Stromal myofibroblasts in oral squamous cell carcinoma and potentially malignant disorders

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Abstract

INTRODUCTION: Concomitant with the progression of a non-diseased epithelium to the pre-cancerous epithelium to carcinoma, the stroma also undergoes modifications. Myofibroblasts are important stromal cells that play a crucial role in carcinogenesis. The current study investigated the presence of myofibroblasts in healthy oral mucosa, potentially malignant disorders (PMDs) and squamous cell carcinoma (SCC).

MATERIALS AND METHODS: The study material consisted of a total of 106 samples categorized into three groups, namely, Group I - Oral SCC (OSCC) (n = 42), Group II - PMDs (n = 32) and Group III - Oral healthy mucosa (n = 32) subjected to immunohistochemical analysis using alpha Smooth Muscle Actin. RESULTS: Among the 42 cases of OSCC, the staining index was negative in 23 cases (54.7%), low in 9 cases (21.4%) and moderate in 10 cases (23.8%). The stroma of cases of verrucous carcinoma, cases of Hyperkeratosis with epithelial dysplasia, 77.5% of the cases of oral Submucous Fibrosis (OSMF) and healthy oral mucosa were devoid of myofibroblasts resulting in a grade of "0" in all cases. Two of the cases of OSMF (12.5%) showed low staining index for myofibroblast. There was a significant difference in the myofibroblasts expression between the Groups (Kruskal-Wallis test P<0.001). CONCLUSION: The findings of the current study justify "myofibroblast" as one among the key stromal element in tumor progression. Future studies involving a larger sample size along with follow up of patients with PMDs are essential to identify the exact stage in which they emerge in the stroma of these lesions.

Key Words: Leukoplakia, myofibroblasts, oral cancer, oral submucous fibrosis

Introduction

Oral Squamous Cell Carcinoma (OSCC) is a major health problem worldwide, especially in the developing countries. Squamous cell carcinoma (SCC) is the most common oral cancer in India with an incidence rate as high as 30-40%[1] and is associated with a high mortality rate. Among the diseases classified under potentially malignant disorders (PMDs),[2] the prevalence of Oral Leukoplakia and Oral Submucous Fibrosis (OSMF) is high in the Indian Subcontinent due to the habit of arecanut chewing. In India, about 5 million people are affected by this disease.[3]

Interactions between epithelial cells and the connective tissue play a major role in many biological processes. From development to maintenance of normal homeostasis, there is a constant reciprocal interaction between these two tissues. This continues even in pathological states where in the two tissues complement each other contributing to the spread of the pathologies. Though the nomenclature “carcinoma” denotes genetic and epigenetic changes of the epithelial cells, they cannot spread or progress without the supportive role of connective tissue. Therefore, the tumor stroma or activated stroma has drawn much attention, with stromal cells namely fibroblasts, myofibroblasts, macrophages, inflammatory cells, which favor the spread of carcinomas with myofibroblasts being a prominent cell type.[4] Transdifferentiation of fibroblasts to myofibroblasts is considered an important event that occurs in the stroma of many invasive carcinomas.[5,6]

Although it has been hypothesized that the presence of myofibroblasts in the stroma is entirely dependent on the OSCC development,[7] Cintorino et al.[8] have identified myofibroblasts being associated with different grades of cervical intraepithelial neoplasia.

Very few studies have independently evaluated the presence of myofibroblasts in normal oral mucosa, Leukoplakia with mild, moderate and severe dysplasia and OSMF (two of the most prevalent PMDs in the Indian Subcontinent), verrucous carcinoma and different histological grades of SCC. Therefore, this study was designed to evaluate the presence of myofibroblasts in PMDs and SCC, that might help in better understanding of the role played by these cells in the oncogenic process from its evolution stage.

Materials and Methods

Sample selection

The study material consisted of a total of 106 samples categorized into three groups, namely, Group I-OSCC (n = 42), Group II – PMDs (n = 32) and Group III – histopathologically normal oral mucosa (n = 32). Formalin fixed paraffin embedded blocks of all the cases were retrieved from the archives of the Department of Oral and Maxillofacial Pathology, between the years 2008-2012. The diagnosis was confirmed based on histopathological examination of Hematoxylin and Eosin stained sections. Epithelial Dysplasia was graded into three groups (mild, moderate and severe epithelial dysplasia) based on the classification proposed by Neville et al.[9] The diagnosis and grading of OSCC was based on the World Health Organization 2005 Classification of head and Neck Tumors.[10] OSMF cases were histopathologically graded into early, moderately advanced and advanced according to Pindborg and Sirsat.[11] The study was approved by the institutional review board.

Group I consisted of 42 cases of SCC with the following subgroups: Well-differentiated SCC (WDSCC) (n = 13), moderately differentiated SCC (MDSCC) (n = 10), poorly differentiated SCC (PDSCC) (n = 09) and a variant of SCC-verrucous carcinoma[12] (n = 10).
Group II consisted of 32 cases of PMDs of which 16 were Hyperkeratosis (clinically, leukoplakia) with mild, moderate and severe dysplasia (5 mild, 5 moderate and 6 severe) and 16 cases of OSMF (3 early, 5 moderately advanced and 8 advanced). The moderately advanced and advanced grades of OSMF also had epithelial dysplasia. Group III consisted of 32 histologically normal oral mucosal specimens.

**Immunostaining procedure**

For immunohistochemical study, 3 µm sections were cut from formalin fixed paraffin embedded blocks, mounted on gelatin coated slides. The sections were deparaffinized in xylene, dehydrated in alcohol and rinse in distilled water. Antigen retrieval was performed using heat induced - epitope retrieval in citrate buffer (pH 6.0) for 10 min in a pressure cooker. Following which endogenous peroxidase was blocked for 5 min, protein block for 5 min. Sections were then incubated with Mouse Monoclonal Alpha Smooth Muscle Actin-SMA-R-7-CE (Leica Microsystems, New Castle, UK) and incubated for 60 min. Detection was performed using Novolink Min Polymer Detection System (Leica Microsystems, Newcastle, UK). The sections were then counterstained with Mayer’s hematoxylin. The slides were then dehydrated and mounted. Negative and positive controls were used in each run. Leiomyosarcoma sections were used as a positive control for alpha SMA and the blood vessels in the sections acted as internal control. Negative controls were achieved by performing the staining procedures with the omission of the primary antibody.

**Evaluation of slides**

Presence of brown colored end product at the site of target antigen was indicative of positive reactivity. Stromal spindle cells that were positive for αSMA were regarded as myofibroblasts. Immunostaining was assessed by the evaluation of the staining intensity and percentage of αSMA-positive cells, according to the method used by Etemad-Moghadam et al.[12] The percentage of immunopositive cells in the non-inflammatory and non-endothelial stromal cells immediately adjacent to the carcinomatous islands, OSMF, dysplastic and normal epithelium was recorded as: 0% = no positive cells, 1% =1–25% cells, 2% =25–50% cells and 3% =50–100% positive cells. Staining intensity was considered 0%, when there was no staining; 1%, in parts where positivity was observed only at a magnification of x400; 2%, in cases where the staining was obvious at x100, but not at x400; and 3%, in fields where immunopositive cells were seen even at x400. Multiplication of the percentage and intensity scores comprised the “staining index” of each specimen. This index was classified as zero (0), low (1, 2), moderate (3, 4) and high (6-9). The immunohistochemical stained sections were analyzed by two independent observers. Any disagreements were resolved using a pentahead microscope.

**Statistical analysis**

All the parameters were tabulated and assessed for statistical significance using the SPSS software (Version 16.0). The differences in the presence of myofibroblasts between groups were statistically analyzed using the Kruskal-Wallis test and Mann-Whitney U test. The Bonferroni approach was used to adjust the P value in multiple comparisons. P < 0.017 was considered to be statistically significant.

**Results**

A total of 106 cases, comprising of 42 cases of OSCC, 32 cases of PMDs, 32 sections of histologically normal oral mucosa were evaluated immunohistochemically for the presence of myofibroblasts. Among the 42 cases of OSCC, the staining index was negative in 23 cases (54.7%), low in 9 cases (21.4%) and moderate in 10 cases (23.8%) [Figure 1]. The staining index of myofibroblasts according to the different histological grades of OSCC is summarized in Table 1.

As far as the distribution of myofibroblasts was concerned they were predominantly around the tumor islands and in areas of desmoplasia. Tumors lacking a fibrous stroma showed minimal or complete absence of myofibroblasts. The stroma of cases of verrucous carcinoma did not show the presence of myofibroblasts [Figure 2]. Moreover, the presence of myofibroblasts was not significantly different between the subgroups of OSCC (Kruskal-Wallis test P < 0.107).

**Table 1: Immunohistochemical staining index for α-smooth muscle actin in different grades of OSCC and verrucous carcinoma**

<table>
<thead>
<tr>
<th>Subgroups of group I</th>
<th>α-Smooth muscle actin staining index</th>
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<tbody>
<tr>
<td></td>
<td>Zero</td>
</tr>
<tr>
<td>WDSCC</td>
<td>6</td>
</tr>
<tr>
<td>MDSCC</td>
<td>4</td>
</tr>
<tr>
<td>PDSCC</td>
<td>3</td>
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<tr>
<td>Verrucous carcinoma</td>
<td>10</td>
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*WDSCC=Well differentiated squamous cell carcinoma; MDSCC=Moderately differentiated squamous cell carcinoma; Poorly differentiated squamous cell carcinoma*
The stroma of all cases of leukoplakia with epithelial dysplasia, 77.5% of the cases of OSMF and healthy oral mucosa were devoid of myofibroblasts resulting in a grade of “0” in all the cases [Figures 3 and 4]. The alpha SMA staining was restricted only to the smooth muscles of the blood vessels. Two of the cases of OSMF (12.5%) showed low staining index for myofibroblast and in both cases, the myofibroblasts were restricted to areas subjacent to the epithelium [Figure 5]. The immunohistochemical staining index for αSMA in normal, dysplastic epithelium, OSMF and OSCC is summarized in Table 2.

There was a significant difference in the myofibroblasts level between the groups (Kruskal-Wallis test \( P < 0.001 \)) indicating a fairly strong relationship between the presence of myofibroblasts among groups. To test which group is more prominently different follow-up tests were conducted to evaluate pairwise differences among the three groups, controlling for Type I error across tests by using the Bonferroni approach. Mann-Whitney U test was employed for these comparisons. However, there was no significant difference in the presence of myofibroblast between the PMDs group and the healthy mucosa group. These results are summarized in Tables 3 and 4.

### Discussion

The present study was aimed to compare the presence of myofibroblasts in a possible continuum of lesions, starting from healthy oral mucosa, leukoplakia and OSMF, verrucous Carcinoma and SCC.

The present investigation demonstrated a phenotypic change in the stroma of OSCC characterized by a gain of αSMA positive myofibroblasts in 45.3% of the cases of OSCC \((n = 42)\). This is in accordance with the previous studies.\(^{[13-17]}\) The basement membrane normally separates the epithelial and the stromal components. The invading tumor cells cause a breach in the basement membrane, thereby bringing the epithelial components in close juxtaposition to the stromal components. This is accompanied by many stromal changes, one of which, that is most important and facilitates tumor progression is the trans differentiation of fibroblasts to myofibroblasts.\(^{[18]}\) This emphasizes the necessity to understand the immensely supportive role played by tumor stroma in order to develop potent therapeutic targets.

Myofibroblasts may stimulate tumor progression by stimulating the growth of cancer cells, sustaining blood vessel formation and lymphangiogenesis, attenuating cancer...
Therefore, myofibroblasts act as key players in collective tumor invasion as they are capable of remodeling the extracellular matrix and providing the mechanical propulsive force that facilitates invasion.[28]

There was no difference in the distribution of myofibroblasts between the three histological grades of SCC in the current study. This is in agreement with the results obtained by Kellermann et al.[28] and Etemad Moghadam et al.[13] These findings indicate that myofibroblasts transdifferentiation is a process that occurs independent of tumor differentiation.[13]

In the present study, the stroma of verrucous carcinoma (n = 10) showed complete absence of myofibroblasts. Verrucous carcinoma, considered to be a low grade variant of SCC,[12] histologically lacks a breach in the continuity of the basement membrane. This histological feature along with our study findings possibly underscores the need for a breach in the basement membrane to evoke a myofibroblastic response. This process is similar to the normal wound healing response where in myofibroblasts are activated as the normal homeostasis between the epithelium and connective tissue is perturbed. Yet another histological feature that can justify the absence of myofibroblasts in verrucous carcinoma is the intense inflammatory cell infiltrate that precedes the advancing front of the tumor. There is a trend for the presence of myofibroblasts to be inversely related to the inflammatory cell infiltration.[29] Taking into account these two features, the lack of myofibroblasts in the stroma of verrucous carcinoma may also be a contributory factor for its low-grade behavior.

The stroma of all cases of leukoplakia with mild dysplasia, moderate dysplasia and severe dysplasia were devoid of myofibroblasts. These results are in accordance with the previous studies in the literature.[5,13,14,16,29] Studies by Costea et al.[18] have shown that stromal cells, especially fibroblasts regulate epithelial morphogenesis. This control of fibroblasts is progressively lost during neoplastic progression. Therefore, dysplasia represents an intermediate step, wherein the stroma is losing its control over epithelial morphogenesis. Once the dysplastic epithelium accumulates further mutations resulting in the invasion, the stroma responds by acting as a co-conspirer. Hence, myofibroblasts differentiation is an event that occurs late in the carcinogenesis process. The literature along with our study findings indicate that stromal myofibroblasts cannot be used as a marker to predict malignant transformation potential of leukoplakia. This relative absence of myofibroblasts in the stroma of Leukoplakia may also reflect its low malignant transformation potential of 6.2%[31] despite its high prevalence.

OSMF is a chronic debilitating PMD with marked fibrosis. In comparison with the world population, the Indian population, in particular the south Indian population has a

cell death and stimulating invasion and metastasis by activating proteolysis.[19,23]

Among the three histological grades of OSCC, 41% of the cases showed absence of myofibroblasts and 59% showed the presence of myofibroblasts in OSCC. Myofibroblasts were present in 7 (53.84%, n = 13) cases of WDSCC, 6 cases (60%, n = 10) of MDSCC, 6 cases (66.6%, n = 9) of PDSCC [Table 1]. This heterogeneity in the presence of myofibroblasts has been previously reported[14,16,24] and could be attributed to the varied secretion of transforming growth factor-β by the tumor cells,[25] which is essential for myofibroblast differentiation.

The arrangement of myofibroblasts was confined to the stroma immediately adjacent to the tumor islands and tumor free stroma was devoid of myofibroblasts. The presence of myofibroblasts directly abutting the tumor cells suggest the possible role played by a tumor cell derived diffusible growth factor that promotes myofibroblast differentiation.[15] This close proximity of tumor cells and myofibroblasts also supports two more hypothesis:

• Myofibroblasts can possibly be derived from the epithelial mesenchymal transition of the tumor cells[16,17,26]

• Myofibroblasts form tunnels that lead the invasive tumor cells in vitro.[27] Therefore, myofibroblasts act as key players in collective tumor invasion as they are capable of remodeling the extracellular matrix and providing the mechanical propulsive force that facilitates invasion.[28]

There was no difference in the distribution of myofibroblasts between the three histological grades of SCC in the current study. This is in agreement with the results obtained by Kellermann et al.[28] and Etemad Moghadam et al.[13] These findings indicate that myofibroblasts transdifferentiation is a process that occurs independent of tumor differentiation.[13]
high prevalence of OSMF (prevalence rate of 1.2-4.57%) due to tobacco chewing habit.\[32\] Apart from being a high risk PMD, with a malignant transformation rate of 4.5%,\[33\] the disease also causes a significant morbidity among the high risk PMD, with a malignant transformation rate of 4.5%.\[34\] The study of myofibroblasts in cases of OSMF might possibly help us to understand its pathogenesis, the similarities; if any, between fibrosis in other organ systems and OSMF and the possible contributory role of fibrosis induced epithelial mesenchymal transition in its malignant transformation. There are previous studies where myofibroblasts have been studied in cases of OSMF.\[34\]

In the current study, two cases of OSMF showed the presence of myofibroblasts.\[35\] In both cases myofibroblasts, were distributed in the connective tissue subjacent to the epithelium. It has been observed that myofibroblasts derived from fibrotic tissues are more capable of promoting tumorigenesis through their interaction with carcinoma cells compared with the normal fibroblasts derived from normal tissues.\[36\] Altered tissue microenvironment, elicited by wounding and fibrosis, significantly increases the risk of tumour incidence and progression as demonstrated in lung, liver and breast.\[35-38\] Co-relating this to OSMF, the possibility of epithelial – mesenchymal interactions resulting in altered keratinocyte phenotype predisposing to the development of malignancy in OSMF has been reported.\[39\] Our study results showed the presence of myofibroblasts subjacent to the epithelium. In one among these cases, patient had developed SCC on the contralateral site. Putting it all together, their presence might represent an actively happening carcinogenic process or a more likelihood for malignant transformation in the near future. However, follow-up studies are required to confirm the significance of this finding.

On studying this continuum of lesions, it is summarized that the absence of myofibroblasts in the stroma of all cases of dysplasia, indicate myofibroblasts differentiation to be an event that occurs late in the carcinogenesis process and is not an ideal marker to relate its malignant transformation potential. As far as OSMF is concerned, the possible role of myofibroblast in the pathogenesis and in malignant transformation has been suggested. However, further research involving larger sample size along with follow-up studies will clearly elucidate our findings. It also confirms the essential role of myofibroblasts in the stroma of SCC promoting tumor progression. The absence of myofibroblasts in the stroma of verrucous carcinoma substantiates its biological behavior as a low grade variant of OSCC.

Future attempts should be directed in identifying the exact stage of occurrence of myofibroblasts in the stroma in PMDs and malignancy. This is quite a challenging task, as dysplasia is not a continuum of process as not all epithelial dysplasias progress to malignancy and malignancy might arise from any grade of dysplasia. The early event in carcinoma is most often missed in routine diagnosis making it difficult to identify these important stromal cells at an early stage.

**Conclusion**

The abundance of myofibroblasts in the stroma of OSCCs may be used as a stromal marker of aggressive behavior as it correlates with poor prognosis. It might help us in identifying a subset of patients who require more aggressive methods of therapy in order to improve their survival rate. Myofibroblasts, if proven to be a marker predictive of malignant transformation in OSMF, might help in an early intervention and patient counselling for prompt follow-up. This might help to curb the carcinogenesis process and provide an improved quality of life to the patients.

**References**

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