Comparison of Myofibroblasts Expression in Oral Squamous Cell Carcinoma, Verrucous Carcinoma, High Risk Epithelial Dysplasia, Low Risk Epithelial Dysplasia and Normal Oral Mucosa

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Received: 30 November 2011 / Accepted: 25 January 2012 / Published online: 6 March 2012
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Abstract The aim was to evaluate and compare the presence of myofibroblasts in oral squamous cell carcinoma (OSCC), verrucous carcinoma (VC), high-risk epithelial dysplasia (HRED), low-risk epithelial dysplasia (LRED), and normal oral mucosa (NOM). The study consisted of 37 OSCC, 15 VC, 15 HRED, 15 LRED and 15 NOM. α -smooth muscle actin (α -SMA) antibody was used to identify myofibroblasts. The α -SMA expression was not observed in NOM and LRED. The α -SMA was expressed in 97.29% of OSCC, 86.66% of VC, 46.66 % of HRED. The α -SMA expression was significantly higher in OSCC than VC ($p = 0.023$) and HRED ($p < 0.000$). The α -SMA expression was significantly higher in VC than HRED ($p = 0.043$). Myofibroblastic expression, as highlighted by α -SMA, is undetectable in NOM and LRED but increases

as the disease progresses from potentially malignant disorders, as HRED to VC to invasive OSCC. Thus, proliferation of myofibroblasts may be used as a stromal marker of oral premalignancy and malignancy.

Keywords Oral premalignancy · Oral epithelial dysplasia · Oral squamous cell carcinoma · Verrucous carcinoma · Myofibroblast · α -smooth muscle actin

Introduction

The cooperation between epithelial and mesenchymal cells is essential for embryonic development and probably plays an important role in pathologic phenomena such as wound healing and tumor progression [1]. The role of the tumor stroma in tumor progression is an important area of current research and has become a potential target for therapeutic intervention [2]. Tumor development is caused by an accumulation of genetic alterations; leading to a heterogeneous clonal dominance of invasion-competent cancer cells [3]. The host commands several responses to neoplasia. As a result of the expression of tumor-associated antigens, the immune system contributes lymphocytes, macrophages, and antibodies—a reflection of immunological surveillance against neoplasia [4]. Tumor neovascularization induced by a tumor-angiogenesis factor represents a secondary response, possibly deleterious, for it may facilitate tumor dissemination [4]. The stromal myofibroblast reaction to many invasive and metastatic carcinomas may constitute a third, albeit more primitive, response [4]. The collagen produced by the myofibroblasts was initially thought to signify an attempt to entrap the neoplasm and impede vascular invasion [5]. A more recently held view is that tumor progression *results from* an

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aberrant communication between cancer cells and their microenvironment. The microenvironment consists of host cells, which in collaboration with the cancer cells, produce the extracellular matrix (ECM), proteinases, and cytokines. However, the cancer cell factors acting upon the microenvironment, the host factors sent to the cancer cells, and their intracellular signaling pathways are largely unknown [2]. The complex cellular tumour stroma comprises immunocompetent and inflammatory cells, endothelial cells, fibroblasts, and a subtype specific to fibroblasts called myofibroblasts [6]. Fibroblasts and myofibroblasts, i.e. activated fibroblasts expressing α -smooth muscle actin, which produce collagen and extracellular matrix proteins, constitute the “desmoplastic reaction” commonly recognized in association with invasive carcinoma [7]. The expression of myofibroblasts has been demonstrated in numerous aggressive and various malignant lesions and have been suggested to represent an important participant in the development of invasion [8–11]. Myofibroblasts are cancer-induced host cells of the microenvironment and are the most prominent stromal cell type. These cells are believed to be derived from normal fibroblasts during times of tissue stress or altered homeostasis and are defined immunohistochemically by the presence of α -smooth muscle actin (α -SMA), vimentin, smooth muscle myosin heavy chain, desmin, calponin, and α 1-integrin. Host cells and the ECM in the tumor may positively or negatively influence tumor progression [5].

Most invasive oral carcinomas are preceded by a pre-invasive stage lasting for many years and the majority of initial alterations of precancerous and cancerous oral lesions are not readily recognizable on clinical and histologic examination. The potentially malignant disorders, such as leukoplakia and erythroplakia, show neoplastic histopathologic changes in epithelia that have been classified as, hyperplastic (nondysplastic), dysplastic, carcinoma in-situ and invasive carcinoma. The term “leukoplakia” should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no risk for cancer [12]. Similar to the definition for leukoplakia, “erythroplakia” is a clinical term that refers to a red patch/plaque that cannot be defined clinically or pathologically as any other condition having a higher risk status [12]. This definition excludes inflammatory conditions that may result in a red clinical appearance. Oral leukoplakia (OL) has an annual malignant transformation rate of 0.1–17% [13–15]. The included cases of potentially malignant disorders were classified according to Warnakulasuriya et al. [16] into two groups ‘no/questionable/hyperkeratosis/mild’ = low risk epithelial dysplasia (LRED) and ‘moderate or severe’ = high risk epithelial dysplasia (HRED). VC is a unique lesion seen in tobacco and snuff eaters. Verrucous carcinoma

refers to a clinicopathologic concept implying a locally aggressive, clinically exophytic, slow growing, low-grade variant of squamous cell carcinoma with minimal metastatic potential, and where the dysplastic changes are limited to the epithelium and there is no breach in the basement membrane [17].

Myofibroblasts are the major constituents of desmoplasia in malignant tumours and are found already in noninvasive precursors, underscoring their putative role in the initiation of invasion [18]. As there is conversion of non-diseased epithelial tissue to pre-cancerous epithelium to carcinoma, the stroma also changes from normal to “primed” to activated or “tumor associated” [19, 20]. Various studies reported the presence of stromal myofibroblasts in invasive breast, throat, and larynx cancers [21–23]. Recently, several studies [24–28] have investigated myofibroblast expression in oral squamous cell carcinoma (OSCC) and few studies [26, 27] have investigated myofibroblast expression in oral premalignant lesions (oral epithelial dysplasia). The aim of this investigation was to assess the possible association between the presence of myofibroblasts and disease progression in the oral mucosa from premalignant conditions, to verrucous carcinoma to invasive squamous cell carcinoma. Thus, in this study, immunohistochemistry was carried out by using α -SMA antibody to demonstrate and compare the distribution of myofibroblasts in normal oral mucosa (NOM), Low risk epithelial dysplasia (LRED), high risk epithelial dysplasia (HRED), verrucous carcinoma (VC) and different histologic and clinical TNM stages of OSCC.

Materials and Methods

The present study was carried out at the Department of Oral and Maxillofacial Pathology and Microbiology, Sharad Pawar Dental College and Hospital, Datta Meghe Institute of Medical Sciences, University, Sawangi (M), Wardha, Maharashtra, India. The study protocol was approved by our Institutional Ethical Committee. The study included 37 cases of OSCC, 15 cases of verrucous carcinoma, 30 cases of potentially malignant disorders and 15 cases of NOM. The study was performed on paraffin-embedded tissue fixed in 10% neutral buffered formalin. The sections were cut serially to 5 μ m thickness for immunohistochemistry to evaluate expression of α -SMA antigen.

Immunohistochemical Method for Detection α -SMA Antigen

For immunohistochemistry, Universal Immuno-enzyme Polymer method was employed. The sections were

deparaffinized with xylene and hydrated. Endogenous peroxidase activity was blocked by incubating the section with 3% H₂O₂ in methanol for 30 min. To prevent non-specific reactions, sections were incubated with 10% serum for 10 min. α -SMA antibody (ready to use prediluted, Monoclonal Mouse Anti-Human α -SMA, Clone: 1A4; Product code: IR611, Dako, Denmark) was incubated at room temperature in a humidifying chamber for 90 min. Pyogenic granuloma showing α -SMA expression was used as a positive control. One section from each positive control was used as the negative control by omitting the primary antibody and by incubating with serum. After the primary antibody and antigen reaction, the sections were rinsed in PBS three times for 10 min each. The HRP Labelled Polymer Antimouse (Dako EnVision+System HRP Labelled Polymer Antimouse, Product Code: K4000, Dako North America Inc.) was incubated at room temperature in humidifying chamber for 30 min. After the PBS washing three times for 10 min each, freshly prepared substrate/chromogen solution of 3, 3' Diaminobenzidine (DAB) in provided buffer (mixing 5 μ l of concentrated DAB in 50 ml of substrate buffer) was used to visualize the antigen-antibody reaction. Finally, the sections were counterstained in Mayer's hematoxylin.

Assessment of Immunohistochemically Stained Sections

The sections stained with α -SMA were examined under Leica DM LB2 (Leica microscope) at $\times 100$ followed by $\times 400$ magnification. Blood vessels present within the connective tissue of the immunostained sections served as a positive internal control, especially in cases of NOM and LRED, and stained with a similar intensity in all the groups. These internal controls confirmed that antigenic expression was correctly maintained in the study sample. Cells (other than non-inflammatory and non-endothelial) positive for α -SMA were regarded as myofibroblasts, if there was intracytoplasmic DAB staining (brown colour) irrespective of the intensity of staining. The evaluation α -SMA expression was done at the invasive front of OSCCs. Immunostaining was assessed by the evaluation of percentage of α -SMA, according to the method proposed by Tuxhorn et al. [29] and applied by Etemad Moghadam et al. [26]. The percentage of immunopositive cells in the noninflammatory and non-endothelial stromal cells (i.e. those considered as myofibroblasts) immediately adjacent to the carcinomatous islands and normal and dysplastic epithelium was recorded as: The percentage of cells positive for α -SMA in the tumor stroma were recorded as: 0 = no positive cells, 1 = 1–33% positive cells, 2 = 34–66% positive cells, and 3 = 67–100% positive cells by the three observers. The same scores obtained by

more than two observers were counted as the final score. Scores 1, 2, and 3 were graded as mild, moderate, and intense expression of α -SMA, respectively. According to Vered et al. [30] the distribution pattern of myofibroblasts, the arrangement of positive-stained cells was classified into 3 groups: 1—Focal: If myofibroblasts had a focal arrangement or had no special arrangement in different areas of connective tissue and stroma. 2—Network: Myofibroblasts with vesicular nuclei and abundant cytoplasm arranged in multiple rows with interwoven network of cytoplasmic extensions forming a network in the stroma of the connective tissue. 3—Spindle: Myofibroblasts arranged in one to three rows in a regular order in the periphery of the neoplastic islands or in the connective tissues with distinctive cell margins around myofibroblasts and malignant tissue.

Statistical Analysis

The data was analyzed statistically using (SPSS 17.0 version software for Windows). Differences among groups were assessed by the Kruskal-Wallis test. The Kruskal-Wallis test was also used for comparison between clinical TNM stages. Subsequent comparisons between groups were carried by Mann-Whitney *U* test and independent student *T* test. The level of statistical significance is at $p < 0.05$.

Results

A wide variation in age was noted between patients with OSCC, potentially malignant disorders PMD, and VC. There was no significant correlation found between the age and sex distributions of patients. All OSCC, PMD and VC patients had a habit of betel nut and tobacco lime quid chewing, with the frequency ranging from five to 20 times a day. Most patients kept the tobacco lime quid for 10–20 min in the oral cavity. Other details regarding the site distribution and risk factors in OSCC, PMD, and VC patients were summarized in Table 1. All PMDs were clinically diagnosed as leukoplakia (60%) and erythroplakia (40%). PMDs were histopathologically diagnosed as hyperkeratosis, mild epithelial dysplasia, moderate epithelial dysplasia, or severe epithelial dysplasia. Included 30 cases of PMDs retrieved from the tissue archives of the department were subcategorized into 15 cases of HRED and 15 cases of LRED or hyperkeratosis without epithelial dysplasia, according to Warnakulasuriya et al. [16] (details are summarized in Table 2).

According to World Health Organization grading system of squamous cell carcinoma of the head and neck, the 37 cases of OSCC were grouped into well-differentiated

Table 1 Demographic data of patients with oral squamous cell carcinoma (OSCC), potentially malignant disorders (PMD) and verrucous carcinoma (VC)

Characteristics	OSCC (n = 37)	PMD (n = 30)	VC (n = 15)
Age			
Mean	51.32	43.60	55.26
SD	8.66	9.76	14.36
Range	35–65	30–70	30–79
Sex			
Male	28 (75.67%)	21 (30.00%)	10 (66.66%)
Female	09 (24.32%)	09 (30.00%)	05 (33.33%)
Site			
Alveolar mucosa	20 (54.05%)	14 (46.66%)	04 (26.66%)
Labial mucosa	03 (08.10%)	03 (10.00%)	05 (33.33%)
Buccal mucosa	11 (29.72%)	11 (36.66%)	04 (26.66%)
Tongue	02 (05.40%)	01 (03.33%)	02 (13.33%)
Palatal mucosa	00 (00.00%)	01 (03.33%)	00 (00.00%)
Floor of mouth	01 (02.70%)	00 (00.00%)	00 (00.00%)
Risk factors			
Tobacco + lime	08 (21.62%)	05 (16.66%)	05 (33.33%)
Tobacco + lime + Betel nut	25 (67.56%)	21 (70.00%)	10 (66.66%)
Betel nut	04 (10.81%)	04 (13.33%)	00 (00.00%)
Duration of habit			
Mean	34.18	23.36	37.46
SD	09.03	9.15	14.48
Range	17–53	7–46	12–61

Table 2 Details of potentially malignant disorders (PMD)

Clinical diagnosis of PMD (n=30)	Histopathological diagnosis of PMD (n = 30)			
	Low risk epithelial dysplasia (n = 15)		High risk epithelial dysplasia (n = 15)	
	Hyperkeratosis	Mild epithelial dysplasia	Moderate epithelial dysplasia	Severer epithelial dysplasia
Leukoplakia (n = 20) (60.00%)	06 (30.00%)	09 (45.00%)	03 (15.00%)	02 (10.00%)
Erythroplakia (n = 10) (40.00%)	00 (00.00%)	00 (00.00%)	03 (30.00%)	07 (70.00%)
Total	06 (40.00%)	09 (60.00%)	06 (40.00%)	09 (60.00%)

squamous cell carcinoma (WDSCC) (20 cases) and moderately-differentiated squamous cell carcinoma (MDSCC) (17 cases). There were no cases of poorly differentiated carcinoma included in this study. According to clinical TNM staging, these 37 cases of OSCC were grouped into clinical TNM stage II (3 cases), stage III (18 cases), and stage IV (16 cases), respectively.

There were no myofibroblasts in the stroma of NOM and LRED (Figs. 1, 2, 3). The focal pattern of positive myofibroblast proliferation was predominantly noted in HRED (Fig. 4). The arrangement of positive cells predominantly appeared in network pattern in OSCC and VC (Figs. 5, 6) as compared to spindle pattern. The α -SMA expression was not found in NOM and LRED. α -SMA expression intensity in all groups are mentioned in Table 3 (Fig. 7). α -SMA

expression was noted in the juxta-epithelial region of HRED (Fig. 4) and was observed mainly adjacent to broad bulbous epithelial rete ridges of verrucous carcinoma (Fig. 5). α -SMA expression was predominantly noted in the tumor invasive front of OSCC (Fig. 6). Kruskal-Wallis test showed statistically significant differences in expression of α -SMA amongst OSCC, VC, and HRED ($p < 0.001$). The α -SMA expression increases from dysplasia to OSCC (Table 4) (Fig. 8). The α -SMA expression was significantly higher in OSCC than VC ($p = 0.023$) and HRED ($p < 0.000$). The α -SMA expression was significantly higher in VC than HRED ($p = 0.043$). The α -SMA expression was significantly higher in MDSCC as compared to WDSCC ($p = 0.047$) (Table 5). The α -SMA expression was significantly higher in clinical TNM stage 4

Fig. 1 Photomicrograph showing negative immunostaining for α -SMA in stromal cells in normal oral epithelium. Strong positive staining of blood vessel walls is seen (internal control) (original magnification $\times 100$)

Fig. 2 Photomicrograph showing negative immunostaining for α -SMA in stromal cells in hyperkeratosis. Strong positive staining of blood vessel walls is seen (internal control) (original magnification $\times 100$)

Fig. 3 Photomicrograph showing negative immunostaining for α -SMA in stromal cells in mild epithelial dysplasia. Strong positive staining of blood vessel walls is seen (internal control) (original magnification $\times 100$)

Fig. 4 Photomicrograph showing α -SMA expression (a, b) noted in the juxta-epithelial region of oral epithelial dysplasia (focal pattern) (original magnification $\times 100$)

($p = 0.045$) and TNM stage 3 ($p = 0.039$) than TNM stage 2. However, no significant difference of α -SMA expression was noted between clinical TNM stage 3 and stage 4 ($p = 0.956$) (Table 6).

Discussion

Transformation of normal oral mucosa to squamous dysplasia and ultimately to oral squamous cell carcinoma represent a complicated process involving numerous etiological factors [18]. There is an increasing amount of interest in the molecular and biological events that occur during the transition of dysplastic epithelium to squamous cell carcinoma. Trans-differentiation of a fibroblast to a myofibroblast is a crucial and early event in tumorigenesis and is mediated by growth factors and cytokines expressed by tumour cells [6, 19]. As genetic and epigenetic factors are capable of affecting both epithelium and lamina propria, it would be possible to assume that carcinogenesis and tumour progression results from a defective response in both compartments [20]. Thus, this study was carried out to assess the possible association between the myofibroblasts expression and disease progression in normal oral mucosa to potentially malignant disorders (LRED and HRED) to VC to invasive OSCC. In the present study, the site in 80% of cases of OSCC, PMD, and VC was the alveolar mucosa/

Fig. 5 Photomicrograph showing α -SMA expression adjacent to broad bulbous epithelial rete ridges of verrucous carcinoma (network pattern) [**a** original magnification $\times 40$]; **b** original magnification $\times 100$]

Fig. 6 Photomicrograph showing **a** α -SMA expression in the invasive tumor front of oral squamous cell carcinoma (network pattern) (original magnification $\times 100$); **b** α -SMA expression adjacent to infiltrated neoplastic islands of oral squamous cell carcinoma (original magnification $\times 400$)

buccal/labial mucosa. Almost all the patients with OSCC, PMD and VC were habitual smokeless tobacco and/or betel nut users, usually keeping it for 10–20 min in the oral cavity especially in the lower vestibular region.

In the present study, α -SMA expression in the stroma of OSCC was higher than its expression in VC and HRED. The α -SMA expression was not found in NOM and LRED. In this study, α -SMA expression was observed

in OSCC (Table 3), which was found to be similar to that of α -SMA expression observed by Kellerman et al. [24], Seifi et al. [27], and Etemad Moghadam et al. [26] in OSCC. The results of the present study for HRED (Table 3) were in accordance to Seifi et al. [27]. In this study, myofibroblast expression was not observed in NOM, which is similar to the findings of Etemad-Moghadam et al. [26]. The α -SMA expression was significantly

Table 3 Positive cases for α -SMA expression with their intensity of expression in all groups

Groups	n	Positive cases for α -SMA	Mild expression of α -SMA	Moderate expression of α -SMA	Intense expression of α -SMA
Oral Squamous cell carcinoma	37	36 (97.29%)	11 (30.55%)	09 (25.00%)	16 (44.44%)
Verrucous Carcinoma	15	13 (86.66%)	07 (53.84%)	04 (30.76%)	02 (15.38%)
High risk epithelial dysplasia	15	07 (46.66%)	04 (57.14%)	02 (28.57%)	01 (14.28%)
Low risk epithelial dysplasia	15	00 (0.00%)	00 (0.00%)	00 (0.00%)	00 (0.00%)
Normal oral mucosa	15	00 (0.00%)	00 (0.00%)	00 (0.00%)	00 (0.00%)

Fig. 7 Graph showing positive cases for α -SMA expression with their intensity of expression in all groups

higher in MDSCC as compared to WDSCC. This is not in agreement with Etemad Moghadam et al. [26] and Kellerman et al. [24]. The α -SMA expression was significantly higher in clinical TNM stage 4 and clinical TNM stage 3 than clinical TNM stage 2. However, no significant difference of α -SMA expression was noted between clinical TNM stage 3 and clinical TNM stage 4. Only three cases of OSCC with clinical TNM stage II were included and no case of clinical TNM stage I could be included, because most of the OSCC patients who visit our tertiary care hospital present with clinical TNM stage III and stage IV. These findings may suggest that the transdifferentiation of myofibroblasts is induced somewhere in the preinvasive or early invasive stage of OSCC, and further loss of tumoral differentiation (increased grade) and increasing clinical TNM stage affects the number of these cells [25]. Thus, results of this study support the Marsh et al. [28] hypothesis that myofibroblast expression can be used in predicting the prognosis and deciding the treatment modalities. Further studies should be carried out with a large cohort to confirm the association of increased myofibroblast expression with further loss of tumoral differentiation and increased clinical TNM.

In VC, an intense inflammatory cell response is observed in the connective tissue adjacent to the epithelium indicative of definitive epithelial-stromal interactions and different growth factors released by these dysplastic epithelial cells. VC showed significant myofibroblastic activity in 13 (86.66%) cases, out of which two (15.38%), four (30.76%) and seven (53.84%) cases showed intense, moderate and mild expression of α -SMA in the stroma, respectively. Interestingly, highly polymorphic ductal carcinoma in-situ of breast and noninvasive urothelial carcinoma of bladder have been shown to induce myofibroblast differentiation in the stroma surrounding the cancer cells [31, 32], demonstrating that factors derived from aggressive tumor cells are able to diffuse through the basement membrane and stimulate myofibroblast transformation. In VC, a distinctive myofibroblast expression pattern, limited to the juxta-epithelial region of the connective tissue, was observed. This suggests that epithelial and connective tissue interactions play an important role in the process of carcinogenesis of VC.

In this study, myofibroblastic expression was not observed in NOM and LRED, but was observed in HRED, VC, and OSCC. This further strengthens the hypothesis based on the “tissue organization field theory” that the mutated epithelial/stromal cells and disturbed stromal-epithelial interactions may be equally responsible for the induction of carcinogenesis [33, 34], emphasizing the importance of the neoplastic microenvironment in oncogenesis. Neoplastic changes that occur in the epithelium are followed by some changes in the stroma that are caused by factors like PDGF and TGF- β 1 from stromal surrounding tumor cells, which in turn promote the differentiation of fibroblasts into myofibroblasts [35]. This happens as a result of induction by a variety of cytokines from cancerous cells [1]. OSCC cells have been shown to secrete high amounts of cytokines and growth factors such as TGF- β 1, TGF- α , IGF-related factors, and interleukins [36–39]. TGF- β 1, in particular, is responsible for the fibroblast to myofibroblast transdifferentiation in experimental models [40]. The arrangement of positive-stained cells was seen predominantly in a network pattern as compared to the spindle pattern in OSCC and VC. The focal pattern of positive myofibroblast was predominantly noted in HRED. These results are in agreement with Seifi S et al. [27] and

Table 4 Descriptive statistics for α -SMA expression in all groups

Groups	N	Mean	SD	Minimum	Maximum
Oral squamous cell carcinoma	37	2.08	0.92	0.00	3.00
Verrucous carcinoma	15	1.40	0.91	0.00	3.00
High risk epithelial dysplasia	15	0.73	0.96	0.00	3.00
Low risk epithelial dysplasia	15	0.00	0.00	0.00	0.00
Normal	15	0.00	0.00	0.00	0.00

Fig. 8 Graph showing α -SMA expression in all groups

Table 5 α -SMA expression in different World Health Organization (WHO) histopathological grades of oral squamous cell carcinoma (OSCC)

WHO histopathological grading of OSCC	N	Mean	SD
Well differentiated squamous cell carcinoma	20	1.80	0.95
Moderately differentiated squamous cell carcinoma	17	2.41	0.79

Table 6 α -SMA expression in different clinical TNM staging of OSCC

Clinical TNM Stages	N	Mean	SD	Minimum	Maximum
Stage 2	3	1.00	0.00	1.00	1.00
Stage 3	18	2.16	0.92	0.00	3.00
Stage 4	16	2.18	0.91	1.00	3.00

further strengthen the hypothesis that the higher the number of myofibroblasts, the more invasive the tumor phenotype. This could be due to the fact that myofibroblasts may stimulate tumor progression by stimulating the growth of cancer cells, sustaining blood vessel formation and lymphangiogenesis, attenuating cancer cell death, and stimulating invasion and metastasis by activating proteolysis [24]. In addition, the myofibroblasts may contribute to OSCC invasion through elevation of synthesis of MMP-1, -2, -9 and -13[41]. Transformed myofibroblasts in oral epithelial dysplasia, VC and OSCC, may play an active role in disease progression by both autocrine effect on tumor stroma and paracrine effect on malignant/dysplastic epithelial cells through tumor-stromal interactions [24].

In conclusion, the presence of myofibroblasts, as measured by α -SMA expression, increases as the disease

progresses from oral mucosa premalignancy to VC and to invasive OSCC. Myofibroblast expression was not seen in NOM and LRED. Thus, the proliferation of myofibroblasts may be used as a stromal marker of premalignancy and malignancy. In the future, it would be worth exploring the autocrine and paracrine effects of transformed myofibroblasts and of the stromal-epithelial interaction as a potential therapeutic approach against cancer progression via drug intervention.

Acknowledgments The authors extend their sincere thanks to Mr. Rupesh Maladhary, Lab Technician, Dept of Oral and Maxillo-facial Pathology, Sharad Pawar Dental College and Hospital, Datta Meghe Institute of Medical Sciences, Sawangi (M), Wardha, Maharashtra, India.

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