Original Article

Evaluation of stromal myofibroblasts in epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study

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ABSTRACT

Background: Among the cancers, oral cancer occupies tenth position, and the incidence is rising alarmingly with more than 300,000 new cases being detected every year. The expression of myofibroblast (MF) has been demonstrated in various malignant lesions and is considered an important participant in the invasive process. We have attempted to analyze the distribution and possible association of MF in epithelial dysplasia and squamous cell carcinoma by immunohistochemistry. **Materials and Methods:** Histopathologically confirmed twenty cases each of epithelial dysplasia and oral squamous cell carcinoma and ten cases of normal mucosa comprised the study group. MFs were detected by immunostaining with alpha-smooth muscle actin (α-SMA). Blood vessels and normal oral mucosa acted as an internal and external control respectively. **Results:** Of twenty cases of epithelial dysplasia, six (30%) were positive, all cases of squamous cell carcinoma and normal mucosa were positive and negative for α-SMA expression respectively. No statistical significance was observed between the patterns of MF distribution. Statistically significant results of α-SMA expression were noted in severe grades of dysplasia ($P = 0.000$) and between epithelial dysplasia and squamous cell carcinoma ($P = 0.000$). **Conclusion:** Analysis of α-SMA expression for MF proliferation can be used as a stromal marker for predicting behavior in oral precancer and cancer.

Key words: Alpha-smooth muscle actin, epithelial dysplasia, immunohistochemistry, myofibroblast, squamous cell carcinoma

INTRODUCTION

Among the cancers, oral cancer occupies tenth position, and the incidence is rising alarmingly with more than 300,000 new cases being detected every year. The estimated survival rate of oral cancer is approximately 50%, but the number of deaths from oral cancer remains unchanged even though there is an overall drop in death rate.[1] Oral squamous cell carcinoma (OSCC) has high mortality rate and is one of the most frequent types of oral malignancy. Among the areas in the oral cavity, lip cancer has the lowest mortality rate (0.04 per 100,000), whereas tongue cancer has the highest rate (0.7 per 100,000).[2] The development of OSCC represents as a complicated process which passes through stages of transformation of normal mucosa to oral epithelial dysplasia (OED) and involves numerous etiological factors. There is an increasing amount of interest in the molecular and biological events that occur during the transition of the dysplastic epithelium to cancer. The tumor stroma and epithelial-mesenchymal interaction plays an important role in the progression of cancer and in the current research, this area has a potential to be used as a target for therapeutic interventions. Genetic alterations lead to the heterogeneous clonal dominance of invasion of competent cancer cells and causes tumor development.[3]

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Recently, research has focused on the fact that tumor progression results from an aberrant interaction between cancer cells and their microenvironment. Host cells, extracellular matrix (ECM), proteinases, and cytokines constitute the microenvironment. The action of cancer cell factors on microenvironment, the host factors sent to the cancer cells, and their intracellular signaling pathways are largely unknown. Immunocompetent and inflammatory cells, endothelial cells, fibroblasts, and a subtype-specific to fibroblast called myofibroblasts (MFs) constitute complex cellular stroma.[4] MFs are cancer-induced host cells of the microenvironment and are the most prominent stromal cell types. They represent an important participant in the development of tumor invasion and expression of the same has been demonstrated in numerous aggressive malignant lesions.[5] MFs play an important role in the initiation of tumor invasion and are the major constituents of desmoplasia in malignant tumors. As there is conversion of nondiseased epithelial tissue to precancerous epithelium to carcinoma, the stroma also changes from normal to "primed" to "activated" or "tumor associated."[6]

Even though many studies illustrate the role of tumor microenvironment in cancer progression, there is still significant knowledge gap with respect to MFs, their identification, appearance, and their exact role in tumor development. Therefore, the present study was attempted to evaluate and compare the role of MFs in normal mucosa, OED, and OSCC and to reinforce the hypothesis that MFs are essentially a part of reactive tumor stroma.

MATERIALS AND METHODS

Study design

This retrospective study was conducted at the Department of Oral Pathology and Microbiology of the Institution after Ethical approval from the Institutional Ethical Committee. Histopathologically confirmed twenty cases each of OED and OSCC were retrieved from the department registry. Ten cases of normal oral mucosa (NOM) obtained from patients undergoing orthodontic extractions served as an external positive control for immunostaining. The study was performed over a period of 10 months. Hematoxylin and eosin-stained sections were used to confirm the histopathological diagnosis. OED and OSCC were graded according to the WHO 2005 classification.[7,8] Fresh unstained paraffin-embedded sections from selected fifty cases were immunostained for alpha-smooth muscle actin (α-SMA) expression by using super sensitive polymer 3'3' diaminobenzidine (DAB) detection kit. Primary α-SMA antibody combines with its corresponding antigen in tissues. Secondary antibodies which have a dextran polymer backbone conjugate with primary antibody. DAB chromogen combines with antigen-antibody complex and a colored reaction product is formed. Immunohistochemically stained sections for α-SMA were evaluated for frequency of expression of MFs. Known positive and negative tissue controls were used to evaluate the specificity of immunoreactions. α-SMA stained blood vessels acted as an internal positive control for each slide. NOM acted as an external positive control. The slides were evaluated by two independent oral pathologists, and the difference of opinion was settled with consensus. Stromal spindle cells positive for α-SMA were regarded as MFs. The staining was assessed for intensity and pattern of distribution.

The intensity of α-SMA expression in MFs was evaluated using Kellermann's criteria.[9]

- Score 0: Negative means 0% positive stained cells
- Score 1: Scanty means 1–40% positive stained cells
- Score 2: Abundant means >40% positive stained cell.

Distribution pattern of α-SMA positive cells in the stroma was determined according to criteria given by Angadi and Krishnapillai[10] as follows:

1. Focal: Localized arrangement of MFs with no special features
2. Network: MFs arranged in multiple rows with interwoven network of cytoplasmic extensions
3. Spindle: MFs arranged in one to three rows in a regular order in the periphery of the neoplastic islands or in the connective tissue with distinctive cell margins around tumor islands and malignant tissue.

The percentage of immunopositive cells among the noninflammatory and nonendothelial stromal cells present in the connective tissue of OED and OSCC were recorded. Fields with immunopositive cells were selected in ×10 objective lens and subsequently observed in ×40 objective lens. In each immunohistochemically stained section, five fields were randomly selected and counts were performed using counting grid containing one hundred squares. The mean number of α-SMA positive cells per section per field for each type of lesion was calculated.

Statistics

The data were collected and subjected to statistical analysis using the software IBM SPSS Statistics for windows, (IBM Corp. released 2011, version 20.0, Armonk, NY: IBM Corp.). "Chi-square test" was performed to assess the significant association between two attributes.

$\chi^2 = \Sigma([O - E]^2/E)$, where O: Observed frequency and E: Expected frequency.

$P < 0.05$ was considered statistically significant.

RESULTS

Gender- and age-wise distribution of selected cases is shown in Table 1.

All ten cases of NOM were negative for α-SMA expression [Figure 1a and b].

Analysis of oral epithelial dysplasia and alpha-smooth muscle actin expression

In the histological grading of OED cases, mild dysplasia was found in seven (35%) cases, moderate in eight (40%) cases, and severe in five (25%) cases, respectively. Among 20 cases of OED, 6 (30%) cases were positive and 14 (70%) were negative for α-SMA expression. Comparison of grades of dysplasia with α-SMA expression in OED group showed that all seven cases of mild dysplasia were negative. Of the eight cases of moderate dysplasia, seven (87.5%) were negative and one case (12.5%) had scanty expression, and all five cases of severe dysplasia showed scanty expression of α-SMA, respectively, and the result was statistically significant only for severe grades of dysplasia (P = 0.00037*) [Figure 2a-c, Tables 2 and 3].

Analysis of oral squamous cell carcinoma and alpha-smooth muscle actin expression

Histological grading of OSCC showed 13 cases (65%) of well-differentiated, six cases (30%) of moderately differentiated, and one (5%) case of poorly differentiated OSCC. Clinical staging of OSCC showed 12 cases (60%) of Stage I, 6 cases (30%) of Stage II, and 2 cases (10%) of Stage III. α-SMA expression was positive in all twenty cases of OSCC. Comparison of grades of differentiation of OSCC with α-SMA expression showed that in well-differentiated OSCC, 12 cases (92.31%) had abundant expression and one case (7.69%) had focal expression. In moderately differentiated OSCC, all six cases had abundant α-SMA expression and also in the only one case of poorly differentiated OSCC [Table 2].

No statistical significance was noted between different grades of OSCC and α-SMA expression (χ^2 = 0.567, P = 0.753). Comparison of α-SMA expression in OED, OSCC, and NOM showed statistically significant difference between OED and OSCC group (P = 0.000) and OSCC versus NOM group (P = 0.000). No statistically significant difference was noted between OED versus NOM group (χ^2 = 26.3, P = 2.8) [Table 3].

Comparison of α-SMA distribution patterns in OED, OSCC, and NOM showed that in OED group, all six (30%) cases had focal pattern [Figure 2a-c], network and spindle patterns were absent in the OED group. Distribution patterns of α-SMA in OSCC group showed one case (5%) of focal [Figure 3a-c], nine cases (45%) of network [Figure 4a and b], and ten cases (50%) of spindle pattern [Figure 5a and b], respectively. There was no statistical significance between patterns of α-SMA distribution in OED, OSCC, and NOM.

DISCUSSION

Epithelial dysplasia denotes histopathologic changes associated with an increased risk of malignant

Table 1: Mean ages and Gender distribution in OED, OSCC and NOM groups

Sex	OED group				OSCC group				NOM group				Total	
		%	Mean	SD		%	Mean	SD		%	Mean	SD	Mean	SD
Male	13	65.00	49.00	13.20	14	70.00	56.14	11.45	4	40.00	30.25	11.98	49.81	14.52
Female	7	35.00	48.00	12.11	6	30.00	54.50	17.87	6	60.00	27.33	18.60	43.53	19.21
Total	20	100.0	48.65	12.51	20	100.0	55.65	13.20	10	100.00	28.50	15.57	47.42	16.56

Table 2: Comparison of grades of dysplasia and differentiation with respect to α-SMA score

In OED and OSCC groups respectively	Alpha-SMA score	Mild dysplasia	%	Moderate dysplasia	%	Severe dysplasia	%	Total
OED group	Negative (0)	7	100.00	7	87.50	0	0.00	14
	Scanty (1)	0	0.00	1	12.50	5	100.0	6
	Abundant (2)	0	0.00	0	0.00	0	0.00	0
	Total	7	100.00	8	100.00	5	100.0	20
Statistically significant result only in severe grades of dysplasia (Chi-square test) P<0.001 *								
	Alpha-SMA Score	**Poorly differentiated**	**%**	**moderately differentiated**	**%**	**Well differentiated**	**%**	**Total**
OSCC Group	Scanty (1)	0	0.0	0	0.0	1	7.69	1
	Abundant (2)	1	100	6	100.0	12	92.31	19
	Total	1	100	6	100.0	13	100.0	20

No Statistical Significance in various Grades of OSCC. Chi-square test=0.567 P=0.753143. The result is not significant at P<0.05.

Table 3: Comparison of α-SMA expression scores in OED, OSCC and NOM groups

α-SMA expression score	OED group	%	OSCC group	%	NOM group	%	Total
Negative (0)	14	70.00	0	0.00	10	100.00	24
Scanty (1)	6	30.00	1	5.00	0	0.00	7
Abundant (2)	0	0.00	19	95.00	0	0.00	19
Total	20	100.00	20	100.00	10	100.00	50

Between OED, OSCC and NOM groups, Chi-square=54.1, The *P*<0.00001* OED group vs OSCC group, Chi-square=38.5, The *P*<0.00001* OED group vs Normal group, Chi-square=26.3, The *P*= 2.8, result not significant at *P*<0.05. OSCC group vs Normal group, Chi-square=36.8 *P*=0.00001* **P*<0.05

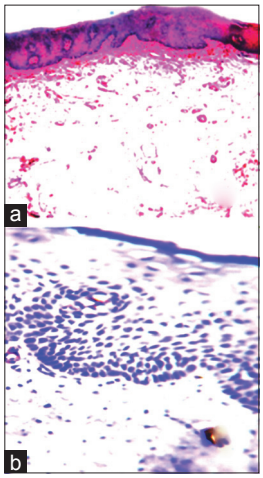

Figure 1: (a) H and E, ×10, section of normal oral mucosa, (b) positive control of alpha-smooth muscle actin expression in blood vessel of normal oral mucosa (×40)

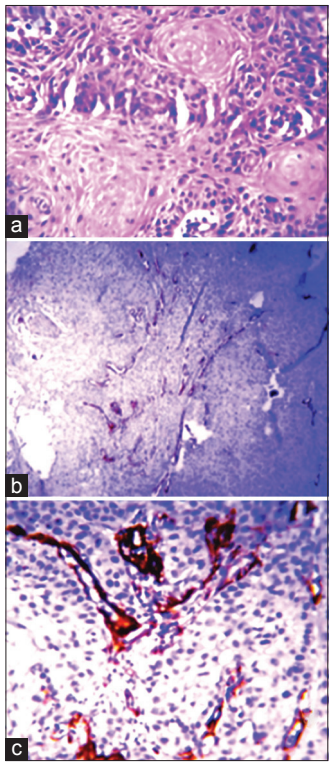

Figure 3: (a) H and E, ×10, section of well-differentiated oral squamous cell carcinoma, (b) alpha-smooth muscle actin expression (focal pattern) in well-differentiated oral squamous cell carcinoma (×10), (c) alpha-smooth muscle actin expression (focal pattern) in well-differentiated oral squamous cell carcinoma (×40)

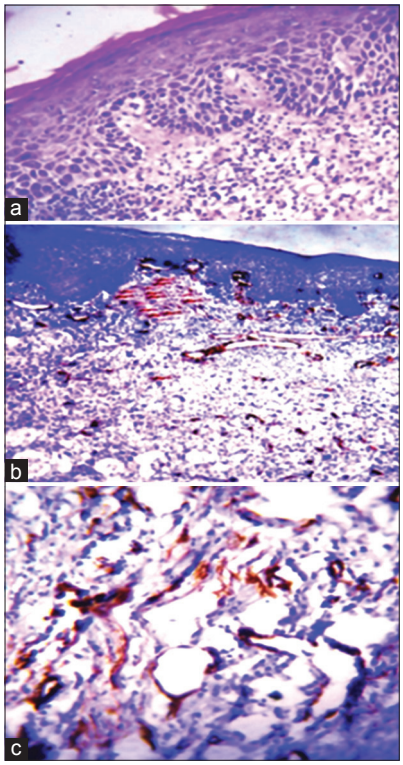

Figure 2: (a) H and E, ×10, section of severe oral epithelial dysplasia, (b) alpha-smooth muscle actin expression (focal pattern) in severe oral epithelial dysplasia (×10), (c) alpha-smooth muscle actin expression (focal pattern) in severe oral epithelial dysplasia (×40)

transformation.[11] Considerable ratio of dysplastic oral epithelial lesions may progress to invasive OSCC. Although the figures are variable, the percentage is directly related to the severity of dysplasia. However, it is noted that in dysplastic lesions the risk of developing oral cancer increases by five times more than that in a normal epithelium. Squamous cell carcinoma has high mortality rate and is one of the most frequent types of oral malignancy. The most common risk factors for developing oropharyngeal cancers are tobacco and alcohol consumption.[12]

The stromal microenvironment is crucial for maintenance of cellular functions, tissue integrity and is altered by the invasion of cancer cells into normal tissue. It has been well documented that the stroma of neoplastic tissue plays an active role in tumor progression. Remodeling of the ECM or stromagenesis is initiated by tumor cells. Cancer

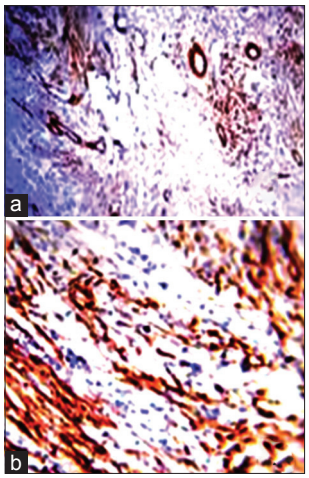

Figure 4: (a) Alpha-smooth muscle actin expression (network pattern) in well-differentiated oral squamous cell carcinoma (×10), (b) alpha-smooth muscle actin expression (network pattern) in well-differentiated oral squamous cell carcinoma (×40)

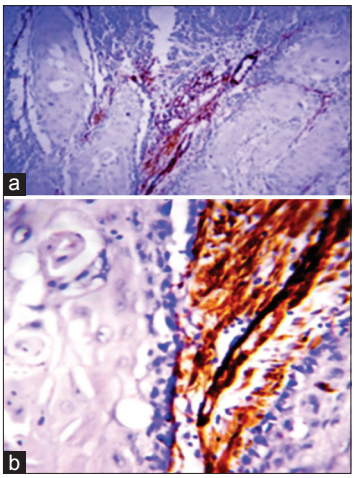

Figure 5: (a) Alpha-smooth muscle actin expression (spindle pattern) in well-differentiated oral squamous cell carcinoma (×10), (b) alpha-smooth muscle actin expression (spindle pattern) in well-differentiated oral squamous cell carcinoma (×40)

invasion may progress when ECM degradation exceeds its synthesis thus preventing complete healing. MF is a type of mesenchymal cell which mediates proteolytic activity in the stroma and may play a key role in cancer invasion. It is also suggested that the fibrous stroma in cancers is a desmoplastic response.[5,13]

It has been reported that transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor, interleukin-4, insulin-like growth factor-II, and several other cytokines induce myofibroblastic differentiation.[14] TGF-β induces the differentiation of fibroblasts to MFs, and it promotes survival of MFs by providing protection against apoptosis by inhibiting nitrous oxide synthetase induction and reducing BCL-2 expression. MFs have been detected at the invasive front in a variety of malignant tumors originating in colon, breast, liver, lung, prostate, pancreas, and oral carcinomas as well.[3] Epithelial-mesenchymal transition predisposes tumors to aggressive behavior which is due to loss of epithelial morphology and acquisition of mesenchymal characteristics and is reflected in the appearance of MFs and tumor desmoplasia. Tumor microenvironment is altered by deposition of matrix metalloproteinases and their inhibitors produced by cancer and stromal cells and has a prognostic significance.[14] Understanding the role of the stromal cells and ECM will enable us to identify more precise diagnostic modalities, prognostic markers and define new therapeutic targets. Literature review reveals few studies on the design and arrangement of MFs and their role in the invasive behavior of tumors. We therefore designed a study to investigate and compare immunoexpression of α-SMA in NOM, OED, and OSCC.

No α-SMA expression was seen in any of the tissues of NOM which is in accordance with the review of literature.[4-6,13,15-20]

Of the 20 cases of OED, 6 (30%) were positive and 14 (70%) cases were negative for α-SMA expression, which is in accordance with the studies done by Etemad-Moghadam *et al*.,[5] Seifi *et al*.,[6] and Chaudhary *et al*.[15] (α-SMA expression in OED in the range 20–37%). Studies done by Zidar *et al*.,[18] Lewis *et al*.,[19] Kellermann *et al*.,[9] Vered *et al*.,[4,20] de-Assis *et al*.,[13] and Sobral *et al*.[17] have shown no expression of α-SMA in the OED group despite any grades of dysplasia.

Our study demonstrated a positive α-SMA expression in all cases of severe dysplasia and in one case of moderate dysplasia. This finding is in accordance with the study conducted by Chaudhary *et al*.,[15] Seifi *et al*.,[6] and Etemad-Moghadam *et al*.[5]

All twenty cases of OSCC were positive for α-SMA expression. This finding is in accordance with the study done by Etemad-Moghadam *et al*.,[5] who found the presence of MFs to be significantly higher in OSCC (more in the tumor invasive front) as compared to both dysplasia and NOM. Etemad-Moghadam *et al*. used α-SMA, vimentin, and desmin markers on 40 samples of OSCC, 15 cases of dysplasia, and 15 cases of normal oral epithelium and concluded that the presence of MFs in the stroma of oral cancer was an expression of their key roles in carcinogenesis. Vered *et al*.[4] have also shown stromal MFs to be significantly associated with tongue carcinomas, whereas their number in premalignant counterpart (hyperplasia and dysplasia) was significantly lower irrespective of their grades of dysplasia. Our findings are in accordance with the study conducted by Seifi *et al*.[6] who have shown greater α-SMA expression in OSCC as compared to epithelial dysplasia and oral hyperkeratosis. Rao *et al*.[21] have demonstrated a progressive increase in the number of α-SMA positive MFs in OSMF without dysplasia to OSMF with dysplasia to OSCC.

Intense staining for α-SMA expression in the stroma of OSCC reflects the positive role of MFs in invasive behavior of OSCC. Since α-SMA expression increased as the disease progressed from severe epithelial dysplasia to invasive OSCC, we propose that the expression of α-SMA in MFs seen in OSCC and OED plays a key role in the progression of tumor in terms of growth and invasion. This finding may also help in predicting lymph node metastasis. The results of our study can be reinforced by including larger sample size and using additional IHC markers. Investigations like tissue culture studies can further ascertain the role of MFs in carcinogenesis, and it could also be possible to target the MFs to develop a therapeutic regimen.

CONCLUSION

NOM was negative whereas OED showed 30% positive expression and focal pattern and all cases of OSCC were positive for α-SMA distribution, respectively. α-SMA expression increases as the disease progresses from potentially malignant disorders of severe epithelial dysplasia to invasive OSCC. Analysis of α-SMA expression for MF proliferation can be used as a stromal marker for predicting behavior in oral precancer and cancer.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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