Original Article

A Comparative Immunohistochemical Study of Presence and Distribution Pattern of Stromal Myofibroblast in Oral Dysplasia and in Different Grades of Oral Squamous Cell Carcinoma

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ABSTRACT

Aims and Objectives: Squamous cell carcinoma (SCC) is a common oral malignancy with a poor survival rate. Early tumorigenesis is marked by transdifferentiation of fibroblasts to myofibroblasts (MFs), which is supported by growth factors and cytokines expressed by tumor cells. The expression of alpha-smooth muscle actin (αSMA) marker correlates with the activation of MFs. This study was undertaken to compare the frequency and distribution of αSMA immunoexpression in oral epithelial dysplasia (OED) and OSCC. **Materials and Methods:** This study was conducted on samples collected from patients with oral epithelial dysplasia and oral SCC who visited Rajarajeswari Dental College and Hospital, Bengaluru. Tissue sections were subjected to Immunohistochemistry using αSMA marker, and cells were counted. The obtained data was subjected to Kruskal–Wallis test and Mann–Whitney U-test. **Results:** On performing Kruskal–Wallis test and Mann–Whitney U-test between the three groups (normal oral mucosa, OED, and OSCC) statistically significant result was found in the frequency between OED and OSCC and between normal tissue and OSCC. On comparing the distribution pattern, statistically significant result was found between OED and OSCC and between normal tissue and OSCC. **Conclusion:** The expression of MFs increases as the disease progresses from high-grade epithelial dysplasia to invasive OSCC. Poorly differentiated SCC showed more attendance of positive MFs in the stroma than other grades of OSCC. The rise in the number of αSMA-positive MFs and change in distribution pattern in OSCC can be associated with tumor invasive characteristics. Thus, the proliferation of MFs may be used as a stromal marker of premalignancy and malignancy.

KEYWORDS: *Alpha-smooth muscle actin, immunohistochemistry, myofibroblasts, oral epithelial dysplasia, oral squamous cell carcinoma*

INTRODUCTION

Oral squamous cell carcinomas (OSCC), an oral malignancy, with high mortality rate are posing a major concern in developing countries. OSCCs are preceded by potentially malignant lesions such as erythroplakia, leukoplakia, speckled leukoplakia, or proliferative verrucous leukoplakia, but it is not uncommon to see them arise in apparently normal oral mucosa.[1] OSCCs are characterized by multitudes of etiopathogenetic agents with tobacco and alcohol as main offenders. DNA viruses, especially human papillomavirus, have been documented to play a role in the initiation or development of these lesions.[2]

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Access this article online

Quick Response Code:

Website: www.jispcd.org

DOI: 10.4103/jispcd.JISPCD_236_18

Interactions or cross-talks between tumor cells and stroma of neoplastic tissue play a very important role in tumor progression. Tumor cells initiate extracellular matrix remodeling and stromagenesis and create a tumor conducive microenvironment. Stromal cells are responsible for the organization of this process. Fibroblasts in the mesenchymal tissue are considered to be playing a pivotal role in tumor progression by undergoing transdifferentiation to myofibroblasts (MFs), a crucial and early event in tumorigenesis, which is mediated by growth factors and cytokines expressed by tumor cells.[3] Transforming growth factor-β (TGF-β), a cancer cell-derived cytokines modulates transdifferentiation of fibroblasts into MFs. TGF-β implicates stimulation of angiogenesis, escape from immunosurveillance and recruitment of MFs.[4]

MFs are known to induce migration and invasion in a number of contexts both in normal development and tumor genesis. MFs not only promote OSCC cell proliferation but are also likely to contribute to subsequent stages of tumor progression.[5] In order of paucity of studies and literature on stromal MF (SMF) in oral dysplasia and OSCC, this study was undertaken to see the presence and distribution of alpha-smooth muscle actin (αSMA)-positive MFs in the stroma of the different grades of OSCC and compare with oral dysplasia and normal mucosa.

MATERIALS AND METHODS

SOURCE OF DATA

20 histologically confirmed cases of OSCC, 20 cases of oral dysplastic epithelium, and 10 cases normal oral mucosa (as control group) were retrieved from the files of the Oral Pathology and Microbiology of Rajarajeswari Dental College and Hospital, Bengaluru. The sample size was derived from the power analysis. For an expected power of 80% with alpha = 0.05, medium effect size (0.5), and number of groups equals 3, the sample size in each group needs to be 14. However, since getting normal tissues is challenging, we decided to restrict the control group size to 10. At the same time, the sample size for each test group was increased to 20. The normal oral mucosa was obtained from patients undergoing tooth extraction for orthodontic purposes after signing written consents. After obtaining the ethical clearance (Ref: RRDC&H/332/2014-2015 dated 26.11.2014), this retrospective study involved the use of paraffin-embedded tissues of previously diagnosed cases of oral epithelial dysplasia and OSCC.

Epithelial dysplasia was graded as:
- Mild: Cytological alterations in basal and parabasal layers of the epithelium
- Moderate: Cytological alterations up to the half of the epithelium and

- Severe: Cytological alterations up to more than the half of the epithelium, based on the classification system suggested by Neville *et al.* 2009.

OSCC was graded into three groups as Grade 1 to 3 based on the system suggested by Neville *et al.*, 2009:
- Grade 1: Well differentiated: Tumor with low cellular and nuclear atypia, high keratin pearls, slow growth and later metastasis
- Grade 3: Poorly differentiated: Tumor with high cellular and nuclear atypia and with no or few keratin pearls, rapid growth, early metastasis
- Grade 2: Moderate differentiated: Tumor with features and behavior between mild and poor.

Clinical information including age, sex, and the site of the lesion was taken from patients' records. Then, 4-μ sections were cut from paraffin blocks, stained using hematoxylin-eosin. The appropriate blocks from each lesion were selected, and 4-μ sections were prepared and again were analyzed by an oral pathologist.

STAINING PROCEDURE

Sections were taken from paraffin-embedded block with semi-automated rotary microtome and placed on poly L lysine coated slide and incubated overnight at 37°C, deparaffinized on slide warmer for 10 min. The sections were deparaffinized in xylene, rehydrated in descending grades of absolute alcohol 2 changes (90% and 70%), 5 min each and the slides were kept immersed in distilled water for 5 min. All incubations were performed in a room temperature in a humidifying chamber. The samples were then inserted into the following solutions in this order: 3% Hydrogen peroxide for 10 min to block endogenous peroxidase, one to two drops of rabbit monoclonal alpha-actin (smooth muscle) antibody for 30–45 min, PolyExcel Target Binder Reagent for 10–12 min, Streptavidin, 3,3′-diaminobenzidine chromogen (in order to see the resulted products), and Mayer's hematoxylin counterstain. The samples were washed in Tris and ethylenediaminetetraacetic acid buffer for 6 min (2 changes) between each stage. The stained samples were analyzed under the light microscope (Olympus, BX51).

Ductal carcinoma of the breast (positive αSMA) was used as positive control to assess the accuracy of the study. Endothelial cells of the blood vessels with αSMA were used as internal positive control [Figure 1]. The αSMA-stained (cytoplasmic stain) MFs in the OSCC islands and epithelial layers in dysplasia were counted in 100 cells at ×40 magnification, and the calculated average number was considered as the percentage of stained cells. The αSMA stained endothelial cells of blood vessel were not included in the calculation.
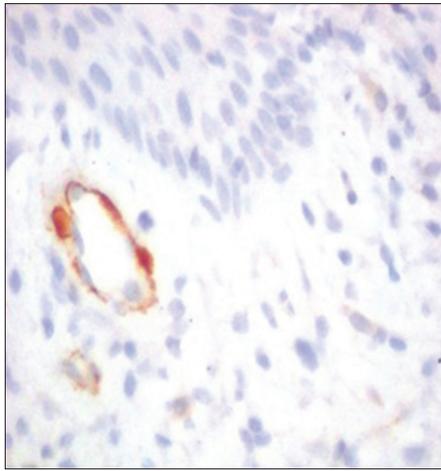
Figure 1: Strong positive staining of blood vessel walls is seen (internal control) ×40(b)
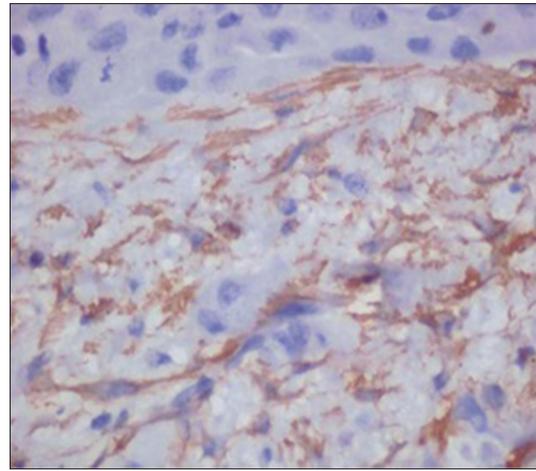

Figure 2: Photomicrograph showing alpha-smooth muscle actin expression noted in the juxta-epithelial region of oral epithelial dysplasia (focal pattern) ×10
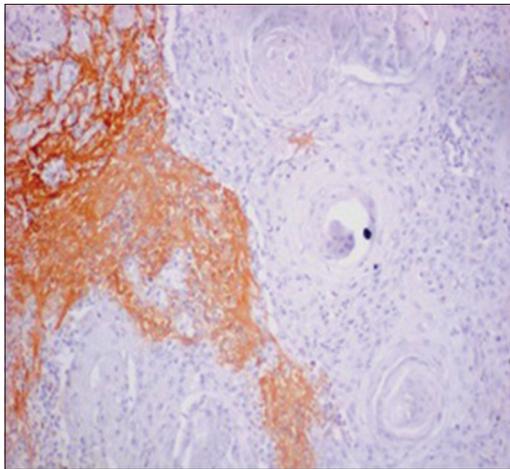

Figure 3: Photomicrograph showing immunohistochemical staining of alpha-smooth muscle actin in well-differentiated squamous cell carcinoma as seen under ×10
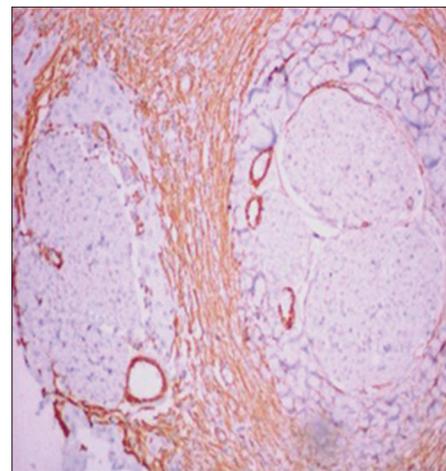

Figure 4: Photomicrograph showing immunohistochemical staining of alpha-smooth muscle actin in oral squamous cell carcinoma showing network pattern of distribution as seen under low power (×10)

The results were scored as follows: (Kellermann *et al.*, 2007):
- Score 1 (−) (Negative): when no staining or if <1% staining with αSMA
- Score 2 (+) (scanty): when more than 1% and <50% were stained with αSMA
- Score 3 (++) (Abundant): when more than 50% of MFs were stained with αSMA.

Considering the distribution pattern of MFs, the arrangement of positive-stained cells was classified into three groups:
1. Focal: Focal MFs with no special arrangement in different areas of tumor stroma
2. Network: Interwoven network arrangement of MFs in the tumor stroma
3. Spindle: Spindled arrangement of MFs in one to three rows in the periphery of the neoplastic islands or the connective tissues (Vered *et al.*, 2009).

The results of the study were analyzed by Kruskal–Wallis test and Mann–Whitney U-test. The statistical significance level was set at 0.05.

Results and Observations

Twenty cases of oral dysplasias, 20 cases of OSCCs and 10 of normal oral mucosal tissue were compared for presence and distribution pattern of SMFs. Positive staining was immunohistochemical brown cytoplasmic color using αSMA marker. The samples of OSCC were made up of large amounts of haphazardly arranged αSMA-positive cells.

The expression of αSMA was mostly observed between and around neoplastic islands. Of 20 cases of epithelial dysplasia [Figure 2], 15 cases (75%) showed score 1(-), 4 cases showed score 2 (+), and 1 case showed score 3 (++). Of 20 cases of OSCC, 5 cases (75%)

showed score 1 (−), 8 cases showed score 2 (+), and 7 cases showed score 3 (++). All normal tissue showed score 1. There was a statistically significant difference seen between the grades of αSMA expression in 3 groups [Table 1]. αSMA was positive in endothelial cells in all lesions.

When comparison of the frequency of αSMA-positive MF in different grades of OSCC was made, Kruskal–Wallis test H was 2.6669. There was no statistically significant difference between the grades of αSMA expression among the three grades [Table 2].

When the distribution pattern of αSMA-positive MFs in normal tissue, epithelial dysplasia, and OSCC was compared, a statistically significant difference was seen [Table 3].

Similarly, when the distribution pattern of αSMA-positive MFs in different grades of OSCC was compared [Figures 3-5], a statistically significant difference seen between the distribution patterns among three groups [Table 4].

DISCUSSION

MFs are a subset of fibroblasts which are metabolically and morphologically distinctive and express αSMA, and when activated by growth factors they play a pivotal role in the development of the fibrotic response.[6] SMFs represent an additional histopathologic feature and contribute to the complex tumor milieu. The direct impact of cancer cell-derived cytokines is now known to alter normal stromal fibroblasts to MFs and is they further facilitate tumor local and distant invasion as well as aid in the suppression of the host immune response.[7] Most human OSCCs show the presence of MFs in the stroma in two dominant patterns, spindle, and network.[8]

In the present study, MFs were seen juxtaposing the tumor islands and seen in cords in the stroma. They were also present in the deep invasive front of the tumors. In few cases, only a few MFs were seen in delicate rows surrounding and abutting the tumor islands, while others showed abundance of MF in the stroma, forming syncytium. In our study, MFs were positive in 15 cases (75%) out of 20 OSCC cases and were negative in 5 (25%) of the cases. These results were in agreement with those of Kellerman *et al.*,[9] Seifi *et al.*,[10] and Kapse *et al.*[11] who have reported the presence of αSMA in 60%, 67%, and 70% of the tumors, respectively.

Studies by Vered *et al.*,[7] Vered *et al.*,[12] Chaudhary *et al.*[13] and Etemad-Moghadam *et al.*[3] have however

Table 1: Comparison of frequency of stage of alpha-smooth muscle actin-positive myofibroblast in normal tissue, epithelial dysplasia, oral squamous cell carcinoma

Stage	Normal tissue (%)	Epithelial dysplasia (%)	OSCC (%)	Total
Score 1 (-)	10 (100.00)	15 (75.00)	5 (25.00)	30
Score 2 (+)	0 (0.00)	4 (20.00)	8 (40.00)	12
Score 3 (++)	0 (0.00)	1 (5.00)	7 (35.00)	8
Total	10 (100.00)	20 (100.00)	20 (100.00)	50

Among three lesions, Kruskal-Wallis test: H=18.8662, P=0.0001*
Between normal tissue versus epithelial dysplasia, Z=−1.0998, P=0.2714
Between normal tissue versus OSCC, Z=−3.2996, P=0.0009*
Epithelial dysplasia versus OSCC, Z=−2.9755, P=0.0029*
*P<0.05. Z=Mann–Whitney U-test applied. OSCC=Oral squamous cell carcinoma, -=Negative, +=Scanty, ++=Abundant
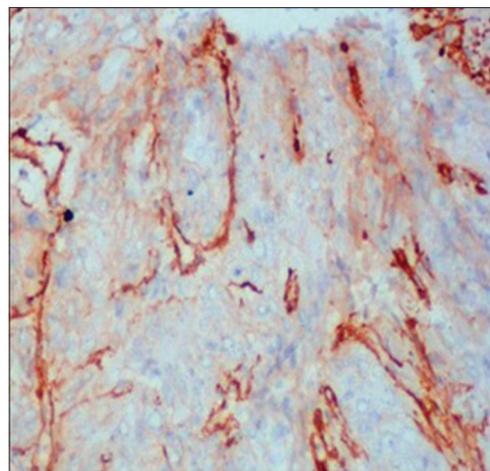

Figure 5: Photomicrograph showing immunohistochemical staining of alpha-smooth muscle actin in oral squamous cell carcinoma showing spindle pattern of distribution as seen under low power (×10)

Table 2: Comparison of frequency of stage of alpha-smooth muscle actin-positive myofibroblast in different grades of squamous cell carcinoma

Stage	WDSCC	Percentage %	MDSCC	Percentage %	PDSCC	Percentage %	Total
Score 1 (-)	2	40.00	2	40.00	1	20.00	5
Score 2 (+)	3	37.50	4	50.00	1	12.50	8
Score 3 (++)	1	14.29	2	28.57	4	57.14	7
Total	6	30.00	8	40.00	6	30.00	20

Among three grades of SCC, Kruskal-Wallis test: H=2.6669
*P<0.05. Z=Mann–Whitney U-test applied, SCC=Squamous cell carcinoma, WDSCC=Well-differentiated SCC, MDSCC=Moderate differentiated SCC, PDSCC=Poorly differentiated SCC, -=Negative, +=Scanty, ++=Abundant

Table 3: Distribution patterns of myofibroblast in normal tissue, epithelial dysplasia, oral squamous cell carcinoma

Distribution pattern	Normal tissue (%)	Epithelial dysplasia (%)	OSCC (%)	Total
None	10 (100.00)	15 (75.00)	5 (25.00)	30
Focal	0 (0.00)	3 (15.00)	0 (0.00)	3
Network	0 (0.00)	2 (10.00)	10 (50.00)	12
Spindle	0 (0.00)	0 (0.00)	5 (25.00)	5
Total	10 (100.00)	20 (100.00)	20 (100.00)	50

Among three lesions, Kruskal-Wallis test: H=21.8733, P=0.0001* between normal tissue versus epithelial dysplasia, Z=−1.0998, P=0.2714
Between normal tissue versus OSCC, Z=−3.2996, P=0.0009*
Epithelial dysplasia versus OSCC, Z=−3.4488, P=0.0005*
*P<0.05. Z=Mann–Whitney U-test applied, OSCC=Oral squamous cell carcinoma

Table 4: Comparison of patterns of myofibroblasts in different grades of oral squamous cell carcinoma

Distribution patterns	PDSCC (%)	MDSCC (%)	WDSCC (%)	Total
Focal	0 (0.00)	0 (0.00)	0 (0.00)	0
Network	4 (40.00)	5 (50.00)	1 (10.00)	10
Spindle	0 (0.00)	1 (20.00)	4 (80.00)	5
Total	4 (26.67)	6 (40.00)	5 (33.33)	15

Among three grades, Kruskal-Wallis test: H=7.1400, P=0.0282*
Between PDSCC versus MDSCC, Z=−0.4264, P=0. 6698
Between PDSCC versus WDSCC, Z=−1.9595, P=0.0500*
Between MDSCC versus WDSCC, Z=−1.7344, P=0.0828
*P<0.05. Z=Mann–Whitney U-test applied. SCC=Squamous cell carcinoma, WDSCC=Well-differentiated SCC, MDSCC=Moderate differentiated SCC, PDSCC=Poorly differentiated SCC

reported 98%, 100%, 97%, and 100% expression of αSMA, respectively.

In the present study, αSMA stained slides of oral epithelial dysplasia (n = 20) were evaluated for frequency of MFs expression and were positive in 5 (25%) of cases of oral epithelial dysplasia (OED). These results were in agreement with those of Seifi *et al.*,[10] Chaudhary *et al.*,[13] and Kapse *et al.*,[11] whereas de-Assis *et al.*,[14] Etemad-Moghadam *et al.*,[3] and Vered *et al.*[12] have reported a complete absence of MFs. In the present study, all cases of OED were of high grade. This might explain the reason for the increased expression of MFs seen in this study and probably suggest more chances of epithelial dysplasia turning in to malignancy.

MFs are undetectable in normal oral mucosa. In cases of epithelial dysplasia 5 (25%) cases showed positivity for MF. Among the three histological grades of OSCC, 25% of the cases showed the absence of MFs and 75% showed the presence of MF in OSCC. MFs were present in 4 cases (66.6%, n = 6) of WDSCC, 6 cases

(75%, n = 8) of MDSCC, and 5 cases (83.3%, n = 6) of poorly differentiated SCC (PDSCC). The expression of MFs was slightly higher in PDSCC than other grades. Kellermann *et al.* in his study found abundance of MFs leads to more aggressive behavior of the SCCs, including an elevated proliferative potential. Shorter overall survival was associated with the presence of abundant MFs, particularly at the invasive tumor front.[15] In our study, 83% of PDSCC showed positivity for αSMA indicating an increased proliferative potential. The change of the distribution of MFs associated with the carcinoma invasion may exhibit the change of the number of MFs or the compression of stromal cells by carcinoma cells.[16]

In 2011, Marsh *et al.* conducted a retrospective study based on 282 OSCC patients for analyzing disease mortality. They considered clinical, pathological, and molecular features and related them to the behavior of OSCC. They suggested OSCC mortality can be predicted strongly based on the SMA-positive myofibroblastic stroma. They also suggested that whether used independently or as part of a prognostic model, SMA identifies a significant group of patients with aggressive tumors, regardless of disease stage.[17] Lúcio *et al.* in 2013, in their literature review on the origin of MFs, established that MFs are an important component of the stroma of oral SCCs, although they are not present in all tumors. They observed that local disease recurrence and decreased patient survival is associated with the presence of abundant MFs.[18] Jayaraj *et al.*, in 2015 compared OSCC and potentially malignant disorders for the presence of SMFs. They observed that there was a significant difference in the MFs expression between the groups and they concluded "MF" as one among the key stromal element in tumor progression.[19] Another study by Dodani *et al.*, in 2016, suggested that MFs create permissive environment for tumor invasion in OSCCs and hence MFs presence can be used as a prognostic marker and that can aid in therapeutics as well by evaluating their frequency in the stroma.[20] Gandhi and Prasad in 2017 evaluated MFs in oral submucous fibrosis and OSCC and concluded that a significant increase in MFs in OSCC as compared to OSMF suggesting a possible role of MFs in the malignant transformation of OSMF.[21]

In this study, different grades of OSCC were considered to evaluate the role of MFs. MFs are a prominent finding in the tumor stroma of 75% but not all OSCC cases. This study supports the theory that MFs play a role in proliferation, progression, and invasion of OSCCs based on the observation that MFs were seen adjoining the tumor islands and cords in the stroma, and often in the deep invasive front of the tumors.

LIMITATIONS OF THE STUDY

This study considered the role of MFs only in epithelial dysplasia, no other premalignant lesions and conditions were considered. Since it is a retrospective study involving archival tissues, biologic behavior or the prognosis of OSCC or dysplasia was not correlated with the frequency or intensity of expression of MFs.

FUTURE RESEARCH

The role of MFs and their downstream secretory proteins need to be understood well by different molecular techniques and culture methods. This may help in future therapeutic protocols of OSCC. Early detection and characterization of these cells may help in reversing dysplasia and hence reducing malignant potential of this premalignant lesion.

CONCLUSION

The increasing expression of MFs with the disease progression from epithelial dysplasia to invasive OSCC suggests that MFs definitely play an important role in tumor progression. Further, understanding the pattern of MFs arrangement in SCC is also of importance as it has been noticed that network pattern represents higher invasive characteristics and weaker prognosis. Thus, the increase in the number of αSMA-positive MFs and altered distribution pattern during oral carcinogenesis process can be used as an indicator in understanding tumor invasive characteristics, and the proliferation of MFs may be used as a stromal marker of premalignancy and malignancy.

FINANCIAL SUPPORT AND SPONSORSHIP

Nil.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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