tumour suppression,[13] with sub-sequent events in stromagenesis generating protumorigenic fibroblasts. Myofibroblasts are contractile cells and arise from many origins.

Mainly, they have been considered as differentiated fibroblasts. They are associated with oncogenesis, fibrosis, inflammation, morphogenesis and wound healing.[14] The appearance of myofibroblasts is not seen much in the normal tissues, but are seen only when there is tissue injury where they bring about fibrogenesis after which they undergo apoptosis. But at the same time, if they remain in the stroma for a longer period of time and due to their excessive contraction and extra-cellular matrix protein secretion, organ function may be impaired.[15]

Invasion and metastasis, which are the hallmarks of malignancy, are caused due to changes in the stroma. Appearance of myo-fibroblasts at the invasion front within the stroma of epithelial tumors is considered as a stromal change and is said to advance progression of cancer invasion.[16] Similar to malignant alterations, myofibro-blasts can also be detected in Oral Potentially Malignant Disorders (OPMDs) in the oral cavity like Oral Submucous Fibrosis (OSMF).

Oral sub-mucous fibrosis is a frequent precancerous disorder of the oral mu-cosa that can affect any area of the oral cavity.

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Furthermore, the steady rise of myofibroblasts from early to late stages implies that they might be used as markers to assess the severity of OSMF.

Also, because myo fibroblasts are responsible for creating a range of components involved in fibrotic processes, they may represent a significant link in the pathogenesis of OSMF.[17] Fibroma, which is a benign soft tissue neoplasm commonly occurring in the oral cavity, occurs by proliferation of benign fibroblasts.

Histopathologically it consists of bundles of interlacing collagenous fibers interspersed with varying members of fibroblasts or fibrocytes. One of the markers used to indicate myofibroblasts is α-SMA, but only fully differentiated myofibroblasts take up this marker. The increase accumulation of Transforming Growth Factor-ß1 (TGF-β1), the presence of important extracellular matrix proteins, and high extracellular stress are three local events required to generate α-SMA positive differentiated myofibroblasts (Figure 1) which is usually evident in malignancy like OSCC.[18]

Thus, fibroblasts, which are the main component of connective tissue stroma, proliferates in both benign and malignant tumors. But, in case of pre malignant condition and in malignant conditions, they transform into myofibroblasts which are also a variant of cancer asso-ciated fibroblasts.

Understanding the involvement of myofibroblasts in oral cancer growth and metastasis with the help of IHC markers will help us better understand the role of the tumour microenvironment in tumour progression and metastasis. This study aims to understand the intensity, location and distribution of myofibroblasts in the stroma of diagnosed cases of OSMF, OSCC and fibroma.

MATERIALS AND METHODS

Tissue Sample

A total of 75 tissue samples were taken from archival blocks from the Department of Oral Pathology and Microbiology, Dr. D. Y. Patil Dental College and Hospital, Pune. The ethical and scientific committee approval for the study was obtained (DYPDCH/IEC/164/168/20). Routine tissue processing along with staining by Hematoxylin and Eosin (H&E) was carried out.

α-SMA marker (Invitrogen, 1:800 dilution) was used for Immunohistochemistry (IHC) staining of the slides using standard protocol.

Study Groups

Seventy five formalin-fixed, paraffinembedded tissue specimens are divided into 3 groups:

- Group I- OSMF (20 samples),
- Group II- OSCC (35 samples)
- Group III – Fibroma (20 samples)

Immunohistochemistry Procedure

α-SMA

The IHC for the detection of α-SMA antigen was performed on polylysine-coated slides with paraffin sections of 4 µm thickness.

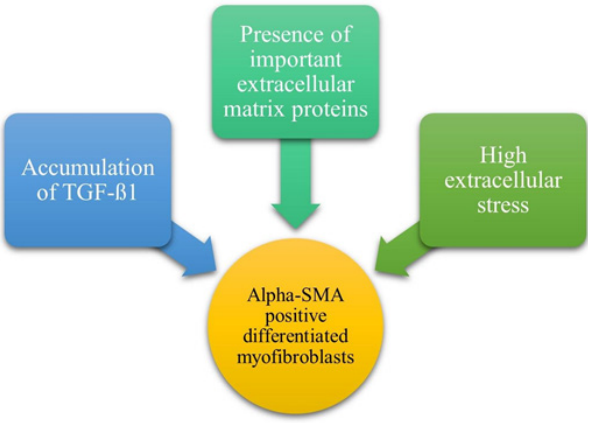
The tissue sections were deparaffinized with xylene thrice (5 mins each) and hydrated

by placing the slides in 100% and 70% isopropanol for 5 mins each. The slides were washed with distilled water thrice for 5 mins each. Slides were then kept in citrate buffer pH6 in a pressure cooker and three whistles were counted.

The sections were allowed to cool down completely for 20-30 mins. The sections were washed with distilled water. Protein blocking serum was added and incubated for 10 mins. These slides were washed with Phosphate buffer solution (PBS) once. A primary antibody (α-SMA antibody, Invitrogen, 1:800 dilution) was added to the specimen and incubated for 60 mins. The sections were washed with PBS thrice. A secondary antibody was added to the section for 30-40 min. Sections were then washed with PBS twice. Diaminobenzidine (DAB) was added for 10 mins. The sections were washed with distilled water and stained with hematoxylin for 2-3 mins.

The slides were then removed, washed with PBS, and then washed with distilled water. The slides were then dried and mounted. Positive and negative controls were conducted concurrently with the study specimens for every staining batch. The internal positive control was the endothelium staining of blood vessels known to be reactive to α-SMA, whereas the primary antibodies were substituted with non-immune mouse serum at the same dilutions for the negative controls. The positive control was normal buccal mucosa tissue.

Figure 1.
Factors responsible for generating α-SMA positive differentiated myofibroblasts



Calculation of staining intensity, distribution of cells and staining index

Staining Intensity (SI)

IHC staining was evaluated by counting the proportion of α-SMA-positive cells and SI). 19 The percentage of immune-positive cells was determined and recorded for each of the four high power fields and for the non-endothelial and noninflammatory cells at the tumour invasive front of OSCC and the non-inflammatory and non-endothelial stromal cells in the subepithelial connective tissue of OSMF.

The entire connective tissue was evaluated in cases of benign proliferation, in fibroma. The evaluation of IHC procedure was done independently by two trained observer in a manual manner.

The staining intensity was calculated as given in Table 1: Percentage of cells (Labelling Index-LI)/ Distribution of cells.

- The labelling index or the distribution of cells was determined by the numbers given in Table 2:

Staining Index

It is calculated by multiplying the staining intensity by the percentage of cells. (SI x LI). The final score is classified as having no stain if it is 0, light if it is 1-2, moderate if it is 3-4, and extreme if it is 6-9.

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Statistical analysis

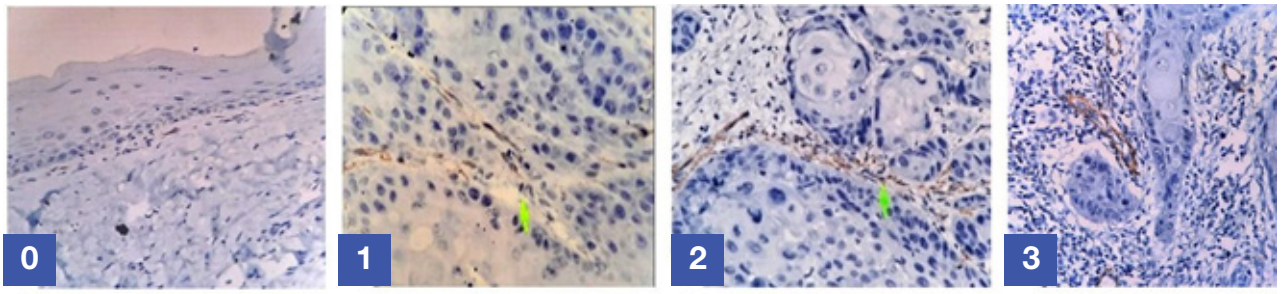
IBM SPSS software was used for statistical analysis (version 21). Statistical significance was defined as $p \leq 0.05$. The Spearman correlation test was used to analyse cell distribution, stain type, and percentage of stained cells across the three research groups.

Kappa statistics value was 0.742 regarding inter-observer variability in stain intensity and percentage of cells stained.

RESULTS

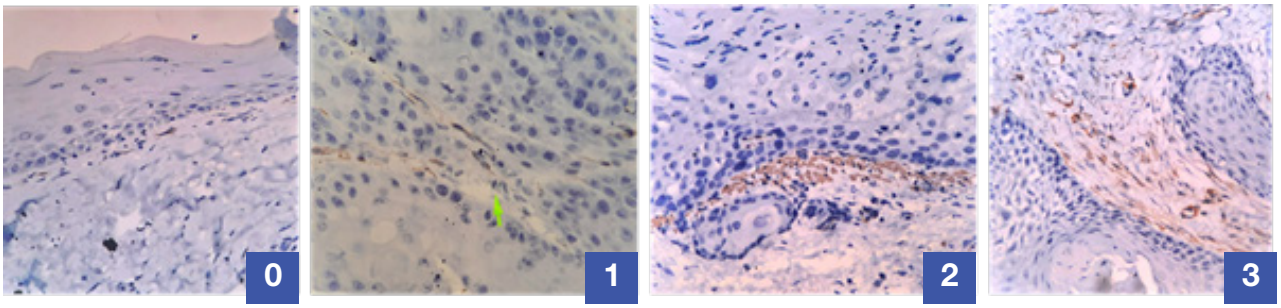
Twenty cases of OSF (Group I), 35 cases of OSCC (Group II) and 20 cases of fibroma (group 3) were analysed for immune reactivity of myofibroblasts. The association of the myofibroblasts with clinicopathologic findings and histologic classification in OSMF and OSCC are shown in Table 4; details can be found in the supplementary materials.

Figure 2.
Staining intensity scoring.



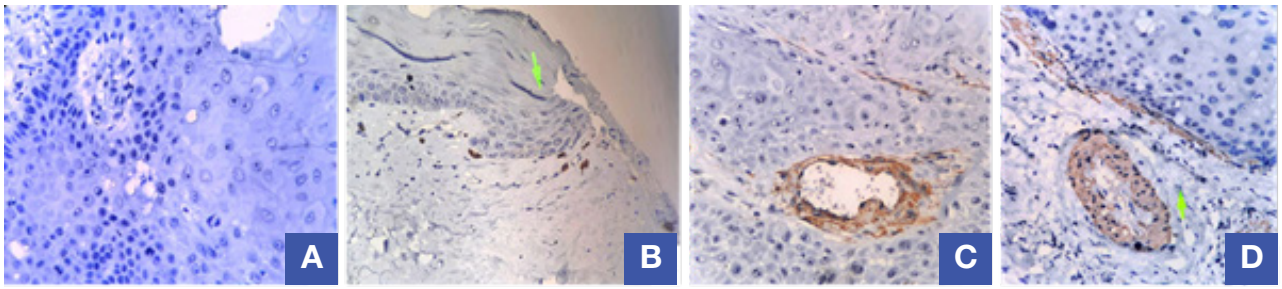
0: No stain visible. **1:** Visible only under 40X. **2:** Visible under 10X. **3:** Visible at 4X.

Figure 3.
Distribution of cells scoring.



0: No stain visible. **1:** 1-25% cells. **2:** 25-50% cells. **3:** 100% cells.

Figure 4.
Staining Index.



A: No Stain. **B:** Mild. **C:** Moderate. **D:** Intense.

Table 1.

Staining intensity calculations.[19]

Staining Intensity [SI]	Stain visibility
0	No stain visible
1	Visible only under 40X
2	Visible under 10X
3	Visible at 4X

Table 2.

Distribution of cells calculation.[19]

[Labelling Index-LI]/ Distribution of cells	% of cells showing staining
0	no staining
1	1-25% cells
2	25-50% cells
3	50-100% cells

Table 3.

Calculation of I x D.[19]

Staining index	Score
No stain	0
Mild	1-2
Moderate	3-4
Intense	6-9

Table 4.

Association of various clinicopathologic findings between OSCC, OSMF and fibroma patients.

		OSCC	OSMF	Fibroma
Mean Age		54	44	61
Gender	Female (n)	9	4	9
	Male (n)	26	16	11
Tumor Location	a. Alveolar mucosa	10		
	b. Buccal mucosa	12	20	18
	c. Gingivobuccal complex	05	---	---
		01	Also	2
	d. Tongue	07	showed	---
	e. Others	---	Tongue involvement	---
Histopathological grading	Well differentiated	22		
	Moderately differentiated	12		
	Poorly differentiated	01		
Lymph node status	Positive	19		
	Negative	16		
TNM staging	Stage I	---		
	Stage II	6		
	Stage III	12		
	Stage IV	17		

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OSCC & α-SMA

Distribution of cases

In a study of 35 cases of OSCC, ages ranged from 32 to 79 years, with a mean age of 53.7. The gender distribution was 74.3% male and 25.7% female, which aligns with existing literature. (Supplementary material).

The distribution of cases by site showed that the alveolar and buccal mucosa accounted for the highest proportion, comprising 54% of the cases. [ST: 3 and SF: 3]. OSCC grading and staging have shown Well Differentiated Squamous Cell Carcinoma (WD-SCC) (62.9%) and Stage IV (48.6%) [ST 4 and 5, SF 4 and 5]. Distribution of patients based on lymph nodes (LN) were 54.3% positive and 45.7% negative; LN were similar. [ST 6 and SF 6].

Association of myofibroblasts with age and Gender and Site

There was no significant correlation between age, gender, site and association of myofibroblast staining index. [ST 7, 8 and 9, SF 7, 8 and 9]

Association of myofibroblasts with Grading and Staging

Though there was moderate to intense staining index in Moderately Differentiated Squamous Cell Carcinoma (MDSCC) and Stage IV cases, there was no significant correlation between grading, staging and staining index in patients. [ST 10 and 11, SF 10 and 11]
LN status also did not show any significant correlation between LN status and staining index. [ST 12 and SF 12]

Association of myofibroblasts and OSMF

Distribution of patients based on staining index in OSMF 70% cases showed mild to intense staining and around 30% did not show any staining [ST 13 and SF 13]

Association of myofibroblast and fibroma

Around 45% patients in the fibroma group did not show staining whereas 55% showed mild to moderate staining with no intense type of staining. [ST 14 and SF 14]

There was a substantial difference in the staining index values between the three groups when the myofibroblast association in OSCC, OSMF, and fibroma was examined. Patients with intense staining are more in OSCC compared to fibroma and OSMF. Mild staining patients are comparable in OSCC and OSMF but less in fibroma.

Moderate staining cases are more in the fibroma group followed by OSMF and oral squamous cell carcinoma. Patients with no stains are least in OSCC and maximum in fibroma group [Table 4] In most of the cases, CAF is seen at the tumor invasion front close to the epithelium.

DISCUSSION

Oral cancer is the fifth common cancer affecting the Indian population. Despite significant advances in the treatment of oral cancer, it continues to have a high mortality rate. Understanding the immunopathogenesis in oral cancer would improve detecting predictability and prognosis of these cases. OSMF is one of the OPMD which has a higher rate of malignancy transformation and is one step before formation of OSCC hence this study group was also included.[20]

In this study of 35 cases of oral squamous cell carcinoma, ages ranged from 32 to 79 years, with a mean age of 53.7 years, and a male prevalence was observed.

This is consistent with the study by Dourado *et al.*,[21] which reported a mean age of 56 years for 34 patients, with an age range of 27 to 89 years, also with male prevalence (74.3% *versus* 25.7% females). Another study reported similar findings, with the majority of patients being male with a mean age of 60 years 22. The site distribution of the cases in this study shows alveolar mucosa and buccal mucosa to be the maximum involved site. The study by Dourado et al noted the most common location to be the floor of the mouth, in 14 cases.[21] In the study conducted by Maqsood *et al.*,[23] the most common site of oral cancer was the buccal mucosa (45%). Freitas-Filho *et al.*,[22] showed that the top sites involved were tongue and the floor of the mouth.

The histological grading in this study showed most cases were of well differentia-ted squamous cell carcinoma[22] followed by moderately differentiated squamous cell carcinoma.[12] Our findings were in accordance with these studies conducted by Dourado *et al.*,[21] and Freitas-Filho *et al.*,[22] A study found histopathological lymph node metastasis in 53.2% of oral cancer patients, which is consistent with the findings of the present study.

In the present study moderately differentiated cases were more and showed staging of cancer ranging between Stage III to Stage IV and maximum number of these cases showed positive lymph node status, which is in agreement with another study in which α-SMA expression was observed more frequently in tumors whose size was more than 4 cms, had advanced T stage and N stage, and less frequently in those with well-differentiated tumors.[4] Another such finding was seen in a study in which myofibroblasts count was significantly higher in OSCC with LN metastasis when compared with OSCC without LN involvement.[25]

There was moderate to intense staining in MDSCC and stage IV cases. LN status and staining intensity showed the same status. Thus, the IHC staining was increased in cases with higher grading. In concordance with findings in previous studies,[26] there was a clear link between higher α-SMA expression and invasion in moderately differentiated tumours, as well as an increased risk of re-currence and LN involvement, indicating that CAFs may contribute to OSCC invasion and metastasis.

In the present study, most of the myo-fibroblasts were seen at the tumor invasive front close to the epithelium. The same observations were noted in another study, where α-SMA was diffusely distributed in 45 oral squamous cell carcinoma cases and identified staining exclusively in the membrane and cytoplasm located adjacent to tumor islets.[27] In this study, OSMF cases showed 70% staining while 30% did not show any staining and showed moderate to intense staining but comparatively less than OSCC cases.

The findings matched with the results of a study conducted by Gupta et al, who reported that the expression of α-SMA was high in oral squamous cell carcinoma without OSMF and in OSCC with OSMF. In another study similar results were reported, in which expression of α-SMA was found to be high in OSCC with OSMF and OSCC without OSMF compared to OSMF group.[28-29]

As demonstrated in OSMF, myofibroblasts play a role in fibrosis. Activated myofibroblasts produce proteolytic enzymes that degrade the matrix, which aids cancer cell invasion

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and metastasis.[14] This study also included a fibroma group, which is a benign tumor. The results showed 55% of cases were positive for α-SMA but the staining was not intense. In a study of 19 cases diagnosed as myofibroma were immunopositive and only one case of desmoplastic fibroma was positive for α-SMA.[30]

Their study concluded that the fibrogenic role of these myofibroblasts was confirmed as their detection was found more in giant cell fibroma, followed by fibromas and fibrous hyperplasia. They also report that these myofibroblasts may play a role in the neoplastic behavior of these lesions as higher concentrations were observed in giant cell fibromas with the appearance of giant myofibroblasts.[30]

In our study, nine cases of fibroma showing mild to moderate staining were of traumatic type of which five cases were diagnosed as traumatic fibroma and four as irritational fibroma. Thus, there is a definite conversion of fibroblasts to myofibroblasts in benign lesions like fibroma but the dynamics completely change in malignant tumors.[31]

Myofibroblasts have a role in the production of fibrous connective tissue in fibromas, which can result in benign tumour growth. In reaction to inflammation or injury, they aid in the synthesis of extracellular matrix components and tissue remodelling.

Myofibroblasts, on the other hand, are known to encourage tumour growth, invasion, and metastasis in oral cancer. They engage in interactions with cancer cells to establish a favourable microenvironment that pro-motes the growth of tumours.

In oral cancer, myofibroblasts can also encourage angiogenesis, immune evasion, and treatment resistance, which can exacerbate the disease's aggressiveness.

CONCLUSION

As myofibroblasts are considered as differentiated fibroblasts that play varied roles in physiological and pathological conditions, it is very important to evaluate their presence and distribution in pathologies.

Cancer-associated fibroblasts are considered as phenotypical similar cells to myofibroblasts or as dedifferentiated myofibroblasts, which can be demonstrated by α-SMA. The α-SMA stain is taken up by fully differentiated myofibroblasts. As such, there may be cells in the tumor stroma which are in process of differentiating into myofibroblasts that are not positive with the α-SMA marker.

Therefore, a thorough investigation of α-SMA for identifying myofibroblasts and their differentiation into cancer-asso ciated fibroblasts is required, considering tumor grading and using a larger sample size to ensure accurate results.

CONFLICT OF INTERESTS

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

ETHICS APPROVAL

The ethical and scientific committee approval for the study was obtained from Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, India [DYPDCH/IEC/164/168/20].

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AUTHORS' CONTRIBUTIONS

Vaibhav Ladke: Conceptualization, Data Curation, Formal Analysis, Investigation, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Writing – Original Draft.

Gauri Kumbhar: Data Curation, Visualization, Writing – Original Draft.

Supriya Kheur: Formal Analysis, Investigation, Supervision, Writing – Review & Editing.

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