ORIGINAL ARTICLE

Stromal myofibroblasts in nonmetastatic and metastatic oral squamous cell carcinoma: An immunohistochemical study

Sudheendra Udyavara Sridhara, Nidhi Choudaha, Sowmya Kasetty, Prathamesh Satish Joshi, Shreenivas Kallianpur, Manisha Tijare

Department of Oral Pathology, People's College of Dental Sciences and Research Centre, Bhopal, Madhya Pradesh, India

Address for correspondence:
Dr. Sudheendra Udyavara Sridhara, Department of Oral Pathology, People's College of Dental Sciences and Research Centre, Bhopal - 462 037, Madhya Pradesh, India.
E-mail: sudheeachar@gmail.com

ABSTRACT

Background and Aims: Myofibroblasts are one of the important components of the tumor microenvironment which could possibly play an important role in tumor progression. The purpose of this study was to compare the presence of α-smooth muscle actin (α-SMA) and CD34 positive fibroblasts in nonmetastatic and metastatic oral squamous cell carcinoma and to evaluate their role in tumor metastasis. **Materials and Methods:** Ten cases each of histologically proven metastatic and nonmetastatic oral squamous cell carcinoma formed the study group. The tissue sections were stained immunohistochemically for α-SMA and CD34. The stromal spindle cells positive for these markers in the study groups were counted and compared. **Results:** α-SMA positive cases were more in the metastatic group and CD34 positive cases were found to be more in the nonmetastatic tumors. **Conclusions:** Though difference in the staining pattern was statistically nonsignificant, the inverse relationship between α-SMA and CD34 positive cells is indicative of dynamic nature and the influence of tumor stroma in tumor progression and metastasis.

Key words: Cancer-associated fibroblasts, immunohistochemistry, metastasis, myofibroblasts, tumor stroma

INTRODUCTION

Myofibroblasts are mesenchymal spindle cells that share features of both fibroblast and smooth muscle cell characterized by the presence of a contractile apparatus. They encompass heterogeneous and multifunctional cell populations exhibiting different phenotypes. They are known to be involved in normal and abnormal wound healing, in diverse reactive proliferative conditions, and in stroma of invasive and metastatic carcinoma.[1]

Stroma driven tissue invasion of epithelial malignancy has increasingly been studied only in the recent years. Tumor stroma is a framework of different connective tissue cells with extracellular matrix and the embedded vasculature. Fibroblasts along with endothelial cells, blood, and lymph and inflammatory cells comprise the cellular component of tumor stroma. Fibroblasts contributing to the tumor stroma have been termed peritumoral fibroblasts, reactive stroma, cancer-associated fibroblasts (CAF), and myofibroblasts.[2]

In carcinomas, several cytokines having both pro- and antitumoral properties are upregulated. Cytokines including platelet-derived growth factor (PDGF), interleukin (IL)-4, insulin-like growth factor II, and transforming growth factor (TGF)-β1 are said to be involved in the transdifferentiation of fibroblasts to myofibroblasts. TGF-β1 secretion by tumoral cells was traditionally known to act as a tumor suppressor. However, recent studies have focused on the ability of TGF-β1 to induce the transdifferentiation of stromal fibroblasts, producing an activated, myofibroblast-rich stromal microenvironment. TGF-β1 is capable of upregulating fibroblast smooth muscle actin (SMA) and collagen both *in vitro* and *in vivo*. The process of activation of fibroblasts is associated with increased proliferation, increased deposition of collagen, spliced variant forms of fibronectin, assembly of vinculin containing fibronexus adhesion complexes, and acquisition of smooth muscle cell characteristics. In fact, it is the expression of SMA that is the hallmark of the myofibroblastic phenotype.[3] Fibroblastic cells adjacent to cancer cell nests express α-SMA and are now recognized to play an important role in tumor invasion and metastasis.[4] Myofibroblastic

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network in carcinomas act as guidance structure directing the invasive tumor cells.[2] These cells are known to release numerous factors like proinvasive proteinases, growth and angiogenic factors, and extracellular matrix components that together promote invasion and growth of neoplastic epithelial cells.[2,4,5]

CD34 is called the human progenitor cell antigen. In normal oral mucosa, it is present in endothelial cells, in perivascular/interstitial dendritic cells mainly in the reticular dermis, and in spindle-shaped cells around the skeletal muscle fibers. CD34+ fibrocytes involved in wound healing, act as antigen presenting cells and secrete a multitude of cytokines. CD34+ fibrocytes are said to play an important role in tumor-associated stromal remodeling. Stromal remodeling precipitated by invasive carcinomas is said to be characterized by a loss of CD34+ expression paralleled by a gain of α-SMA expression in stromal cells, resulting in a phenotype change from CD34+ fibrocytes towards α-SMA positive myofibroblasts. A reduction of antigen presenting CD34+ fibrocytes might constitute a step in escaping the host immune control directed against invasive carcinoma cells.[5-7] However, the role of α-SMA myofibroblasts and CD34+ fibrocytes in determination of biologic behavior of oral squamous cell carcinoma is little understood.

The aim of our study was to assess and compare the immunoexpression of α-SMA and CD34 in the stromal cells of metastatic and nonmetastatic oral squamous cell carcinoma in an attempt to evaluate the role of stromal cells in the metastasis of oral squamous cell carcinoma.

MATERIALS AND METHODS

- Materials for the present study consisted of archival biopsy specimens of oral squamous cell carcinoma from the Department of Oral Pathology and Microbiology, of our college with the detailed clinical history of the same
- Only those lesions occurring in the gingiva and buccal mucosa were included in the study. Seventeen cases involved patients >40 years of age and only three cases were in patients of <40 years
- The lesions were grouped as metastatic (n = 10) and nonmetastatic (n = 10) lesions based upon histological confirmation following radical neck dissection. Metastatic group included cases that showed level I and II node involvement
- Five normal oral mucosa samples were also taken for immunostaining
- Four micrometer sections were made from formalin fixed, paraffin embedded tissue blocks, one section was stained with hematoxylin and eosin (H and E) for the confirmation of the diagnosis and two other sections were stained immunohistochemically with α-SMA and CD34.

Staining procedure

Immunohistochemistry was performed on the paraffin embedded tissue sections using a standard avidin-biotin complex procedure using 3,3'-diaminobenzidine as a chromogen. Briefly sections were incubated for 1 h at 60°C followed by deparaffinization in xylene and dehydration in serial gradient concentration of alcohol; 3% H_2O_2 for 5 min was used to block endogenous peroxidase activity. After washing the sections with phosphate buffer saline for 5 min followed by overnight incubation with rat monoclonal antibodies against CD34 (clone QBend/10, Biogenex, USA) and α-SMA (clone 1A4, 1:100 solution, Biogenex, USA); sections were then incubated with biotinylated anti-mouse immunoglobulin (IgG) followed by an avidin-biotin peroxidase complex. Sections were counterstained with Mayer's hematoxylin. Positive and negative controls were run simultaneously with the study specimens. Positive controls were obtained from the normal colon tissue for α-SMA. Staining of blood vessels was used as an internal positive control for CD34. The primary antibodies were replaced by nonimmune mouse serum at the same dilutions for negative controls.

Immunohistochemical analysis

α-SMA and CD34 were checked in noninflammatory and nonendothelial stromal spindle cells, wherein cytoplasmic and/or membranous staining was considered positive. The areas between and adjacent to the tumor islands and the connective zone immediately adjacent to the invasive tumor front were considered for counting. Number of cells in randomly selected 10 fields was counted in each section under high power field (HPF; ×400). The scoring of immunopositive stromal cells was recorded quantitatively as:[8] Score 1 = no positive cells/<20 cells, score 2 = 21-100 positive cells, score 3 = 101-400 positive cells, and score 4 = 401 or more positive cells. The scores obtained were further calculated for mean positive cells per case and per study group.

Statistics

Statistical significance of differences in α-SMA and CD34 expression were tested using the unpaired t-test. A $P < 0.01$ was considered statistically significant.

RESULTS

α-SMA

None of the normal mucosal tissue showed stromal cells positive for α-SMA. Of the carcinoma cases, both metastatic and nonmetastatic, none of the tumoral cells were α-SMA positive. Of the 10-nonmetastatic cases, half were negative for α-SMA, three cases showed score 2 (21-100 cells), and only two cases were of score 3 (101-400 cells) [Figure 1 and Table 1]. Whereas, among the metastatic group, seven cases

were positive for α-SMA with four cases showing score 3 (101-400 cells) [Figure 2]. The mean number of α-SMA positive cells were also more in the metastatic group [Figure 3].

CD34

Most of the cases in the nonmetastatic group showed either score 2 (n = 4) or score 3 (n = 5) [Figure 4]. Three cases in the metastatic group showed negative staining for CD34 [Figure 5] and the mean number of CD34 positive cells were more in the nonmetastatic group [Figure 6]. The inverse differences noted in the staining pattern of α-SMA and CD34 though, was statistically insignificant [Table 2].

DISCUSSION

The role of nonneoplastic stromal cells in tumor progression and spread has been studied in epithelial malignancies occurring in various anatomical locations. Myofibroblast is one such nonneoplastic cell which has been implicated in tumor growth and spread. Role of myofibroblasts have been studied in a variety of tumors including oral squamous cell carcinomas. The number of α-SMA positive myofibroblasts has been

Table 1: Results of immunoexpression with α-smooth muscle actin

No. of positive cells	Metastatic (n=10)	Nonmetastatic (n=10)
Score 1: Negative/<20 cells	3	5
Score 2: 21-100 cells	3	3
Score 3: 100-400 cells	4	2
Score 4: 410 and more cells	0	0

Table 2: Results of immunoexpression with CD34

No. of positive cells	Metastatic (n=10)	Nonmetastatic (n=10)
Score 1: Negative/<20 cells	3	1
Score 2: 21-100 cells	4	4
Score 3: 100-400 cells	3	5
Score 4: 410 and more cells	0	0

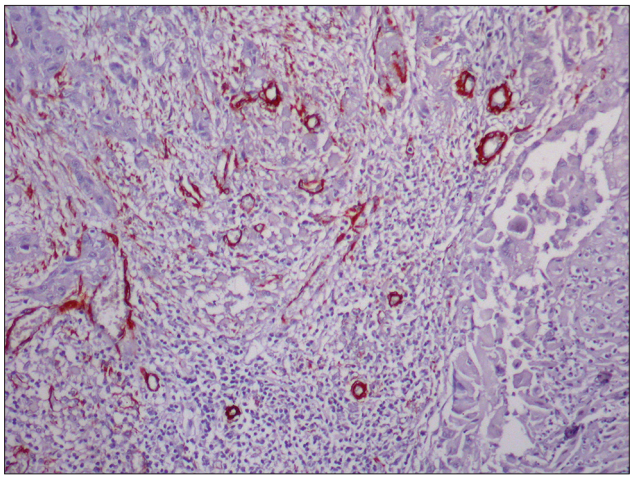

Figure 1: α-Smooth muscle actin (α-SMA) expression in nonmetastatic oral squamous cell carcinoma. Positivity seen in endothelial cells (IHC stain, ×200)

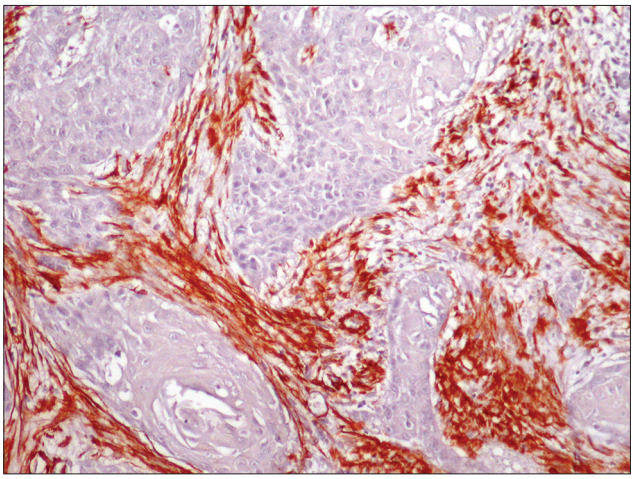

Figure 2: α-SMA expression in metastatic oral squamous cell carcinoma (IHC stain, ×400)

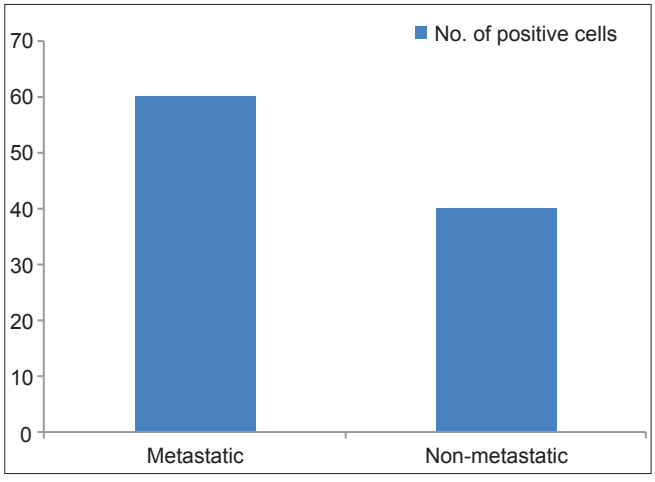

Figure 3: Mean number of α-smooth muscle actin (α-SMA) positive cells

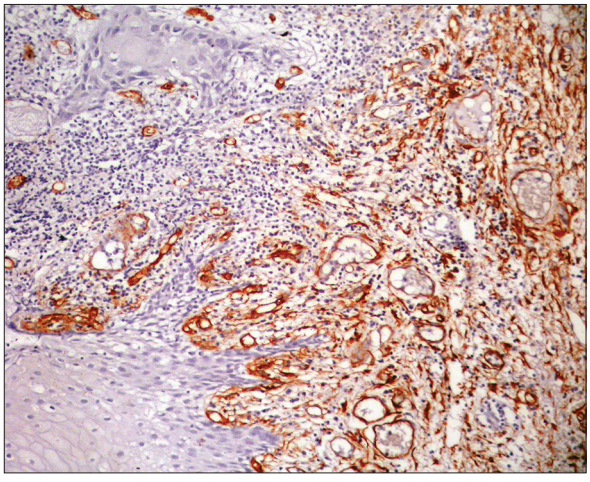

Figure 4: CD34 expression in nonmetastatic oral squamous cell carcinoma. Positivity seen in stromal and endothelial cells (IHC stain, ×200)

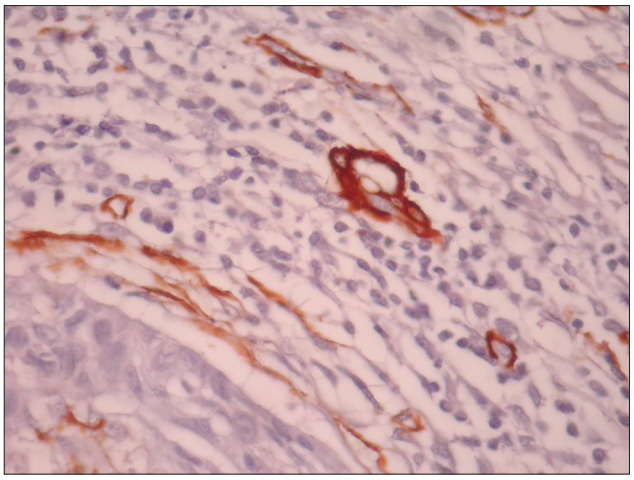

Figure 5: CD34 expression in metastatic oral squamous cell carcinoma (IHC stain, ×400)

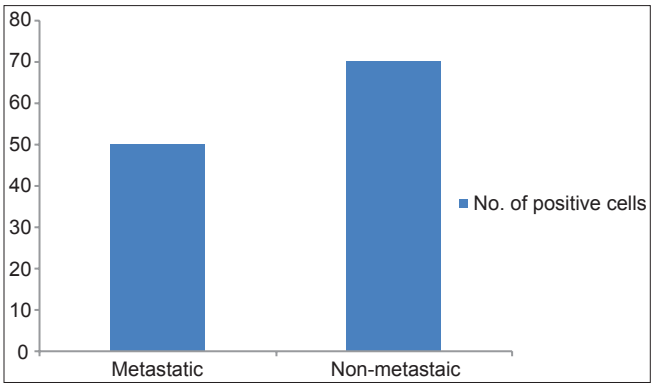

Figure 6: Mean number of CD34 positive cells

shown to be increased in oral squamous cell carcinomas in comparison to normal and dysplastic epithelium.[9-11] There are very few studies focusing on the relationship between CD34 and α-SMA and correlating myofibroblasts with metastasis of oral squamous cell carcinomas.[12-14]

Our results show an increase in the number of α-SMA+ myofibroblasts in metastatic lesions in comparison to nonmetastatic lesions. This is in agreement to the findings of a study by Kellerman, *et al*., wherein an abundance of myofibroblasts in the overall stroma or at the invasive tumor front was significantly correlated to pathologically confirmed lymph node metastasis. Their results also included significant correlation with other parameters of biologic behavior such as lymphatic or vascular invasion and extracapsular nodal invasion.[12] The difference among the metastatic and nonmetastatic group in our study was not statistically significant. Considering that the present study involved cases of oral squamous cell carcinoma of the same age, grade, and site cohorts; the finding of increase in α-SMA+ myofibroblasts has to be further pursued for its role in metastasis and correlation with different levels of nodal involvement and overall survival rate.

Experimental studies have shown that α-SMA is upregulated in CD34+ fibrocytes exposed to TGF-β.[3,13] This has been postulated to be the primary event of stromal remodeling, followed by a complete loss of CD34 expression. Their number is also shown to be downregulated in carcinomas in comparison to normal and *in situ* lesions of the oral cavity, larynx, pharynx, and breast;[5-7] and the CD34+ fibrocytes have been shown to exist in an inverse proportionality to α-SMA+ myofibroblasts in epithelial malignancies.[6]

Though the CD34+ spindle cells in the present study were more in nonmetastatic when compared to metastatic tumors in direct contrast to the findings of α-SMA+ myofibroblasts, the difference was not statistically significant. However, it has to be noted that the inverse relation between α-SMA and CD34 was present in the study sample. Areas with lymphocytic infiltration also have exhibited a loss of CD34+ fibrocytes.[7] In the present study, both the study groups consisted of tumors with inflammatory reactions ranging from mild to severe and the influence of inflammation to CD34 expression could not be clearly elucidated. Though the loss of CD34+ fibrocytes have been considered valuable tools in distinguishing benign from malignant lesions, it has also been demonstrated in radial scars of the breast.[6] The above findings do seem to suggest the limitation of CD34 as a prognostic indicator in epithelial malignancies.

Tumor microenvironment changes as evidenced by altered expression of α-SMA and CD34 in the stromal cells provide an exciting prospect in understanding tumor biology and further studies need to be carried out to clarify their role as prognostic indicators in malignancies and for designing therapeutic strategies employing them.

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