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# MYOFIBROBLASTS IN ORAL SUBMUCOUS FIBROSIS, ORAL SQUAMOUS CELL CARCINOMA AND NORMAL MUCOSA- AN IMMUNOHISTOCHEMICAL ANALYSIS

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## ABSTRACT

**Introduction:** Myofibroblasts are spindle shaped smooth muscle like fibroblast involved in the physiological repair of tissue structure can also cause pathological remodeling of tissue by forming fibrosis. They express smooth muscle actin which can be evaluated for their biological behavior in the physiological and pathological conditions. **Objective:** To evaluate the expression of Myofibroblasts in oral submucous fibrosis, oral squamous cell carcinoma and normal mucosa by Immunohistochemical analysis. **Methods and materials:** It is a cross-sectional study done to evaluate the expression of Myofibroblasts in oral submucous fibrosis (Group-I), oral squamous cell carcinoma (Group-II) and normal buccal mucosa (Group-III) using immunohistochemistry using alpha smooth muscle actin in formalin fixed, paraffin embedded tissue specimens. The staining intensity, labeling index and staining index was calculated among the three groups and compared. **Results:** The gender distributions in the study were in favor of males. It was observed

that staining intensity and labeling index was seen more in group I than in group II and progressively increased from early to advance stage of oral submucous fibrosis. Higher staining intensity was observed in well differentiated oral squamous cell carcinoma. **Conclusion:** Myofibroblasts are important in normal healing process, but can induce fibrosis. In oral submucous fibrosis, they can be used to assess the severity of the lesion by their increased expression of alpha smooth muscle actin. In oral carcinogenesis increase in the number of alpha smooth muscle actin can change the distribution pattern results in tumor invasive characteristics.

**Keywords: Alpha smooth muscle actin, Myofibroblasts, Oral Squamous Cell Carcinoma, Oral Submucous Fibrosis, Staining intensity**

## INTRODUCTION

Myofibroblasts are spindle shaped cells which produce collagen and having contractile properties like smooth-muscle, hence known as smooth muscle like fibroblast. They express alpha smooth muscle actin (α SMA) **[1, 2]**. They primarily involved in wound healing by forming fibrous tissue and secrete extracellular matrixes helps in the repair process by reducing the physical size of the damage by their contractility properties. Once the tissue is repaired they disappear by apoptosis. Apart from the physiological repair process, they are involved in the pathological remodeling of the tissue in which they persist and form tissue deformation seen as hypertrophic scars **[3]**.

Fibrosis is a progressive disease characterized by accumulation of scarring extracellular matrix proteins, which disrupt normal tissue architecture **[3]**. Oral Submucous Fibrosis (OSF) is one such disorder characterized by exuberant deposition of sub epithelial collagen in response to chronic areca nut chewing resulting in mucosal rigidity that leads to limitation in mouth opening. In various fibrotic disorders, the key cellular mediator of fibrosis is myofibroblasts, which when activated serves as primary collagen-producing cell **[4, 5]**.

Oral squamous cell carcinoma (OSCC) is the malignant epithelial tumor commonly occurring in the oral cavity. Myofibroblasts are involved in the cancer progression by stimulating the microenvironment stromal cells.The activated Myofibroblasts express α-smooth muscle actin which represents the majority of tumor stromal cells **[5, 6]**. Thus, it is necessary to understand the expression of myofibroblast in the molecular mechanism of

oral submucous fibrosis and oral cancer progression **[7]**.

**Aim**

The aim of the study was to evaluate the expression of myofibroblasts in oral submucous fibrosis, oral squamous cell carcinoma and normal mucosa by immunohistochemical analysis using alpha smooth muscle actin.

**MATERIALS AND METHODS**

The study material comprised of 50 formalin fixed, paraffin embedded tissue specimens from archival blocks. The samples were divided into 3 groups namely: Group I- Oral submucous fibrosis (20 samples), GroupII-Oral squamous cell carcinoma (20 samples) and Group III – normal buccal mucosa (10 samples) taken as control group. Oral submucos fibrosis was graded in according to Pindborg classification to grade I, II and III. Squamous cell carcinoma was graded according to World Health Organization as well, moderately and poorly differentiated carcinoma.

**Methodology:**

Tissue samples were taken from archival blocks. Immunohistochemical staining was done with α-smooth muscle actin as primary antibody (Biogenex – synthetic NH2 terminal decapeptide of α-smooth muscle actin, Mouse monoclonal category and IgG2a immunoglobulin) and Secondary antibody used were Biogenex-super sensitive IHC detection system kit - Poly Horse Radish Peroxidase –pretitrated anti-species immunoglobulin labeled with enzyme polymer, super enhancer reagent, anti-mouse monoclonal negative control serum, and liquid DAB- Diamino-benzidine-chromogen) The technique for Immunohistochemical analysis was done as per the standardized method **(Figure-1).**

The staining intensity, labeling index and staining index was calculated and compare between the three groups and also between the grades of oral submucous fibrosis and oral squamous cell carcinoma. The whole slide was assessed by two examiners. The expression of alpha smooth muscle actin in the endothelial cell and the salivary acini were excluded. The calculation was done as per the following criteria.

**STAINING CRITERIA AND STAINING INDEX CALCULATION METHODS:**

***Calculation of staining intensity (SI):***

The calculation of staining intensity was considered 0 (SI 0) when no stain visible. If the staining visible only under 40X is calculated as 1 (SI 1), staining visible under 10X as 2 (SI 2) and staining visible even at 4X considered as 3 (SI 3).

***Calculation of percentage of cells (Labeling Index-LI)***

The calculation of percentage of cells, labeling index was done as there is no positive cells it was considered as 0 (LI 0), if 1-25% positive cells was calculated as 1 (LI 1), 25-50% positive cells was calculated as 2 (LI 2) and 50-100% positive cells was seen as 3 (LI 3).

***Calculation of staining index***

It is derived from multiplication of staining intensity and percentage of cells. (SI xLI)

The final score is grouped into no stain if the score is 0, mild if the score is 1-2, moderate if the score is 3-4 and intense categories if the score is 6-9.

**Statistical analysis:**

Statistical analysis was done using SPSS ™ software (version 11.5). $p \leq 0.05$ was considered to be statistically significant.

- Kruskal Wallis and Mann-Whitney test was done to compare the intensity of stain and the percentage of cells stained among the three study groups.
- The inter-observer variability for the staining intensity and labeling index was assessed using kappa statistics.

**RESULTS**

Twenty cases of OSF (Group I), 20 cases of OSCC (Group II) and 10 cases of normal mucosa (Group III) were analyzed for immune reactivity of myofibroblasts. All the samples were taken from the buccal mucosa.

***DISTRIBUTION OF GENDER AMONG GROUPS***

The males were predominant in the study compromising 95% in group I, 75% in group II and 80%in group III.The age groups were divided into 20- 40 years, 41 - 60 years and 61+ years. In Group I, 65% belonged to age Group 20-40 years and 35% belonged to age Group 41-60 years. In Group II, 10% belonged to age Group 20-40 years and 65% belonged to age Group 61+years. In Group III 90% belonged to age Group 20-40 years and 10% belonged to age Group 41-60 years.

**I.STAINING INTENSITY**

***Distribution of Staining Intensity (SI) Of α-SMA (Alpha Smooth Muscle Actin) Among 3 Groups:***

α-SMA revealed positivity in group I, II and III. In Group I , the positivity showed 85% with the expression of myofibroblast (**Figure-2,3),** in Group II cases showed 85% staining for α-SMA **(Figure-4,5)**, whereas in Group III, positive staining was observed 30% (**Figure -6)**.

The highest score in Group I and Group II cases was observed in SI 1(35%) and In Group III 70 % had a score of 1 (SI 1). The p

values was 0.156 among the groups which is statistically insignificant (**Figure-7A)**.

***Distribution of Staining Intensity among Different OSF Grading:***

In 20 cases of OSF, 6 cases belonged to Grade I, 10 cases belonged to Grade II and 4 cases belonged to Grade III. In Grade I 50% had a score of 1 (SI 1). In Grade II 40 % had a score of 1 (SI 1) and in Grade III 50 % had a score of 3 (SI 3). The p value was 0.182 (**Figure-7B)**.

***Distribution of Staining Intensity among Different OSCC Grading:***

In 20 cases of OSCC, 13 cases belonged to well differentiated, 3 cases belonged to moderately differentiated and 4 cases belonged to poorly differentiated. In well differentiated 30.8 % had a score of 3 (SI 3) which is the highest. In moderately differentiated group 66.7 % had a score of 1 (SI1) and 33.3 % had a score of 2 (SI 2). In poorly differentiated group 50 % had a score of 1 (SI 1) and 50 % had a score of 2 (SI 2). The p value was 0.405 **(Figure-7C)**

**II. PERCENTAGE OF IMMUNOPOSITIVE CELLS-LABELING INDEX (LI)**

***Distribution of Labeling Index (LI) Of α-SMA (Alpha Smooth Muscle Actin) Among 3 Groups:***

α-SMA revealed positivity in group I, II and III. In Group I and Group II cases showed 85% staining for α-SMA, whereas in Group III, positive staining was observed 40%.

The labeling index LI 1 was seen highest in Group I (40%) and Group II (85 %). In Group III out of 10 normal cases 60 %had a score of 0 (LI0) and 40 % had a score of 1 (LI1).the p value was statistically significant 0.000 (**Figure-8A)**.

***Distribution of Labeling Index (LI) Among Different OSF Grading:***

Total 20 cases of OSF, 6 cases belonged to Grade I, 10 cases belonged to Grade II and 4 cases belonged to Grade III. In Grade I and Grade II ,LI 1 was seen in 50% of the cases .In Grade III 50 %had a score of 0 (LI 0) and 50 % had a score of 2 (LI 2).the p value was 0.242. **(Figure-8B)**

***Distribution of Labeling Index (LI) Among Different OSCC Grading:***

Total 20 cases of OSCC, 13 cases belonged to well differentiated group, 3 cases belonged to moderately differentiated and 4 cases belonged to poorly differentiated. In well differentiated group, 23.1% had a score of 0 (LI 0) and 76.9 % had a score of 1 (LI 1). In moderately and poorly differentiated group all had a score of 1 (LI1). The p value was 0.387 **(Figure-8C)**.

**KAPPA STASTISTIC VALUE**

The inter-observer agreement for the staining intensity of stain and labeling index for all the 3 groups was arrived using kappa statistics and kappa value is 0.689.

**III. STAINING INDEX**

***Distribution of Staining Index among the Groups.***

In group I 15% had no stain, 40% had mild stain,20%had moderate and 25% had intense stain. Using Kruskal-Wallis test the comparison among groups derived, there is a significant difference with p value 0.004 in the expression of myofibroblasts. (**Table-1)** (**Figure-9A)**

***Comparison Of Staining Index Between Group I And Group III***

In OSF, 15% of the cases showed no staining while 40% mild staining, 20% moderate staining and 25% of the cases had intense staining. In normal mucosa, 40% exhibited mild staining and no staining was seen in 60% of the cases. The result is statistically significant with p value 0.005 (**Table-2)**.

***Comparison Of Staining Index Between Group II And Group III***

In OSCC, 15% of the cases showed no staining while 65% mild staining and 20% moderate staining. In normal mucosa, 40% exhibited mild staining and no staining was seen in 60% of the cases. The result is statistically significant with 0.019. (**Table-3)**

***Comparison Of Staining index Between Group I And Group II***

In OSF group, 15% of the cases showed no staining while 40% mild staining, 20% moderate staining, and 25% of the cases had intense staining. In OSCC group, 15% of the cases showed no staining while 65% mild staining and 20% moderate staining. As both the lesions expressing myofibroblasts, the result is statistically not significant with p value 0.157. (**Table-4)**

***Comparison of Staining index In Different OSF Grading***

In Grade I, 16.7% showed no stain, 50% showed mild stain 16.7% moderate and 16.7 % showed intense staining. In Grade II 50 % showed mild staining 20 % showed moderate and 30% showed intense staining. In Grade III 50 % showed no stain, 25% showed moderate and 25 % showed intense staining. The p value was 0.309 (**Table-5) (Figure-9B)**.

***Comparison of Staining index In Different OSCC Grading***

In well differentiated group 23.1% showed no stain, 46.2% showed mild stain and 30.8% showed moderate staining. In OSCC moderately differentiated 100% showed mild staining. In OSCC poorly

differentiated 100% showed mild staining. **9C)**.

The p value was 0.215 (**Table-6) (Figure-**

**Table 1 : Distribution of Staining Index Amongthe Groups (n=50)**

| STAINING INDEX | | NO STAIN | MILD | MODERATE | INTENSE | p VALUE |
|---|---|---|---|---|---|---|
| GROUP I | n=20 | 3 | 8 | 4 | 5 | 0.004* |
| | % | 15 | 40 | 20 | 25 | |
| GROUP II | n=20 | 3 | 13 | 4 | 0 | |
| | % | 15 | 65 | 20 | 0 | |
| GROUP III | n=10 | 6 | 4 | 0 | 0 | |
| | % | 60 | 40 | 0 | 0 | |

**Table 2: Comparison Of Staining Index Between Group I And Group III**

| STAINING INDEX | NO STAIN (%) | MILD (%) | MODERATE (%) | INTENSE (%) | p VALUE |
|---|---|---|---|---|---|
| Group I (n=20) | 15 | 40 | 20 | 25 | 0.005* |
| Group III (n=10) | 60 | 40 | 0 | 0 | |

**Table 3: Comparison Of Staining Index Between Group Ii And Group III**

| STAINING INDEX | NO STAIN (%) | MILD (%) | MODERATE (%) | p VALUE |
|---|---|---|---|---|
| Group II (n=20) | 15 | 65 | 20 | 0.019* |
| Group III (n=10) | 60 | 40 | 0 | |

**Table 4: Comparison Of Staining Index Betweengroup I And Group II**

| STAINING INDEX | NO STAIN (%) | MILD (%) | MODERATE (%) | INTENSE (%) | pVALUE |
|---|---|---|---|---|---|
| Group I (n=20) | 15 | 40 | 20 | 25 | 0.157 |
| Group II n=20 | 15 | 65 | 20 | 0 | |

**Table 5: Distribution Of Staining Index Among Different Osf Gradings (n=20)**

| STAINING INDEX | | NO STAIN | MILD | MODERATE | INTENSE | p VALUE |
|---|---|---|---|---|---|---|
| OSF GRADE I | n=6 | 1 | 3 | 1 | 1 | 0.309 |
| | % | 16.7 | 50 | 16.7 | 16.7 | |
| OSF GRADE II | n=10 | 0 | 5 | 2 | 3 | |
| | % | 0 | 50 | 20 | 30 | |
| OSF GRADE III | n=4 | 2 | 0 | 1 | 1 | |
| | % | 50 | 0 | 25 | 25 | |

**Table 6: Distribution Of Staining Index Among Different Oscc Gradings (n=20)**

| STAINING INDEX | | NO STAIN | MILD | MODERATE | p VALUE |
|---|---|---|---|---|---|
| OSCC-WELL DIFFERENTIATED | n=13 | 3 | 6 | 4 | 0.215 |
| | % | 23.1 | 46.2 | 30.8 | |
| OSCC MODERATELY DIFFERENTIATED | n=3 | 0 | 3 | 0 | |
| | % | 0 | 100 | 0 | |
| OSCC POORLY DIFFERENTIATED | n=4 | 0 | 4 | 0 | |
| | % | 0 | 100 | 0 | |

**IHC PROCEDURE FLOW CHART**

- APES coated slides with 2 paraffin embedded tissues
- Placed in xylene thrice (5 minutes each)
- Placed in 100% isopropanol (5 minutes)
- Placed in 70% isopropanol (5 minutes)
- Washed in distilled water thrice (5 minutes each)
- Placed in 3% hydrogen peroxide (20 minutes)
- Kept in citrate buffer at pH 6 and autoclaved for antigen retrieval and bench cooled for40 minutes
- Washed in distilled water thrice (5 minutes)
- Protein blocking serum added and incubated for one hour
- Primary antibody added to the specimen and incubated for one hour
- Washed in PBS thrice (5 minutes each)
- Secondary antibody added and incubated in an enclosed hydrated container (30 minutes)
- Washed in PBS thrice (5 minutes each)
- Avidin biotin enzyme reagent added and incubated (30 minutes)
- Washed in PBS thrice (5 minutes each)
- DAB added and incubated in an enclosed hydrated container (5 minutes)
- Washed in PBS thrice (5 minutes each)
- Stained with haematoxylin (20 seconds)
- Washed in tap water
- Placed in 70% isopropanol (1 minute)
- Placed in 90% isopropanol (1 minute)
- Placed in 100% isopropanol (1 minute)
- Placed in xylene (1 dip)
- Slides were mounted using DPX
- Slides were observed under the LM and graded.

**Figure 1: Immunohistochemistry protocol**

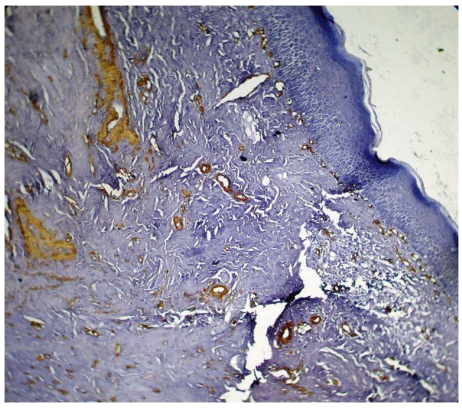

**Figure 2: Immunohisto stained section showed myofibroblast in OSF-10x**

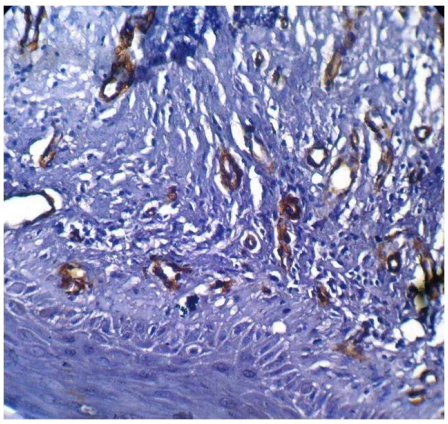

**Figure 3: Immunohisto stained section showed myofibroblast in OSF -40x**

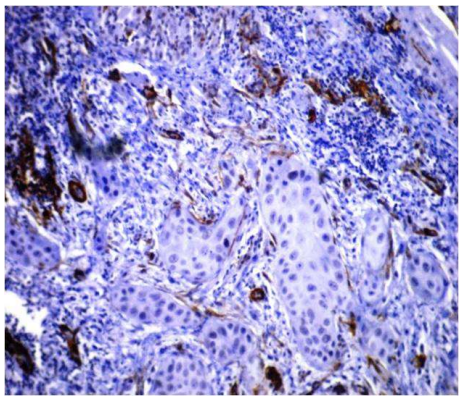

**Figure 4: Immunohisto stained section showed myofibroblast in OSCC-10x**

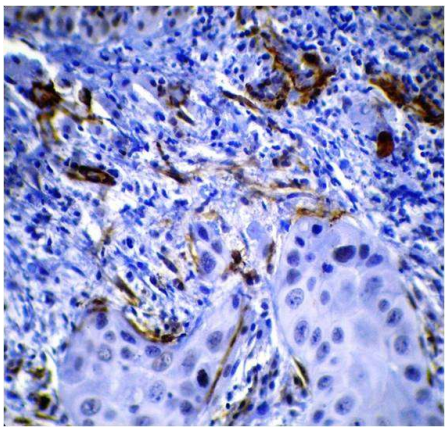

**Figure 5: Immunohisto stained section showed myofibroblast in OSCC -40x**

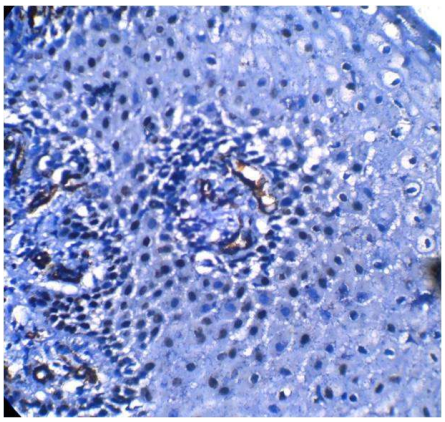

**Figure 6: Immunohisto stained section showed myofibroblast in normal tissue-10x**

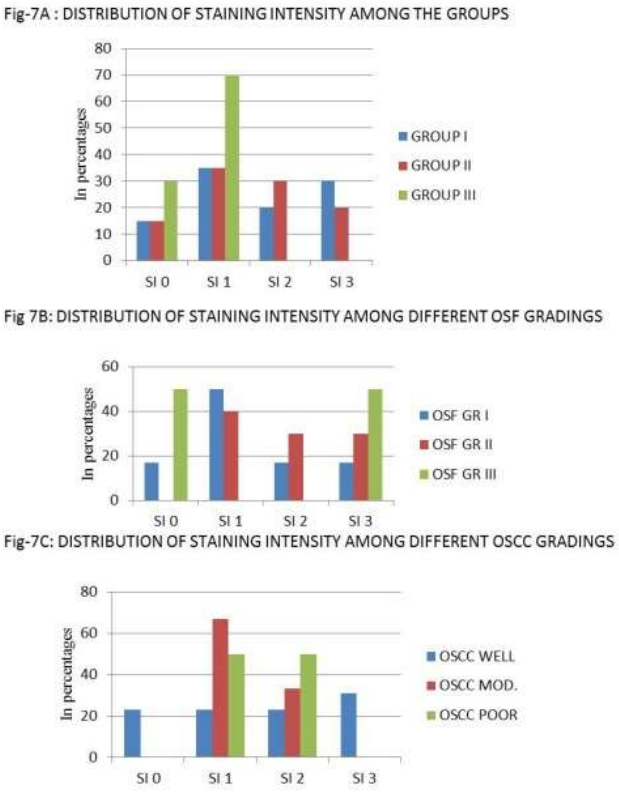


**Figure 7: Staining Intensity**

Fig-8A -DISTRIBUTION OF PERCENTAGE OF CELLS AMONG THE GROUPS

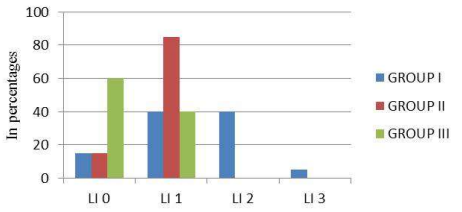


Fig-8B: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSF GRADINGS

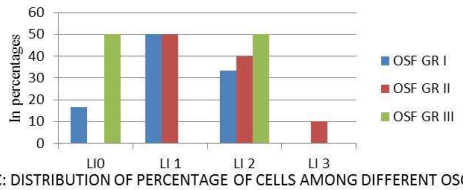


Fig 8C: DISTRIBUTION OF PERCENTAGE OF CELLS AMONG DIFFERENT OSCC GRADINGS

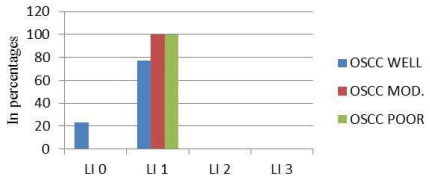


**Figure 8: Labeling Index**

Fig-9A : DISTRIBUTION OF STAINING INDEX AMONG THE GROUPS

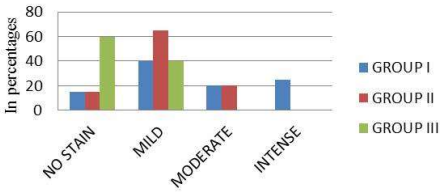


Fig 9B: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSF GRADINGS

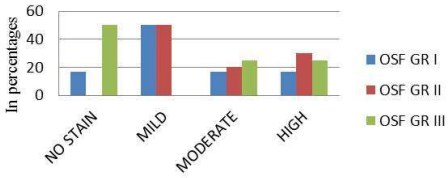


Fig 9C: DISTRIBUTION OF STAINING INDEX AMONG DIFFERENT OSCC GRADINGS

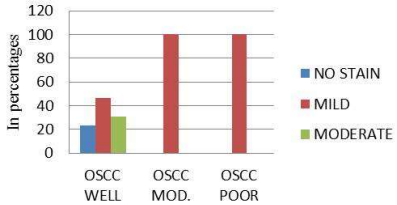


**Figure 9: Staining index**

## DISCUSSION

Myofibroblasts are smooth muscle like fibroblasts and one of the phenotypic heterogeneity among the fibroblasts. They can alter the inflammatory response by secreting the soluble mediators of inflammation. Furthermore myofibroblasts is suggested to be induced by TGFβ, a potent pro-inflammatory and pro-fibrotic cytokine related to imbalance between collagen deposition and degradation in Oral Submucous Fibrosis **[7]**. Hence the role of myofibroblasts in the repair process has varying functions in the physiological and pathological conditions

The distribution of genders in the study groups ranged 4:1 in favor of Males in the control group and 19:1 in the OSF group. The male preponderance among OSF has been documented in several studiesand can be due to the habit of using tobacco **[8]**.

The age distribution of OSF is higher between 20-40 years as similar to other studies reported in the literature.[7] Similarly the occurrence of OSCC commonly occurs among older age groups as reflected in our study.

***Staining intensity:***

Staining intensity between the three study groups was not statistically significant and the expression was seen more in group I than in group II (20%). The staining intensity progressively increased from early OSF (16.5%) to advance OSF (grade III 50%). This indicates that OSF represents a failed wound healing process of the oral mucosa after chronic sustained injury resulting in scarring and fibrosis. This could be also in response to the hypersensitivity caused by arecoline and the resultant persistent juxtra epithelial inflammatory response in OSF, which acts as an initiating factor leading to a defective inflammatory response and activation of fibroblasts culminating in fibrosis **[9, 10]**.

The well differentiated OSCC showed higher staining intensity indicating that myofibroblasts are stimulated during the repair of extra cellular matrix and are capable of augmenting and down-regulating the inflammatory response by secretion of these soluble mediators of inflammation **[9, 10]**.

***Labeling index:***

The labeling index indicates the more percentage of cells stain with α SMA in group I than in group II or III and can be due to chronic inflammation and continuous tissue remodeling in OSF as well as chronic sustaining injury to factors such as arecoline, micro trauma could cause the transient or continuous differentiation of fibroblast from

undifferentiated stem cell or a differentiated fibroblast pool **[11]**.

The labeling index among the various grades of OSF shows an increase from grade I to III. This indicates with increase of stimulus and disease grade occurs on more amount of myofibroblasts differentiation **[12]**. However the difference between the grades were not statistically significant in OSCC with varying grades of differentiation, no significant difference could be found in the labeling index. Hence the expression of α SMA indicates that differentiation of tumor does not appear to influence the differentiation of myofibroblasts **[13]**.

***Staining index:***

There was statistical significant difference in the pattern of staining index among the study group. Intense staining of α SMA was observed only in OSF while moderate staining was seen in OSF and OSCC only. In control group had only the effect of inflammation and hence had a mild staining index in 40% of cases while no staining was seen in 60% of cases. The intense staining in group I was similar to the findings in the literature **[13]**. Though 65% cases of OSCC showed a mild staining index and 20% cases had moderate, none had intense staining index. The absence of intense staining index in OSCC needs to further analyze.

On comparing the Staining Index between OSF and normal tissue, it is observed that there was a wide difference between them. The uniform mild expression in both cases probably is a result of the mild continuous inflammation in both the condition **[15, 16]**.

On comparing the staining index between OSCC and normal tissue, it is observed that there is only a mild difference between the grades of Staining Index. However it was statistically significant. The uniformity in expression probably relates to the chronic inflammation that exists between both the conditions **[16]**.

There is no significant difference between OSF and OSCC; However 24% of cases exhibited intense staining in OSF while no cases of OSCC had intense Staining Index. This indicates that OSF had more myofibroblasts expression than OSCC **[17, 18]**.

The staining index did not considerably vary within the grades of the OSF .This indicates that the staining intensity as well as labeling index is crucial factor for the staining index. In this cross sectional study the expression of staining intensity increased with increasing grades of OSF **[19,**

**20]**. While no such observation was seen in labeling index. Low number of cells expressed SMA positivity while the staining intensity varied. This indicates that with grades of OSF increasing only a small subset of fibroblast population expressed intensive staining while the number of cells expressing αSMA never altered **[21, 22]**.

## CONCLUSION

Persistent, chronic inflammation leads to formation of a subset of fibroblastic population-myofibroblasts. The progressive increase of myofibroblasts in the Oral submucous fibrosis can be used as a marker in evaluating their severity. Myofibroblasts stimulate tumor development and constitute the tumor stroma and their presence in the invasion front of the tumor suggests they are associated with tumorgenesis **[23, 24]**. Clinical evidence suggests that the presence of myofibroblasts is associated with a 2poor prognosis have to be evaluated **[25, 26]**.

## Declarations

**Conflicts of interest:** The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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