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Myofibroblasts and Tumor Micro-environment in Oral Squamous Cell Carcinomas – A Histochemical and Immunohistochemical Analysis

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Abstract

Oral squamous cell carcinoma is a multi-factorial disease and no single causative factor has been primarily held responsible. Its pathogenesis is a multistep process involving initiation, promotion and tumor progression. The tumor micro-environment, particularly the collagen characteristics, and mesenchymal cells like myofibroblasts are increasingly implicated. The present study aims to determine the myofibroblast distribution and stromal characteristics in different grades of oral squamous cell carcinoma. Total of 46 specimens of different grades of oral squamous cell carcinoma were selected from departmental archives and subjected to immunohistochemical evaluation of myofibroblasts using α-smooth muscle actin marker. Parallel sections were subjected to van gieson staining to determine the characteristics of the collagen of the tumor stroma. Statistical analysis was performed for comparison between the different grades of oral squamous cell carcinoma. 80% of squamous cell carcinoma specimens were positive for α-smooth muscle actin. Poorly differentiated carcinoma specimens consistently demonstrated higher concentration of α-SMA positive myofibroblasts and a dense stroma. There was a positive correlation between myofibroblasts and the stromal density. The interesting feature was the higher concentration of myofibroblasts in the stroma away from the tumor islands the results indicate that the effect of the myofibroblasts on the tumor stroma may play a role in the fundamental cellular processes essential for tumor progression. It is well known that TGF-β dependent accumulation of collagen type 1 in tumor microenvironment is related to increased tumor invasiveness. Therefore, myofibroblasts may play a role in tumor growth and invasion.

Keywords*: Myofibroblasts, Oral Squamous Cell Carcinoma, Tumor Microenvironment.*

Introduction

Oral cancer is the eleventh most common cancer in the world with geographic difference in its occurrence. It accounts for 3-4 percent of head and neck cancers. The most common type of oral cancer is squamous cell carcinoma

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(OSCC), constituting about 90% of all the oral malignancies and causing more death than any other oral disease [1].

Oral squamous cell carcinoma is a multifactorial disease and no single causative factor has been clearly defined and accepted. Malignant transformation is attributed to the action of various intrinsic and extrinsic factors [2].

The development of oral carcinoma is a multistep process involving initiation, promotion and tumor progression. In reality the cancer is not just a disease of the cell; the supporting stroma is also an active participant. During tumor progression stroma is associated with series of dyanamic changes and its crucial role is undeniable. Stromal tissue consists of resident fibroblasts, newly formed blood vessels, extracellular matrix and immune components [3]. The reaction of the tumor stroma can be quite different, including the lysis of connective tissue, production of collagen and other constituents causing desmoplasia. Desmoplastic stroma, perhaps is the best-characterized stromal phenotype and is associated with many invasive cancers including oral cavity [4-9]. Several lines of evidences demonstrated that such tumor stroma associated with an increased number of a specific subtype of fibroblast called myofibroblast which plays a pivotal role in promoting the growth of cancer cells [10, 11,12,13,14,15]. It is seen that stromal myofibroblasts express smooth muscle actin (α SMA) [11]. They are the chief source of extracellular matrix including collagen 1, collagen 3, various growth factors, cytokines and proteolytic enzymes. Myofibroblasts are also involved in epithelial mesenchymal interaction and transition in tumor stroma [11,16,17] thus orchestrating the various events of carcinogenesis including initiation, progression and metastasis as well as the resistance of tumor to therapies [12]. Though the role of these cells is well known, they are not fully elucidated.

The present study is focused on the myofibroblast distribution in the stroma of oral squamous cell carcinoma and metastatic lymph nodes using immunohistochemical detection of smooth muscle actin antigen. Furthermore, the tumor stroma was evaluated for its collagen content using histochemical stain, Van-Gieson.

Materials and Methods

The study group comprised of 56 formalin fixed paraffin embedded sections which included 46 specimen oral squamous cell carcinoma, 5 specimens of non-specific ulcer and 5 specimens of normal buccal mucosa as control. Among the samples of oral squamous cell carcinoma, 21 cases of well differentiated carcinoma (WDSCC), 19 cases of moderately differentiated carcinoma (MDSCC) and 6 cases of poorly differentiated carcinoma (PDSCC) were included.

The site in all the selected squamous cell carcinoma cases belonged to lining mucosa. Out of the 46 specimens, 43 cases were from buccal mucosa and 3 cases from alveolar mucosa and floor of mouth were also included.

All the sections were subjected to immunohistochemical staining using monoclonal α smooth muscle actin antibody. The expression of α smooth muscle actin corresponding to the blood vessels served as the internal control. The negative control was taken after omission of primary antibody during immunohistochemical staining. The intensity of staining was graded as weak and strong by comparing with the internal positive control. Two observers viewed all the slides to eliminate the bias.

The sections were employed for special staining using Van Gieson stain for comparison of the collagen content and its distribution in the underlying stroma in the study and control groups. The stroma was graded as dense and loose. In case of normal mucosa, the stroma was described as

fibrocellular with collagen bundles arranged parallel to the overlying mucosa.

Methodology

The paraffin embedded tissue blocks were sectioned at 5 micron thickness, the sections floated on to the APES coated slides and were kept in incubator for 24 hours. The sections taken for special staining were floated on the microscopic slides and incubated at 48o C on the slide warmer for one hour to ensure the adhesion of the section on the slides. Sections were then deparaffinized in Xylene and hydrated through decreasing grades of alcohol and then taken to water. Immunohistochemical (IHC) staining was done by using Novolink polymer detection system. The reagents for IHC staining were obtained from Novacastra Lab. Private Limited.

Evaluation

Assessment of antigen – expressing cells was performed by using light microscope. Blood vessels and the myoepithelial cells which express α smooth muscle actin were considered as internal positive control. Special stain employed for stromal collagen evaluation was Van Gieson stain (Figures 1-3). Sections of different grades of squamous cell carcinoma were evaluated for myofibroblast cells in the stroma expressing α smooth muscle actin. The intensity of staining was graded as weak and strong by comparing with the internal positive control. Two observers viewed all the slides to eliminate the bias.

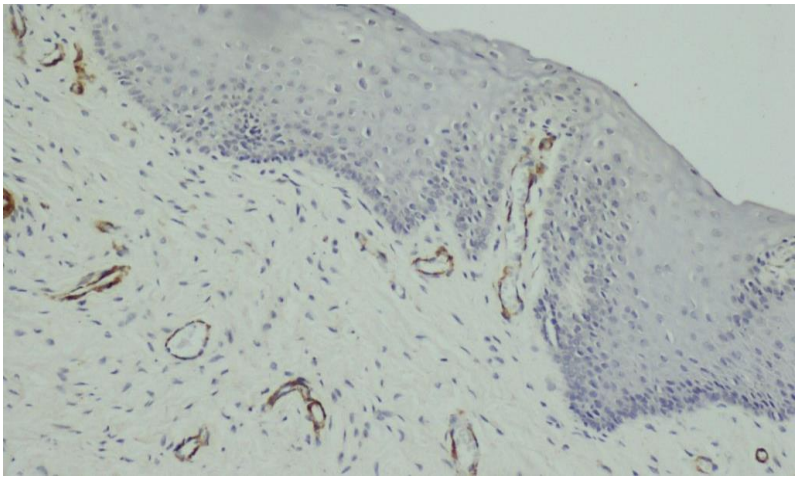

Figure 1. The Normal Mucosa with Negative α SMA Expression in the Connective Tissue. The Intrinsic Positive Areas Seen with Respect to Blood Vessels (4x magnification)

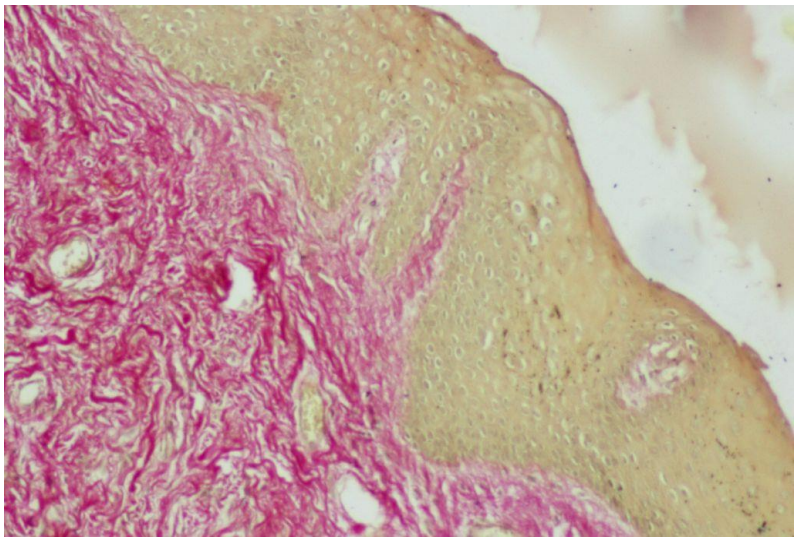

Figure 2. Normal Mucosa. Vangieson stain (4x magnification)

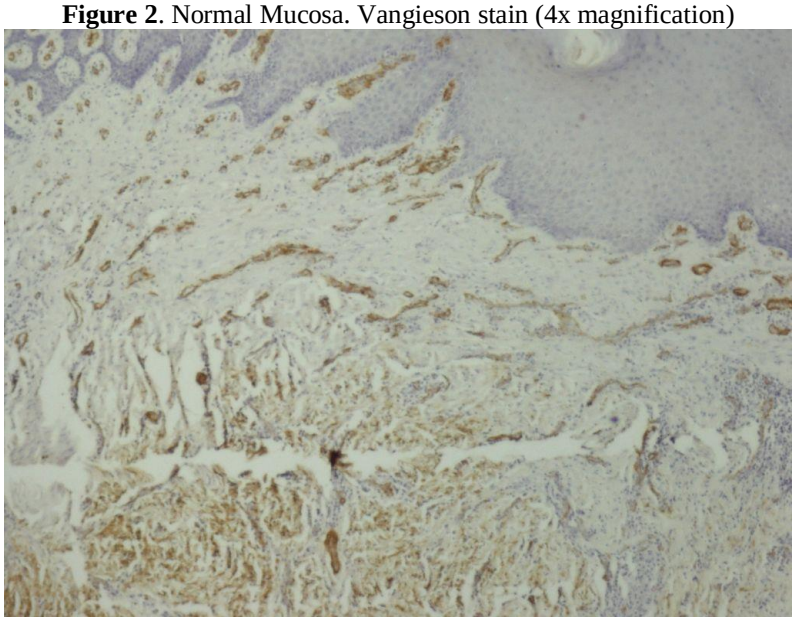

Figure 3. Shows α SMA Positive Cells in Nonspecific Ulcer (4x magnification)

Results

The study samples of oral squamous cell carcinoma were evaluated for the expression of α smooth muscle actin. Among 46 cases of oral squamous cell carcinoma 37 cases were positive and 9 cases were negative. Total 37 positive cases of oral squamous cell carcinoma comprised of 15 well differentiated, 16 cases of moderately differentiated and 6 cases of poorly differentiated carcinoma. (Table 1, Figures 4, 5)

Table 1. Comparison of Different Study Groups for Expression of α Smooth Muscle Actin Expression

Type	**Positive Actin Expression**	**Negative Actin Expression**	**P value**
Normal	0 (0%)	5 (100%)	0.001
Non-specific ulcer	3 (60%)	2 (40%)	0.001
Squamous cell carcinoma	37 (80.4%)	9 (19.6%)	0.001
Total	40 (71.4%)	16 (28.6%)	0.001

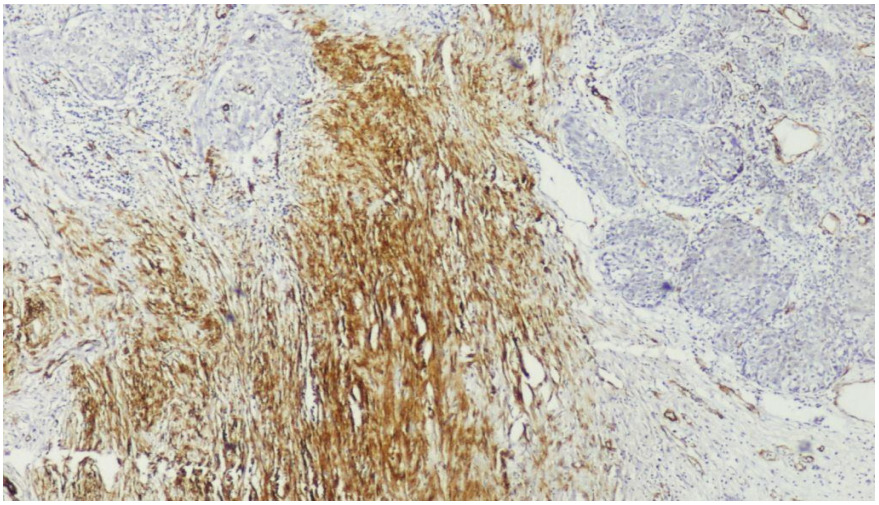

Figure 4. α SMA Positive Cells in the Stroma of Oral Squamous Cell Carcinoma. (4x magnification)

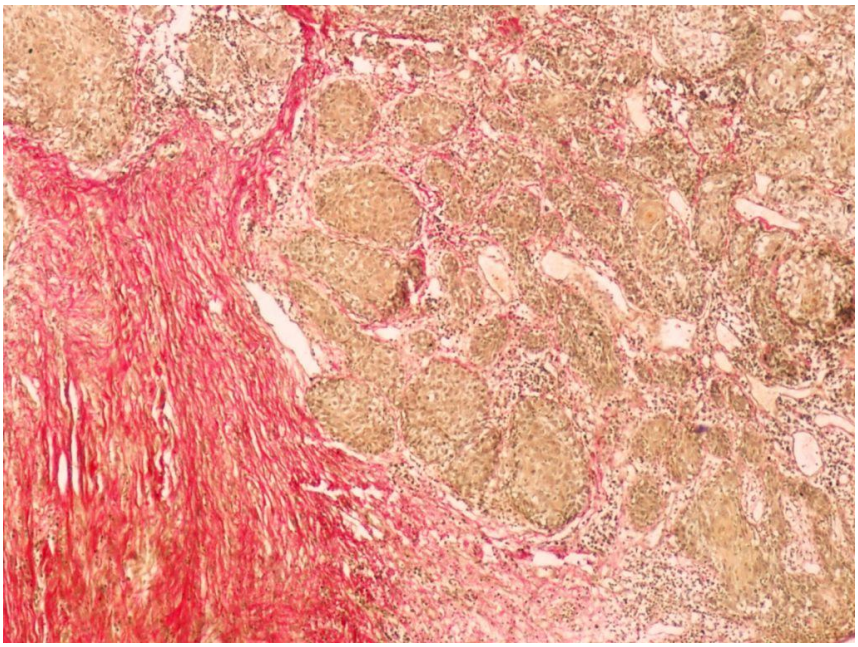

Figure 5. Van Gieson Positive Collagen in the Stroma of Oral Squamous Cell Carcinoma (4xmagnification)

The results of immunohistochemical staining of squamous cell carcinoma groups are given in table 1. Actin expression in different grades of squamous cell carcinoma was not significantly different between the grades (p=0.257).

The observation of the location of positive areas has revealed that in majority of cases of oral squamous cell carcinoma the stromal expression of α smooth muscle actin antibody was found to in the stroma away from the tumor islands. On applying Chi-Square test a highly significant "p" value was obtained. (P < 0.001). (Table 2)

Table 2. Comparison of Location of Myofibroblast in Squamous Cell Carcinoma

	Observed N	**Expected N**	**Residual**	**P value**
No expression	9	11.5	-2.5	<0.001
Stroma away from tumour islands (dense stroma)	33	11.5	21.5	<0.001
In the vicinity of tumour island	2	11.5	-9.5	<0.001
Tumour invading borders	2	11.5	-9.5	<0.001
Total	46			

Van Gieson staining demonstrated that collagen content of stroma in squamous cell carcinoma was seen mainly as dense and thick bundles arranged in stratified layers. In such areas staining intensity was bright red, while in few cases has shown loose fibrillar collagen where the staining intensity was pink. Among the cases of oral squamous cell carcinoma 12 cases of well differentiated squamous cell carcinoma has shown dense deposition of collagen in the tumor stroma was seen in 12 well differentiated, 13 moderately

differentiated and 6 poorly differentiated squamous cell carcinoma. While 9 cases of well differentiated and 6 cases of moderately differentiated tumors showed loose fibrillar deposition.

The comparison of the collagen content in the different study groups revealed that in greater number of oral squamous cell carcinoma specimens, the stroma was dense with thick bundle of collagen. Further it was seen that all the cases of ulcerated tissue had loosely arranged fibrillar collagen content. The connective tissue in control cases of normal mucosa was found to be matured fibrous with parallel arrangement of collagen bundles to the overlying mucosa. On applying Chi-Square test for the above comparison a 'P' value of highly significance was found. (P<0.001) (Table 3, Figure 5 and 6)

Table 3. Comparison of Distribution of Collagen in Different Study Groups

Study groups	**Normal Collagen**	**Dense Collagen**	**Loose Collagen**	**P value**
Normal	5 (100%)	0 (0%)	0 (0%)	<0.001
Non-specific ulcer	0 (0%)	0 (0%)	5 (100%)	<0.001
Squamous cell carcinoma	0 (0%)	31 (67.4%)	15 (32.6%)	<0.001

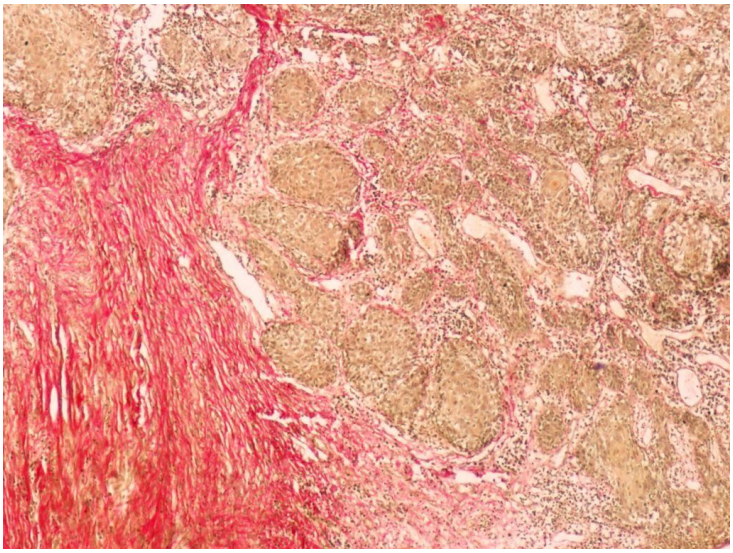

Figure 5. Van Gieson Positive Collagen in the Stroma of Oral Squamous Cell Carcinoma (4xmagnification)

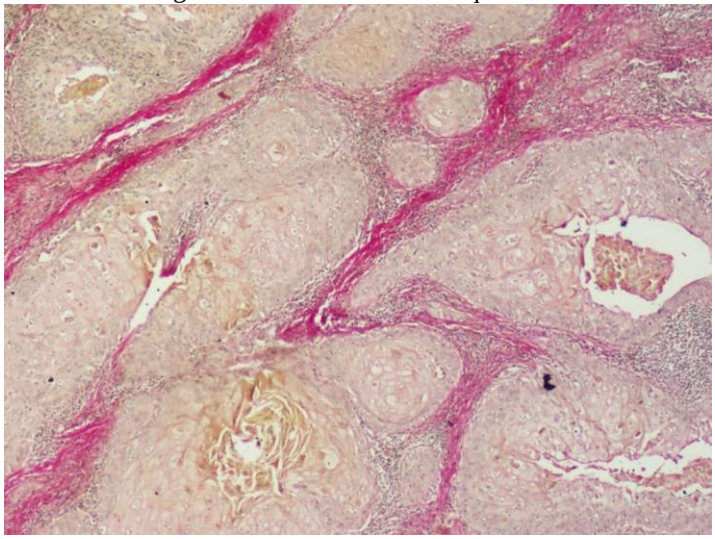

Figure 6. Van Gieson Positive Collagen in the Tumor Stroma Surrounding the Islands (magnification 4x)

Out of 21 cases of well differentiated squamous cell carcinoma 12(57.1%) cases have shown dense stroma. Among 19 cases of moderately differentiated squamous cell carcinoma 13 (68.4%) has dense stroma. And in poorly differentiated cases all of them (100%) had dense stroma and in case of ulcer the stroma was loose type (100%). The application of Chi-Square test for above comparison showed high significance and p value of 0.007 was obtained. (Table 4)

Table 4. Comparison of Distribution of Collagen in Nonspecific Ulcers and Different Grades of OSCC

Grade	Dense Collagen	Loose Collagen	P value
Non specific ulcer	0 (0%)	5 (100%)	0.007
Well differentiated	12 (57.1%)	9 (42.9%)	0.007
Moderately differentiated	13 (68.4%)	6 (31.6%)	0.007
Poorly differentiated	6 (100%)	0 (0%)	0.007

Further an attempt to compare the presence of α smooth muscle actin positive myofibroblast in the stroma and the collagen content was done. The application of chi-square test revealed highly significant p value. (Table 5, Figure 7,8).

Table 5. Comparison of α Smooth Muscle Actin Positive Myofibroblast and Distribution of Collagen

Actin Expression	Normal Collagen	Dense Collagen	Loose Collagen	P value
Positive	0 (0%)	31 (77.5)	9 (22.5%)	<0.001
Negative	5 (31.3%)	0 (0%)	11 (68.8%)	<0.001
Total	5 (8.9%)	31 (55.4%)	20 (35.7%)	<0.001

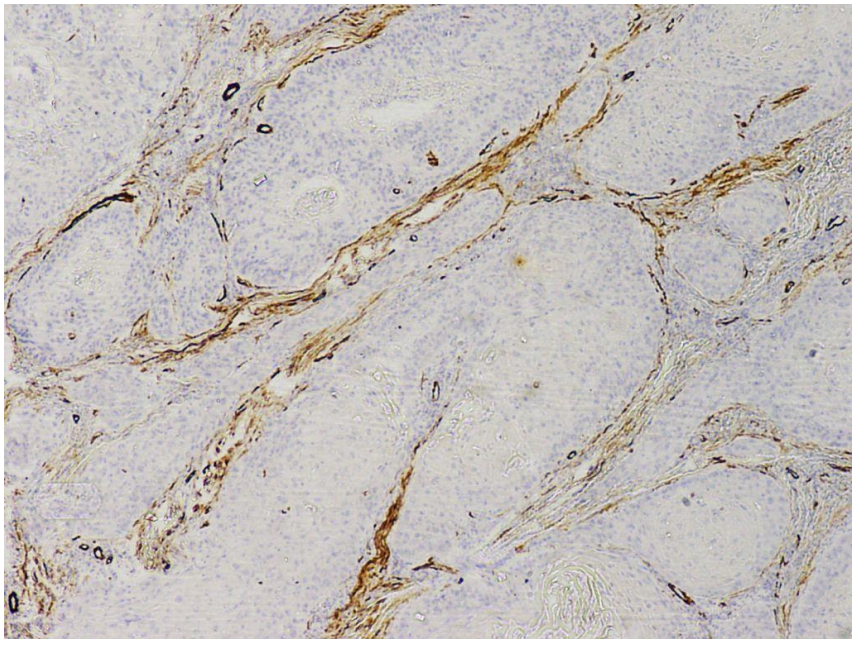

Figure 7. α SMA Positive Myofibroblast in the Vicinity of Tumor Islands (4x magnification)

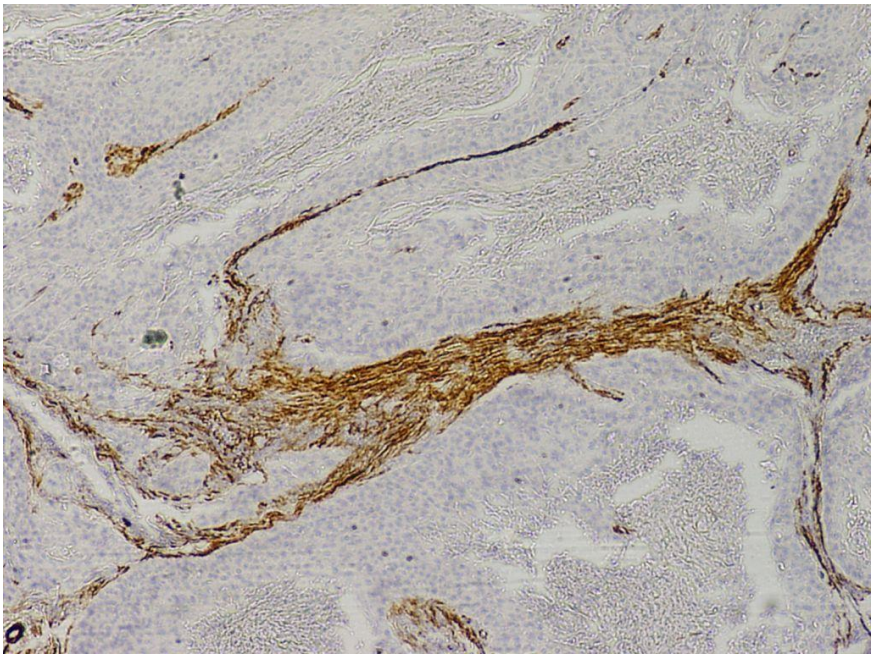

Figure 8. α SMA Positive Myofibroblast in the Vicinity of Tumor (Magnification 4x)

Using Fisher's exact test, a comparison was made within 3 different histological grades of squamous cell carcinoma for its myofibroblastic proliferation (i.e α smooth muscle actin positive or negative) and the distribution of collagen (i.e dense or loose). We found that among 21 well differentiated squamous cell carcinoma cases, 12 α smooth muscle actin positive cases (80%) showed dense distribution of collagen, whereas 6 α smooth muscle actin negative cases showed loosely distributed collagen. the P value obtained here was highly significant. Among 19 moderately differentiated cases the comparison revealed that, 13 α smooth muscle actin positive cases had dense stroma (81.25%) and 3 α smooth muscle actin positive cases had loose stroma, while rest 3 cases lacking α smooth muscle actin in stromal cells showed loose stroma (18.75%). The p value obtained here was significant. Among poorly differentiated squamous cell carcinoma all the 6 cases revealed α smooth muscle actin positive myofibroblast and the dense stroma. (Table 6, Figure 9,10)

Table 6. Comparison of Actin Expression with Distribution of Collagen in Groups of OSCC

Grade	**Actin Expression**	**Dense collagen**	**Loose collagen**	**P value**
Well Differentiated	Positive Actin Expression	12 (80%)	3 (20%)	0.002
Well Differentiated	Negative Actin Expression	0 (0%)	6 (100%)	0.002
Well Differentiated	Total Expression of Actin	12 (57.14%)	9 (42.86%)	0.002
Moderately Differentiated	Positive Actin expression	13 (81.25%)	3 (18.75%)	0.021
Moderately Differentiated	Negative Actin Expression	0 (0%)	3 (100%)	0.021
Moderately	Total Actin	13 (68.42%)	6 (31.58%)	0.021

Differentiated	Expression			
Poorly Differentiated	Positive Actin Expression	6 (100%)	Nil	Nil
Poorly Differentiated	Total Actin Expression	6.00 (100%)	Nil	Nil

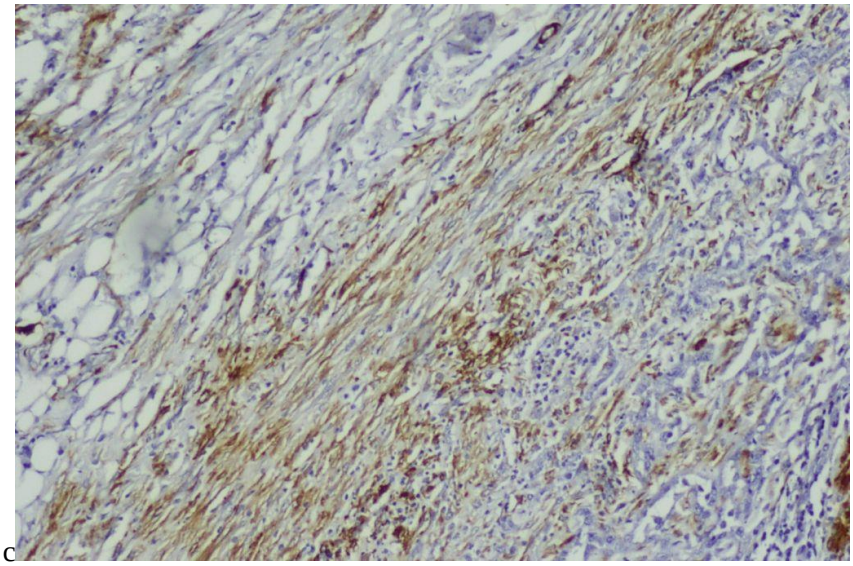

Figure 9. αSMA Positive Myofibroblast at the Tumor Invading Borders (Magnification 4x)

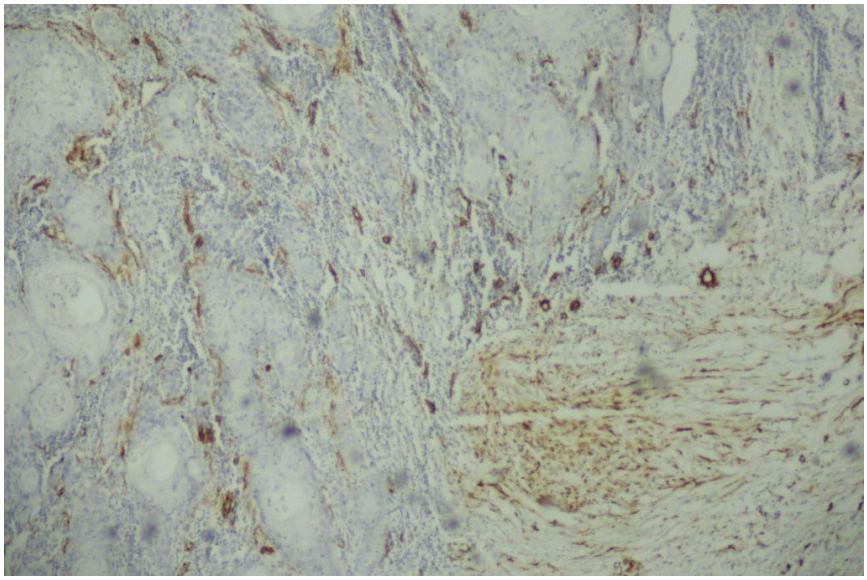

Figure 10. α SMA Myofibroblast at the Tumor Invading Borders. (Magnification 4x)

Discussion

Tumor development and progression has been considered to be a consequence of an imbalance between apoptosis and proliferation of transformed cells. Proliferation is the result of genetic aberrations which trigger the activation of oncogenes and/or loss of tumor suppressor genes. However, progression towards a malignancy requires a dynamic interaction between tumor cells and the environment in which they thrive. This interaction promotes changes in the tumor stroma, including the appearance of desmoplasia [18].

In oral carcinogenesis, several experimental studies have focused on tumor-microenvironment and the presence of tumor associated fibroblast with extracellular matrix.

This cellular microenvironment with extracellular matrix directly modulates tissue architecture, cell morphology, and cell fate. In the present study normal mucosa cases were negative for α SMA actin which is in agreement with those reported by Zidar et al [20] who also found the absence of myofibroblast in normal and dysplastic laryngeal epithelium. We assume that in case of normal mucosa the tissue lacks the trans-differentiation of fibroblast to myofibroblast.

In present study α smooth muscle actin was seen sparsely in 3 cases of nonspecific ulcers.

During wound healing, mast cells, neutrophils, and monocyte/macrophages are the first inflammatory cells that migrate to the site of injury. These cells are the main source of mitogen, cytokines, and proteinases affecting the proliferation of capillary associated cells, epithelial cells and mesenchymal cells in the local environment, as well as the proteolytic enzymes that modulate matrix architecture further facilitating growth and angiogenesis. Normal tissue repair involves the replacement of damaged tissue with appropriate amount of newly synthesized matrix. This process involves interaction between multiple cell types and cytokines and myofibroblasts have been recognized to be the key cells involved in the deposition of granulation tissue.

In the present study few biopsy sections from nonspecific ulcer showed the smooth muscle actin positive fibroblasts in the connective tissue. The intriguing possibility is that during healing the activated inflammatory cells begin to secrete profibrotic cytokines and growth factors such as TGF-β, IL-13 and PDGF which further activate the macrophages and fibroblasts. The activated fibroblasts transform into α – smooth muscle actin expressing myofibroblasts which promote wound contraction.

A tumor consists of cancer cells and tumor associated host cells. The latter comprises of blood and lymph endothelial cells, inflammatory cells, immunocytes, macrophages and fibroblasts. Together the stromal mass makes up about half of most of the malignant tumor.

The composition of tumor stroma can vary significantly between tumor types and between different locations creating a structural heterogeneity. This suggests that formation depends upon complex set of interactions between cancer cells and non-malignant cells and intercellular matrix. Moreover, the interaction between the stroma and the tumor involves several growth factors. Fibroblasts are ubiquitous cells that provide mechanical strength to tissue by providing supporting framework of extracellular matrix. However not all fibroblasts are the same. Their properties may change in wound healing in developing process or in tumor. There is increasing evidence to indicate the tumor microenvironment exerts major modulatory effect in epithelial tumor and the stroma makes an important contribution to the processes of tumor progression. In vitro studies have demonstrated that tumor cell fibroblast interaction enhances tumor cell invasion. The result of the present study showed that α-SMA positive in 37 specimens of OSCC. However, the frequency of these positive cells was not uniform in 15 cases of WDSCC and 16 MDSCC and 6 cases of PDSCC. The stromal myofibroblast constituted the major component in the stroma. In most of the sections of WDSCC and MDSCC the stromal myofibroblast displayed a stellate shaped or fusiform architecture with cytoplasmic projections and were organized in to bundles that mostly surrounded the tumor islands or nests. While in few cases of PDSCC the myofibroblast were amalgamated with the tumor cells and were organized in a syncytium-like cellular network.

Although we studied small number of cases, not all sections examined presented a homogenous pattern of stromal myofibroblasts. These differences in layout of

myofibroblasts might have an impact on defining the biological aggressiveness of tumors. These myofibroblasts are the source of ECM degrading proteases such as MMPs. MMPs probably allow the cancer cells to cross the tissue boundaries by degradation of ECM proteins for migration, invasion and escape of tumor cells from the primary site [21]. In the present study some of the stromal myofibroblasts had an epitheliod appearance-mainly at epithelial connective tissue interface. While in PDSCC some of the tumor cells which had lost the cohesiveness and had acquired spindle shaped, a fibroblastoid appearance probably in response to the stimuli from microenvironment. This has been explained by Maeda G & Prime SS et al that the cancer derived transforming growth factors interfere with intercellular junction of tumor cells mainly E-catherin and β catenin, whereby E-catherin mediated sequestration of β catenin at the cell membrane is abolished which will downregulate E-catherin expression and lead to loss of intercellular adhesion [22,23].

Studies provide evidence of cancer promoting role by stromal myofibroblasts. Studies have also been directed to compare the effects of activated myofibroblasts with normal fibroblasts19, indicating that the latter inhibit cancer progression. Supported by studies on breast epithelium and prostrate neoplasias wherein the loss of signaling of TGF-β in fibroblasts indicating a suppression of carcinogenic event. Furthermore, it has been described by Silzie et althat fibroblasts are significant source of immune modulatory cytokines, such as interferon γ, IL-6 and TNF-α which in turn can influence the mobilization of cytotoxic T-lymphocytes, NK cells and macrophages and thus the normal fibroblasts help to prevent apoptosis of T-cells. Although the results of the present study on αSMA do not fully understand the mechanism regulating the activation of fibroblasts and their accumulation in tumor tissue [24].

In the present study we also evaluated the tumor stroma for its collagen content and were compared with that of nonspecific ulcer. To evaluate the collagen content Van Gieson was employed. Based on the result it was seen that majority of the tumor stroma had increased mature collagen content in the form of tightly packed dense collagen bundles which had stained intensely with Van Gieson stain. As opposed to this all the cases of nonspecific ulcer have shown loose fibrillar arrangement of the same in its connective tissue stroma. It is well established that collagen type I, the major ECM component produced by myofibroblasts, not only functions as a scaffold for the tissue but also regulates the expression of genes associated with cellular signaling and metabolism, gene transcription, and translation, thus affecting fundamental cellular processes that are essential for tumor progression. Collagen type I accumulation has been observed at the tumor-stroma boundary, and considered to be dependent on TGF-β activation of neighboring stroma cells. Collagen type I has been hypothesized to be a signal for invasion, and its intratumoral expression level has been associated with increased tumor invasiveness [19].

Further not only collagen I is said to be increased but reports mention the increased production of type III collagen in tumor stroma by myofibroblast [25]. In the present study we also appreciated the presence of fibers positive for silver deposition in the tumor stroma when the sections were subjected for Gomoris silver impregnation for reticulin fibre. These reticulin fibres were seen mainly surrounding the tumor island, in the areas where tumor mass was invading the underlying connective tissue and it was also seen intermingled with mature collagen fibre. This observation was confirmed by the studies of other authors. Nobuyoshi and Okamura reported similar stromal characteristics in hepatocellular carcinoma. They found the increased content of reticulin fibres which was

visualized by using reticulin stain and further it was confirmed by increased immunohistochemical expression of collagen III antibody. L. David and V. Dulong mentioned the direct role of collagen I and III in tumor growth and invasion as these proteins (types I and III collagens) have the ability to modify different cellular activities [26].

Another interesting finding in present study was the α smooth muscle actin positivity of histologically confirmed metastatic lymph nodes of two specimens of oral squamous cell carcinoma cases. Studies have indicated that activated fibroblasts at metastatic sites promote proliferation of cancer cells similar to cancer-associated fibroblasts in primary tumor [27, 28]. Such metastasis-associated fibroblasts could represent a variant of cancer associated-fibroblasts. However, this observation made in the present study needs further clarification by including good number of biopsy specimen from metastatic sites, to find a link between the SMA positive fibroblasts and the metastasis-associated fibroblasts.

Conclusion

The present study indicates that myofibroblasts may play a crucial role in the behavior of oral squamous carcinomas. The state of the tumor stroma as demonstrated by both immunohistochemical and histochemical staining probably reflects the aggressiveness of the malignancy. Further studies on the constituents of tumor microenvironment, epithelial-mesenchymal transition and the phenotypic characteristics of myofibroblast in the primary site are essential for considering α SMA as a diagnostic marker although several studies have been done to identify novel biomarkers for oral squamous cell carcinoma [29-31].

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Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

None

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