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ORIGINAL ARTICLE

Myofibroblast presence in apparently normal mucosa adjacent to oral squamous cell carcinoma associated with chronic tobacco/areca nut use: evidence for field cancerization

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Abstract

Objective. Myofibroblasts are primary cellular components of activated tumor stroma, associated with poor prognosis in oral squamous cell carcinoma (OSCC). However, their role in field cancerization has not been addressed. This study aims to evaluate the presence of myofibroblasts in patient-matched histologically normal mucosa adjacent to oral squamous cell carcinoma (HNMAOSCC) and OSCC tissues. ***Materials and methods.*** Fifty patient-matched tissues of OSCC and HNMAOSCC associated with chronic areca nut/tobacco use were subjected to immunohistochemistry using α-SMA for detection of myofibroblasts. Normal oral mucosa ($n = 15$) were stained as controls. ***Results.*** The number of α-SMA stained myofibroblasts in OSCC and HNMAOSCC were significantly increased as compared to that of the normal controls ($p < 0.001$). Further, a significant correlation was established for the presence of myofibroblasts in the stroma of OSCC and HNMAOSCC. ***Conclusions.*** Myofibroblasts are an early stromal change in the HNMAOSCC, highlighting the possible role of myofibroblasts as likely mediators for field cancerization and their potential use as a field effect marker.

Key Words: *areca nut, apparently normal mucosa adjacent to oral squamous cell carcinoma, field cancerization, myofibroblasts, oral squamous cell carcinoma, tumorigenesis, tobacco*

Introduction

Most oral cancers usually recur within 2 years of treatment of primary tumors. In spite of histopathological confirmation of complete excision and major advances in the management modalities, the risk of developing a recurrence or a second primary tumor is ~20% [1,2]. This risk associated with considerable morbidity and mortality has been predominately attributed to the field cancerization phenomenon [2,3]. The concept of field cancerization was originally described by Slaughter et al. [3] several decades ago. They proposed that genetic alterations in response to exposure to carcinogens may be spread broadly across the mucosa within an affected field and oral carcinoma developed in a multi-focal pattern within this altered field [2,3]. Several studies have also corroborated that a field of genetically altered but clinically normal tissue extends 1 cm or more beyond the margins of oral squamous cell carcinoma (OSCC) [4,5]. However, the molecular mechanisms governing field change remains unclear and the extent, composition and the biologic significance of this altered field is still ambiguous [4–6]. The evidence for field cancerization to date has been by studies that have established several molecular changes in apparently normal tissue adjacent to OSCC as compared to normal tissues. These include differences in the proliferative index, morphometric variation of nuclear and cytoplasmic areas, chromosomal aberrations, microsatellite alterations, *P53* expression, altered CK expression, etc. in the epithelial cells which emphasize the possibility that these molecular alterations can provide mechanisms for limitless replicative capacity and genomic instability that support tumor initiation and progression [4,6]. However, the

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possible role of the surrounding stromal components in field cancerization has been less explored.

It is now widely recognized that tumor development is a prolonged and circuitous process involving a continuous reciprocal interaction between the tumor cells and their surrounding microenvironment/stroma analogous to normal development and during wound healing [7]. Tumor stroma composed of inflammatory cells, capillaries, fibroblast, myofibroblasts and extracellular matrix components reflect disturbed interactions between the neoplastic cells and the surroundings that support tumor initiation, progression and metastases [8–12]. Coincident with the conversion of non-diseased epithelial tissue to pre-cancerous epithelium to carcinoma, the stroma also changes from 'normal' to 'primed' to 'activated or tumor associated' [11,13]. Fibroblasts are considered to be the most important stromal cells and transdifferentiation of fibroblast to myofibroblast is a crucial and early event during tumorigenesis [14,15]. Myofibroblasts characterized by α-SMA positivity, originally identified in granulation tissue and wounds, are considered to be the critical cells in an activated/desmoplastic stroma of many primary and metastatic epithelial malignancies [14,15]. They are named variably as stromal myofibroblasts, carcinoma associated fibroblasts, activated fibroblasts and peritumoral fibroblasts [14,16]. These cells are considered to be main effectors for the tumor needs in terms of angiogenesis, production of desmoplastic reaction due to increased deposition of collagen and ECM proteins, release of metalloproteinases for collagen breakdown and tissue remodeling that promotes invasion and modulation of host immune response [8,16,17]. The presence of myofibroblasts has been studied in OSCC and has been correlated with increased aggressiveness, metastasis and recurrence, especially in tongue carcinomas [17,18]. Recently, myofibroblasts have been demonstrated in histologically normal tissue adjacent to breast carcinomas as likely mediators of field cancerization [13]. Currently, there have been no attempts made to elucidate the presence and role of myofibroblasts in apparently normal mucosa to assess oral field cancerization. The present study aims to evaluate the presence of myofibroblast in pair-matched primary tumors and apparently normal mucosa adjacent to OSCC to elucidate its role in field effects.

Materials and methods

Tissue material

After obtaining the institutional ethical clearance, the material for the study included 50 patient-matched formalin-fixed paraffin-embedded tissue blocks of histologically proven cases of oral squamous cell carcinomas along with the apparently normal mucosa 1 cm beyond the lesion, i.e. beyond the surgical margin adjacent to OSCC (ANMAOSCC). The lesional tissue as well as the ANMAOSCC were obtained from entire surgical excision and radical neck dissection specimens available in our department. The ANMAOSCC included specimens taken 1 cm beyond the surgical margins which were evaluated for clearance from tumor cells and absence of dysplasia in the epithelium. The patients were all predominantly males (45) and five females in the age range of 35–65 years. All these patients had a history of areca nut and tobacco chewing for varying duration ranging from 3–21 years, with a frequency of 5-times a day on an average. The OSCC were predominantly located in buccal mucosa (27), tongue (10) and the maxillary and mandibular gingivo-buccal sulcus (13) sites. Fifteen tissue blocks of normal oral mucosa obtained from gingival and vestibular mucosa after extraction of impacted teeth were used as controls. Diagnosis was confirmed by two oral pathologists using hematoxylin and eosin stained sections. These cases of OSCC, ANMAOSCC, along with normal oral mucosa were subjected to immunohistochemistry for α-SMA for detection of myofibroblasts.

Immunohistochemistry

Paraffin embedded tissue blocks were cut into 4–5 μm thickness and mounted onto 2%, 3-aminopropylethoxysilane solution (APES) (Sigma Aldrich, St. Louis, MO) adhesive coated slides. The sections were de-paraffinized and rehydrated through xylene and descending grades of alcohol. Antigen retrieval was performed using a commercial microwave antigen retrieval system where the sections were placed in a container containing 10 mM sodium citrate buffer (pH = 6.0) at 96°C for three cycles of 6 min each (EZ-Retriever System, Biogenex life sciences, San Ramon, California, USA). After rinsing in PBS, the sections were treated with 3% H_2O_2 in water for 15 min, followed by a power block for 20 min to block any endogenous peroxidase activity and non-specific antigenic sites. The sections were subsequently incubated at room temperature for 1 h with optimally prediluted antibody against α-SMA (Biogenex: Clone 1A4). After rinsing with PBS, the sections were then exposed to One-Step Polymer-HRP reagent for 30 min. Visualization was performed using freshly prepared DAB (di-amino benzadine tetrahydrochloride) chromogen. The slides were counterstained with Harris hematoxylin, subsequent to which sections were dehydrated, cleared and mounted with DPX. For each batch of staining, positive and negative control were run simultaneously with the study specimens. Normal salivary gland tissue served as a positive control and the endothelial staining of blood vessels known to be reactive to α-SMA was used as the

internal positive control; while the primary antibodies were replaced by non-immune mouse serum at the same dilutions for the negative controls.

Immunohistochemical analysis

Stromal spindle cells positive for α-SMA were regarded as myofibroblasts. Immunostaining was assessed by the evaluation of α-SMA positive cells, according to the method used by Kellermann et al. [19]. The presence of immunopositive cells in the non-inflammatory and non-endothelial stromal cells in the sub-epithelial connective tissue throughout the section were classified as 0 = negative,1 = Scanty (1–25%) and 2 = abundant (>25–100%). All hematoxylin/eosin and immunohistochemical stained sections were evaluated by two observers to eliminate inter-observer bias and any disagreements were resolved with a penta-head microscope.

Statistical analyses

The differences in the presence of myofibroblasts in different groups were analyzed using Kruskal-Wallis and Chi-square tests. p-values of less than 0.05 were considered to be statistically significant. Correlation of myofibroblast presence in patient-matched OSCC and ANMAOSCC was evaluated using the Spearman correlation coefficient test.

Results

Fifty patient-matched histologically proven cases of OSCC and apparently normal mucosa adjacent to OSCC were stained with hematoxylin and eosin (H&E). On histopathological evaluation, they belonged to well differentiated (33 cases) and moderately differentiated carcinoma (17 cases) grades. All these cases and the normal oral mucosa (15 cases) were stained with α-SMA.

Myoepithelial cells exhibited α-SMA positivity in the salivary gland tissue that served as positive control and the blood vessels within the connective tissue served as positive internal control stained with intensity in all the cases. These internal controls confirmed the accuracy of the staining protocol. The normal mucosa, OSCC and ANMAOSCC demonstrated immunostaining at varying levels for α-SMA, as demonstrated in Table I.

A statistically significant difference was noted in the presence of myofibroblasts between normal, OSCC and ANMAOSCC (Kruskal Wallis test; $p < 0.001$) (Table I and Figures 1,2,3). Furthermore, statistical significance at $p < 0.001$ was observed between normal and OSCC, normal and ANMAOSCC and between OSCC and ANMAOSCC using Chi Square test (Table II). Additionally, a positive correlation was identified between the presence of myofibroblast in patient matched OSCC and ANMASCC ($r = 0.335$, $p = 0.017$) (S) (Figures 2 and 3).

Table I. Immunohistochemical staining for myofibroblasts in normal, histologically normal mucosa adjacent to OSCC and OSCC.

		α-SMA staining			
Groups of cases	Total cases	0	1	2	p-value
Normal Oral Mucosa	15	11	3	1	
HNMAOSCC	50	7	34	9	
OSCC	50	3	23	24	0.0010*

*Kruskal-Wallis Test; α-SMA; Alpha Smooth Muscle Actin; OSMF, Oral Squamous cell carcinoma; HMAOSCC, mucosa adjacent to squamous cell carcinoma.

Discussion

Field cancerization is induced by carcinogens that act on a large area of tissue and cause molecular alterations that may not be obvious clinically and histologically, but can increase the risk of development of

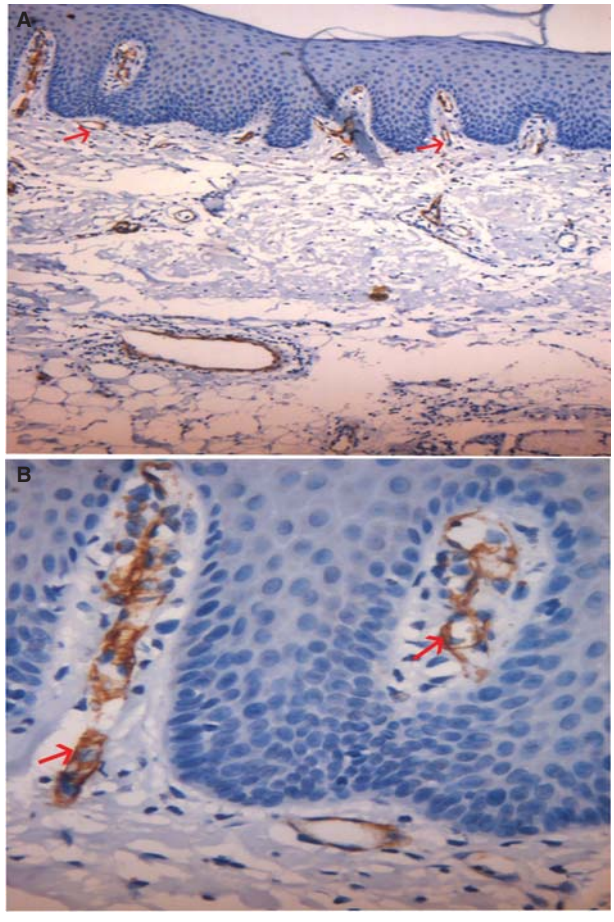

Figure 1. Normal mucosa depicting α-SMA positivity in the endothelial cells (red arrows) with absence of myofibroblasts (score 0) (A $\times$100, B $\times$250).

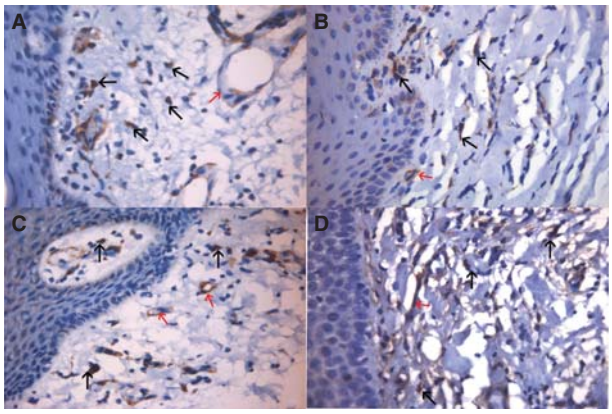

Figure 2. α-SMA expression demonstrating scanty myofibroblasts (score 1) (A, B, C ×250; black arrows) and abundant myofibroblasts (score 2, black arrows) (D ×250) in stroma of histologically normal mucosa 1 cm beyond the margins of oral squamous cell carcinoma. Red arrows depict endothelial cells showing α-SMA positivity.

malignancy [2,20]. Historically, most studies on neoplastic transformation and progression have focused on the tumor cells. However, in addition to the transformed cells, tumors are composed of host stromal tissue comprising of fibroblast, newly formed vessels and inflammatory components. Although stroma was initially thought to support tumor development passively, there is increasing evidence to suggest that it actively contributes to malignant progression [21,22]. The tumor microenvironment has significant similarities to an inflammatory or a wound healing stromal response [22,23]. Within this microenvironment, fibroblasts constitute the majority of cells, which in turn are often activated and transdifferentiated to myofibroblasts or carcinoma-associated fibroblasts [18]. These stromal myofibroblasts are known to stimulate angiogenesis by secreting pro-angiogenic factors, release various matrix metalloproteinases, regulate the immune system by secreting chemokines and mediate direct effects on the tumor

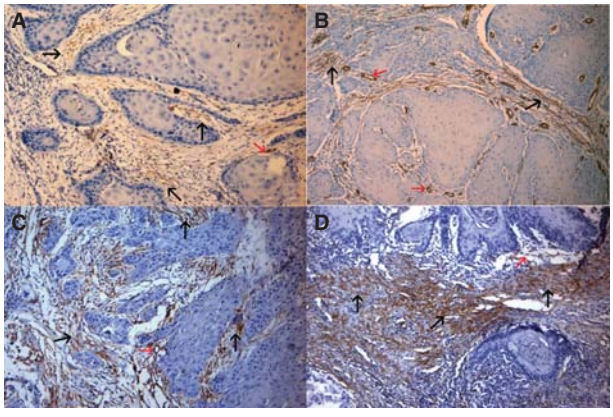

Figure 3. Depicts scanty (score 1, black arrows) (A, B ×100) to abundant myofibroblasts (score 2, black arrows) (C, D ×100) in stroma of oral squamous cell carcinoma. Red arrows depict α-SMA positive endothelial cells (A–D).

Table II. Chi-square test for comparison of α-SMA staining in the various study groups.

Groups of cases	Total cases	α-SMA staining 0	1	2	*p*-value
NOM	15	11	3	1	0.001 (S)
HNMAOSCC	50	7	34	9	
NOM	15	11	3	1	< 0.001 (S)
OSCC	50	3	23	24	
HNMAOSCC	50	7	34	9	0.005 (S)
OSCC	50	3	23	24	

α-SMA, Alpha Smooth Muscle Actin; NOM, Normal Oral Mucosa; OSCC, Oral Squamous cell carcinoma; HNMASCC, histologically normal mucosa adjacent to squamous cell carcinoma; S, Significant.

cells that render them hyper-proliferative and pro-invasive, contributing to tumor initiation, invasion and progression [24].

In the present study, we have demonstrated that myofibroblasts are present in the tumor stroma of most of the OSCC cases studied (94%). This is in accordance with previous studies in oral squamous cell carcinoma by Moghadam et al. [15] (100%), Vered et al. [17] (100%) and Kellerman et al. [19] (60%). Moreover, several studies have correlated its expression with tumor recurrence, survival and increased incidence of lymph node metastases [17,19,25,26].

Tumor-derived cytokines are the best candidates to generate fibroblast diversity and myofibroblast differentiation [23]. Both *in vivo* and *in-vitro* OSCC cells have been shown to secrete increased amounts of cytokines and growth factors such as IGF, TGF-α, PDGF and various interleukins [18]. TGF-β, in particular, has been deemed responsible for the transdifferentiation of fibroblast to myofibroblast [18]. Lewis et al. [21] and Kellerman et al. [25] have shown OSCC induced transdifferentiation of fibroblast to myofibroblast via secretion of TGF-β1. Emerging evidence implies that the malignant epithelial cells themselves could be a source of myofibroblasts by epithelial mesenchymal transition (EMT) phenomenon [27], as demonstrated by Vered et al. [28] in tongue carcinomas.

Further, our study also showed that the myofibroblasts were not only present in the stroma surrounding the neoplastic cells, but extended well beyond the margins and were evident in the connective tissue below the apparently normal mucosa adjacent to the lesion. Although the magnitude of their occurrence was predominantly scanty in ANMASCC as compared to their abundance in the neoplastic stroma ($p < 0.005$); nevertheless their presence is a striking finding, since the normal mucosa from non-cancer patients (control group) demonstrated relative

absence of myofibroblasts ($p < 0.005$). This raises the possibility that myofibroblasts may be an early stromal change and may contribute to the field cancerization effect. It evokes a provocative possibility that the field of abnormal stroma precedes or initiates tumorigenesis.

This prospect is substantiated by an elegant experiment which established that knocking out the TGF-β II receptor in stromal fibroblasts in a transgenic mouse model resulted in spontaneous squamous cell carcinoma of the stomach and pre-neoplastic lesions of the prostate. This implies that an underlying abnormality of fibroblasts can initiate and advance epithelial cancers [29]. Further, the role of myofibroblast in tumor initiation was illustrated by Olumi et al. [30], who showed that prostate epithelial cells were not tumorigeneic *in vivo*, when transplanted alone or with normal human prostate fibroblasts, but on co-transplantation with activated myofibroblasts/CAF, the prostate epithelial cells formed large solid tumors [30]. Thus, myofibroblasts have been implicated in epithelial/stromal signalling, even before the onset of invasion in several carcinomas such as breast [31], cervix [32] and colon [33], and have been detected even during the pre-malignant stages before invasion in these tumors. However, presence of myofibroblasts in normal, hyperplastic and dysplastic epithelium in the oral cavity is contradictory in *in vivo* [15,34] as well as *in-vitro* reports [35] and generally an increase in myofibroblasts has been evidenced concomitant with invasion [18,35]. Thus, it appears the OSCC cells influence the myofibroblast transdifferentiation in the tumor stroma which in turn reciprocally aids in further tumor progression. These carcinoma associated myofibroblasts are considered to be activated and retain their tumor promoting effects after passage for 10 population doublings *in vitro*, even when they are no longer in continuous contact with the tumor cells [16,36]. Therefore, it can be stipulated that, initially, these myofibroblasts develop under the influence of carcinoma cells and create an altered microenvironment. Since these myofibroblasts retain their active trait and represent a motile mesenchymal phenotype [36], they may easily spread to the surrounding submucosal compartment beyond the limitation of the cancer margins and are consequently detected in ANMAOSCC in the present study. Here, the myofibroblasts might serve as a switch for development of overt malignancy and provide 'fertile soil for development of second primary tumors (SPT), multiple primary tumors (MPT) and recurrent tumors of field cancerization' [13,37,38]. This possibility is supported by the positive correlation observed for the myofibroblast presence in the primary tumors and their matched ANMAOSCC. Myofibroblasts are also considered to be primary mediators of angiogenesis and their presence in ANMAOSCC correlates well with El-Gazzar et al.'s [12] observation of a prominent angiogenic profile in the normal mucosa adjacent to OSCC as compared to the normal mucosa. In breast carcinomas, myofibroblast presence has been reported in tumor adjacent mucosa 1 cm beyond the margins and has been attributed to have arisen from the tumor cells via EMT [13]. This suggests another intriguing prospect that these myofibroblasts could actually represent carcinoma cells themselves which have undergone EMT. However, additional studies are needed to elucidate the origin and precise mechanisms involved in field effects to define the true prognostic significance of myofibroblast presence in ANMAOSCC.

Ge et al. [37] hypothesized that, in field cancerization, the carcinogens in tobacco and areca nut not only cause genomic alterations in the epithelial cells but may also affect the stromal cells to mediate field effects [37]. The evidence regarding the likelihood of stromal cells harboring genetic alterations is rather mixed and controversial [39–43]. Several studies have demonstrated loss of heterozygosity and somatic mutations of TP53, PTEN alterations and genetic instability in the stroma of breast cancer and ovarian cancer [39,40]. Specifically, carcinoma associated myofibroblasts have been shown to demonstrate epigenetic modifications, such as DNA methylation, which may pre-dispose to their tumor promoting phenotype [9,41]. However, in contrast studies have also failed to detect significant genetic alterations in the stromal myofibroblasts [42,43]. Nevertheless, if these myofibroblasts are arising via EMT, there is a likelihood of these cells harboring the genetic alterations present in the carcinoma cells. This highlights the need for additional studies related to the presence of genetic/epigenetic alterations within the tumor stroma and specifically to evaluate the cellular and molecular mechanisms that influence the tumor promoting phenotype in myofibroblasts, especially in oral squamous cell carcinoma.

Conclusion

This is the first report demonstrating the presence of myofibroblast in ANMAOSCC, highlighting the possible role of myofibroblast as a likely mediator for field cancerization and its potential use as a field effect marker. The current investigation also reveals interesting insights into the complex, reciprocal interactions between the tumor and the surrounding stroma, even before the onset of overt carcinoma. Resection margins need to be evaluated for genetic and epigenetic alterations, not only in the epithelial cells but also in the myofibroblasts for risk assessment. Further, anti-stromal agents targeting myofibroblast recruitment and activation can be introduced as chemopreventive/therapeutic modalities to combat field effects.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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