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ORIGINAL RESEARCH


# Quantitative Assessment of Myofibroblast in Severe Dysplasia, Microinvasion and Oral Squamous Cell Carcinoma: An Immunohistochemical Study

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## ABSTRACT

Myofibroblast are essential for the integrity of human body by virtue of its role in wound healing and pathological organ remodeling. Myofibroblast is a universal cellular component in mammalian lesions, but not a typical component of normal un-traumatized tissues. Therefore its presence in abundance in case of cancer is a matter of concern. Tumor microenvironment plays a pivotal role in tumor progression. These so called cancer associated fibroblast or myofibroblast are the major components and occur in stromal tissue during carcinogenesis processes. This study is a quantitative assessment of presence and distribution of myofibroblast in severe dysplasia, microinvasion and oral squamous cell carcinoma (OSCC).

**Keywords:** Myofibroblast, Vimentin, α-SMA, OSCC, Severe dysplasia, Microinvasion.

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## INTRODUCTION

Myofibroblast harbors' within itself two phenotypes—an amalgamation of fibroblasts and smooth muscle cell. Ultrastructurally these cells disclose irregular, stellate outlines with numerous long cytoplasmic connections connected by intermediate or adherens junctions and are connected to the extracellular matrix by cell-to-stroma attachment sites through fibronexus. They contain bundles of cytoplasmic filaments arranged parallel to the long axis of the cell, a well-developed rough endoplasmic reticulum, golgi and indented nucleus with prominent nucleoli.[1] The transforming growth factor beta 1 (TGF-1) brings about the transdifferentiation of fibroblasts into MF. Platelet-derived growth factor (PDGF) is responsible for their maturation.

The myofibroblast is an unusual and an interesting cell for a number of reasons in that it can have a benign or a malign influence depending on circumstances. By virtue of its role in wound-healing, it can promote health, but it can also endanger it by promoting the progression of tumors. Understanding the biological complexity of this cell has been of major interest for pathologist and scientists investigating the mechanism of various diseases.

Myofibroblasts have been reported in many locally aggressive odontogenic lesions like odontogenic keratocyst and solid ameloblastomas.[2] In oral submucous fibrosis there was significant increase of myofibroblasts when compared to that of the normal control and could be used as markers for evaluating the severity of oral submucous fibrosis.[3] The abundant myofibroblasts at the invasion front of oral squamous cell carcinomas contribute in growth of the tumor cells by secreting angiogenic molecules, growth factors and invasion by secretion of matrix metalloproteinases and suppression of host immune response.[4]

Stromal myofibroblasts have the potential to facilitate progression of neoplastic epithelial cells that could contribute to their biological behavior. In cancer, MF perpetuates inflammatory process resulting in improper tissue repair that disrupts organ and tissue function. Uncontrolled TGF-β regulation leads to MF proliferation in carcinogenesis. Double Paracrine mechanism has been demonstrated between squamous carcinoma cells and MF.[5] The first evidence of MF has been a matter of controversy and debate but it has been suggested that a greater number of MF the poorer the prognosis.

## MATERIALS AND METHODS

Archive samples between 2009 and 2012 from Department of Oral and Maxillofacial Pathology in MA Rangoonwala

Dental College were used for the study. Clinical information including age, sex and the location of the lesion were extracted from patients' files.

Paraffin embedded blocks of 21 cases of each histopathologically diagnosed severe dysplasia, microinvasion and oral squamous cell carcinoma were retrieved. Five cases of colon were taken as control for α-SMA and human tonsil was used for vimentin. Sample specifications based on grades of OSCC were not elaborated as cases were randomly taken and majority samples were incisional biopsies; determination of the exact differentiation of grades and their comparison was not considered.

4-micron and 3-micron sections were prepared from each paraffin block and were respectively used for routine hematoxylin-eosin staining and immunohistochemical staining (Standard Avidin Biotin Peroxidase method) (Fig. 1).

Cases of severe dysplasia included in the study were reviewed based on classification of epithelial dysplasia given by Neville et al (2009). According to him severe epithelial dysplasia is when the cytological aberrations extend from basal and parabasal layers to more than the half of the epithelium.[6] Similarly cases of microinvasion included in the study were reviewed based on Susan and et al (1997) description as a single focus of invasive carcinoma ≤2 mm or up to three foci of invasion, each ≤1 mm in greatest dimension.[7]

## Staining Procedure

The 4-micron sections were floated onto albumin coated slides and stained according to routine H & E protocol.

Immunostaining was performed according to the manufacture's instructions, using primary mouse monoclonal antibodies against α-SMA (clone 1A4; DAKO, Denmark) and vimentin (clone V9; DAKO). In brief, 3-micron sections were floated onto 3-aminopropyltriethoxysilane coated slides; dewaxed in xylene and hydrated in descending grades of alcohol. Endogenous peroxidase activity was blocked by incubation in $H_2O_2$ (6%) for 10 minutes followed by rinsing in distilled water.

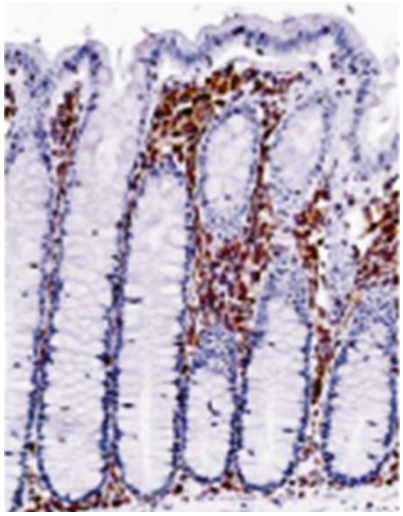


**Fig. 1:** Colon-control for α-SMA

Heat-induced antigen retrieval was accomplished using microwave oven (56°C, 20 min). α-SMA was treated with 10 mM tris buffer, 1 mM EDTA at a pH of 9; and Vimentin with 10 mM citrate buffer at a pH of 6. All slides thereafter were incubated with α-SMA (1:100) for 1 hour and with vimentin (1:50) for 120 minutes at room temperature. The sections were washed in phosphate buffer saline (PBS) for 10 minutes and stained with the streptavidin—biotin peroxidase detection kit (DAKO), followed by rinsing with PBS and development in 3, 3c-diaminobenzidine. Mayer's hematoxylin was employed for counterstaining. Positive and negative controls were run simultaneously with the study specimens. Positive controls were obtained from the normal colon tissue for α-SMA and human tonsil was used for vimentin. The primary antibodies were replaced by non-immune mouse serum at the same dilutions for negative controls. Staining of endothelial cells of the blood vessels with α-SMA was used as internal positive control. Five cases of normal oral mucosa with α-SMA served as control group. Immunohistochemical analysis confirmed of stromal spindle cells positive for α-SMA and vimentin, which were regarded as myobroblasts (MF) (Table 1).

**Table 1:** Statistical evidence of the positivity of α-SMA and Vimentin in severe dysplasia, microinvasion and OSCC

| *OSCC* | | *Severe dysplasia* | | *Microinvasion* | |
|---|---|---|---|---|---|
| *vimentin* | *α-SMA* | *Vimentin* | *α-SMA* | *Vimentin* | *α-SMA* |
| 16 (91%) | 16 (70%) | 18 (75%) | 01 (3%) | 15 (90%) | 2 (3.7%) |
| 5 (9%) | 5 (30%) | 3 (81%) | 20 (0.01%) | 6 (84%) | 19 (0%) |

## RESULTS

Histomorphometric evaluation of α-SMA and vimentin stained sections were done. Representative fields were randomly selected and seen under high power with the help of counting grid containing 25 squares.

Ten fields were chosen for each section, 100 cells at 40-time magnification were taken and the calculated average number was considered as the percentage of stained cells. The α-SMA stained endothelial cells of blood vessel were not included in the calculation. Each section was counted twice done by another pathologist afterward. Results are presented as the mean number of vimentin and α-SMA positive cells. Differences in the mean number of Vimentin and α-SMA positive cells per field among all types of lesions and the differences among the groups were analyzed using one-way ANOVA test and unpaired t-test (Fig. 2).

Statistical significance was at $p < 0.05$ (Figs 3 to 5).

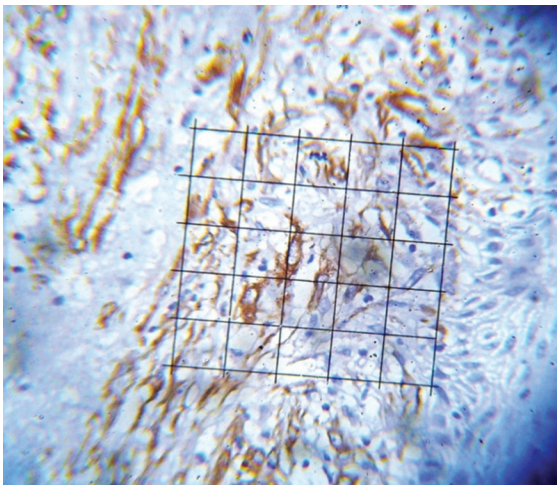
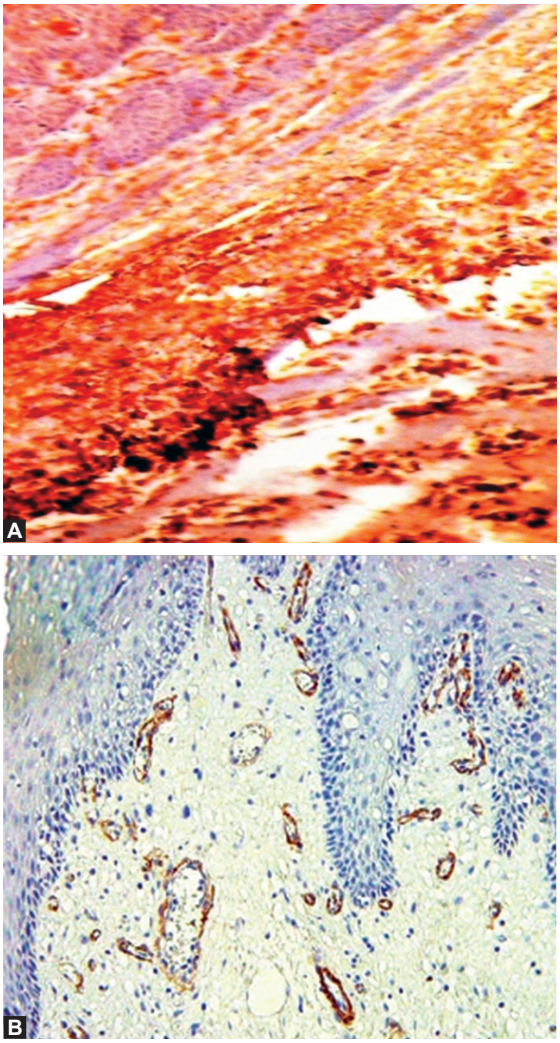
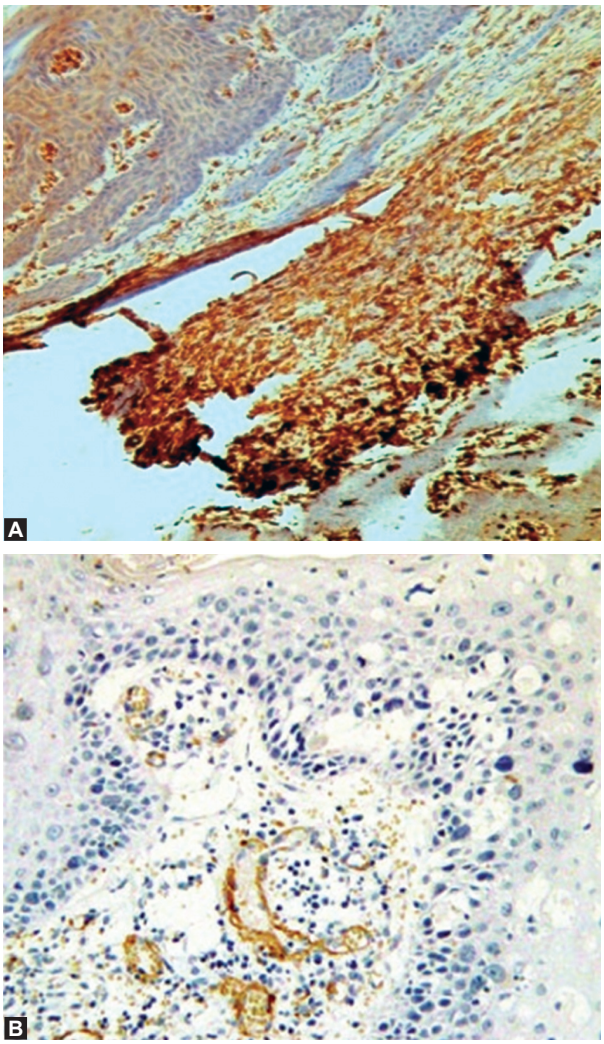


**Fig. 2:** Histomorphometric evaluation of α-SMA and Vimentin under high power with the help of counting grid containing 25 squares




**Figs 3A and B:** Severe dysplasia: (A) Exaggerated Vimentin positivity; (B) α-SMA marked negativity is seen except for the prominently stained blood vessels

Severe dysplasia and squamous cell carcinoma was statistically significant ($p = 0.000$). Considering the myofibroblasts distribution, microinvasion occurred in one case, three cases of severe dysplasia showed scanty and focal



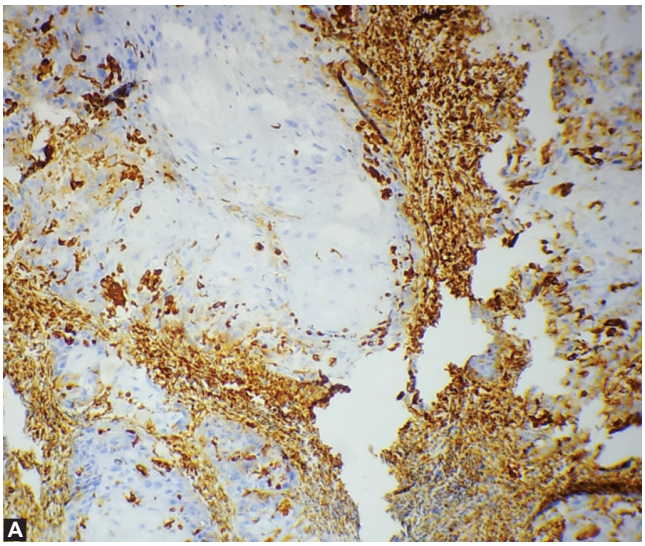

**Figs 4A and B:** Microinvasion: (A) Exaggerated vimentin positivity; (B) α-SMA negativity is seen except for the prominently stained blood vessels

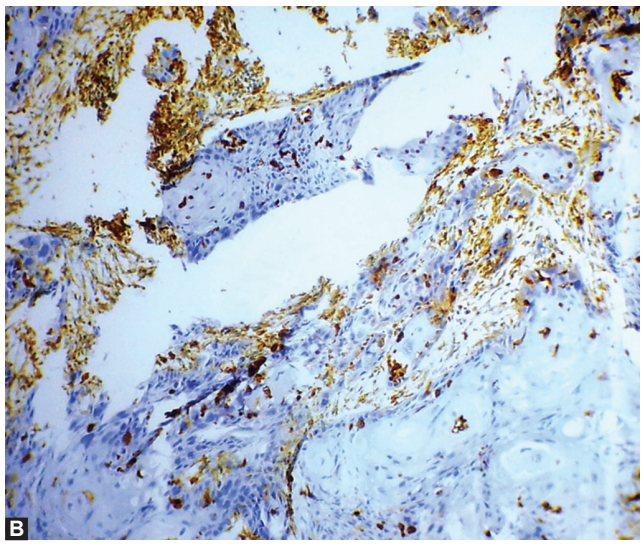
myofibroblasts in the round blood vessels. However, network and spindle arrangements occurred in maximum cases of squamous cell carcinoma respectively. The relationship between cellular distribution and the lesion type was statistically significant ($p < 0.05$) (Fig. 6).

## DISCUSSION

One of the major components of the tumor stroma is the myofibroblast. This a transdifferentiated fibroblast is known to modulate various aspects of tumor progression. They produce cytokines and growth factors that enhance tumor proliferation and angiogenesis. They also secrete pro-migratory extracellular matrix (ECM) components and up-regulate the expression of serine proteases and matrix metalloproteinases (MMP) that degrade and remodel the extracellular matrix thereby promoting tumor progression.[9]

Considering that genetic and epigenetic factors are capable of affecting the entire tissue (epithelium and lamina




**Figs 5A and B:** OSCC: Prominent Vimentin and α-SMA positivity is seen in A and B stained section

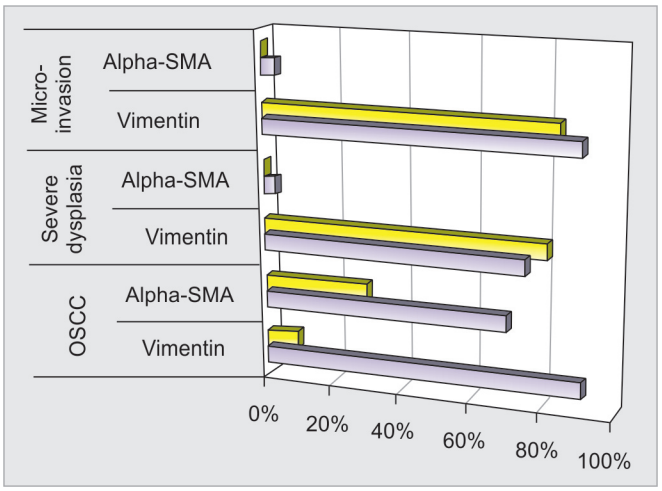



**Fig. 6:** Frequency of Vimentin and α-SMA positive MF in OSCC, severe dysplasia and microinvasion

propria), it would be logical to assume that carcinogenesis and tumor progression result from a defective response of both compartments;[8,9] suggesting that altered epithelial cells of OSCC would not be solely responsible for carcinogenesis, and different stromal factors participate in its development via communication with the epithelial elements. Albini and Sporn suggested a revision of the nomenclature of malignant epithelial tumors.[10]

Several studies have confirmed the important role of the carcinomatous stroma in tumorigenesis, invasion and metastasis.[11,12] However, the exact mechanism by which different stromal cell types, such as myobroblasts can influence neoplastic cells remains unclear. Trans-differentiation of broblasts to myofibroblasts is considered an important event that occurs in the stroma of several invasive carcinomas.[15,16] According to the results obtained in this study, the presence of myofibroblasts was signicantly higher in OSCC compared to dysplastic epithelium and microinvasion, which were both devoid of myofibroblasts.

A study done in Brazil demonstrated an elevated number of myofibroblast in oral squamous cell carcinoma, which are frequently associated with the deep invasive front of the tumors in contrast there were no α-SMA positive myofibroblasts in the stroma of normal oral mucosa and premalignant oral lesions. Thus, suggesting that a close contact is needed with tumor cells to induce myofibroblasts transdifferentiation and demonstrated that an abundance of myofibroblasts leads to more aggressive behavior of oral squamous cell carcinoma including an elevated proliferative potential. They also demonstrated that the abundant presence of myofibroblasts in oral squamous cell carcinoma were significantly associated with shorter disease free survival.[13]

Some recent studies mention that lymph node metastasis occurs more frequently in the myofibroblast-positive group and the survival rate are significantly poorer in this group.

There is growing evidence that myofibroblasts promote tumor development, and act in concert with neoplastic cells. This conflicts with the early idea that abundant matrix synthesized by the myofibroblast formed a physical barrier inhibiting tumor cell movement and amounted to a protective measure for the host.[9,14-16]

Myofibroblast expression, as highlighted by α-SMA, is undetectable in normal oral mucosa and low resistant epithelial dysplasia but increases as the disease progresses from potentially malignant disorders as high grade epithelial dysplasia to verrucous carcinoma to invasive OSCC. Thus, proliferation of myofibroblasts may be used as a stromal marker of oral premalignancy and malignancy.[17]

Results of this study showed an increase in the number of α-SMA-positive myofibroblasts and change in distribution pattern during oral carcinogenesis process which can be an expression of their role in tumor invasive characteristics. It is suggested that OSCC represented higher invasive characteristics and weaker prognosis. Concluding

that the presence of MF is a prognostic marker and evaluation of the frequency in the stroma can be used as therapeutic targets.

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