Journal of Clinical and Translational Research 2022; 8(1): 80-85

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Role of cancer-associated fibroblasts in oral squamous cell carcinomas, surgical margins, and verrucous carcinomas: An immunohistochemical study

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ARTICLE INFO

Article history:
Received: August 18, 2021
Revised: December 01, 2021
Accepted: January 08, 2022
Published online: January 25, 2022

Keywords:
cancer-associated fibroblasts
squamous cell carcinoma
tumor microenvironment
tumor margins
verrucous carcinoma

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ABSTRACT

Background and Aim: Cancer-associated fibroblasts (CAFs) are among the key tumor microenvironment components that determine tumor invasion, progression, and resistance to cancer therapeutics. Histologically normal mucosa adjacent to oral squamous cell carcinoma (OSCC) has been shown to harbor CAFs which aid in the loco-regional recurrence of the lesion. Verrucous carcinoma (VC), a low-grade variant of squamous cell carcinoma, has a better clinical outcome. However, few VCs show an aggressive biological course and necessitate wide excision with strict follow-up. Scarce literature is available regarding the role of CAFs in VCs. Thus, our study aimed to evaluate the frequency of CAFs in OSCC, normal mucosa adjacent to OSCC, and VC.
Methods: Thirty cases of squamous cell carcinoma, normal mucosa adjacent to OSCC, and VC each were included in the study. The sections were stained with an antibody against alpha-smooth muscle actin protein and CAF frequency was evaluated.
Results: The CAF frequency was highest in squamous cell carcinoma, followed by VC, and least in normal mucosa adjacent to OSCC (P<0.001).
Conclusion: CAF frequency progressively increases with an increase in the grade or biological behavior of the lesion. Thus, screening CAF frequency in these benign and malignant oral lesions is necessary for better treatment outcomes.
Relevance for Patients: The immunohistochemical screening for CAFs in OSCC and VC can serve as an integrated approach for the development of a directed treatment plan that leads to a better patient prognosis. Routine assessment of CAF frequency in surgical margins can serve as an adjunct in determining clear margins and possible locoregional recurrence. Furthermore, target therapy for CAFs can be used to minimize possible recurrence and distant metastasis.

1. Introduction

Cancer and its microenvironment are a conglomerate of numerous factors that self-support the growth and progression of cancer cells. Cancer-associated fibroblasts (CAFs) form one such integral part of the microenvironment that drive tumorigenesis and metastasis [1,2]. CAFs can be derived from local fibroblasts, endothelial cells, hemopoietic stem cells, preadipocytes, and tumor epithelial cells by epithelial-mesenchymal transition [1-3]. These CAFs interact with epithelial cells and other connective tissue cells and regulate tumor invasion and angiogenesis

DOI: http://dx.doi.org/10.18053/jctres.08.202201.007

[4-12]. They modulate the stroma primarily by the production of factors such as metalloproteinase, growth factors, cytokines, and chemokines [10-14]. CAFs are a prominent feature of the tumor stroma of many, but not all oral squamous cell carcinomas (OSCCs) [4]. When present, CAFs are associated with resistance to immunotherapy and poor disease-free survival in OSCC [4,14-16]. In addition, the CAFs have also been found in potentially malignant conditions and histologically normal mucosa adjacent to oral squamous cell carcinoma (HNMOSCC) [17-21].

Benign tumors such as Verrucous Carcinomas (VCs) generally have a better clinical outcome; however, 1/5th of VCs co-exist with an OSCC. Such hybrid tumors may remain histologically unidentified and have a greater tendency to recur locally [21]. Hence, it is essential to differentiate these VCs from the conventional ones at the time of diagnosis for improved treatment outcomes. Understanding the molecular differences between VCs and OSCCs has been the focus of various studies [21-29]. However, most of these studies concentrate on the differences between the malignant epithelial components but the stromal component of VCs is relatively unexplored. Thus, the present study aimed to evaluate the role and difference in the CAF frequency in OSCCs, HNMOSCC, and VCs.

2. Methods

In the present retrospective study, histologically diagnosed tissue blocks of OSCCs, VCs, and HNMOSCC (30 each) were obtained from the department archives. The study was exempt from the institutional ethical committee review because of its retrospective nature. The patient-matched OSCC lesional tissue and HNMOSCC were obtained from the surgical excision and radical neck dissection specimens available in the archives. The HNMOSCC included were taken one centimeter beyond the surgical margin of OSCC; they were histologically tumor-free and the epithelium was devoid of dysplasia. The patient-matched OSCC and HNMOSCC tissues consisted of 26 male and four female patients. The age range was 35-90 years. The most common site involved was buccal mucosa (12 cases), followed by the tongue (seven cases), alveolar mucosa (three cases), retromolar area (three cases), gingivobuccal sulcus (three cases), and palate (two cases). VC had 16 male and 14 female patients in the age range of 35-80 years. VCs were predominantly located on the buccal mucosa (18 cases) followed by labial mucosa (four cases), two cases of buccal vestibule and gingivobuccal sulcus; and one case each of retromolar area, tongue and alveolar mucosa and palate. Tissue sections of 4 µm thickness were taken on 3-aminopropyl triethoxy silane-coated slides. One section was stained using Harris hematoxylin-eosin and the other with αSMA (ready to use, Monoclonal Mouse Anti-Human αSMA, Clone 1A4; catalog no AM128-5M BioGenex Lab). The αSMA antibody is widely used as an immunohistochemical marker for the identification of differentiated fibroblasts/CAFs [12]. Immunohistochemically stained sections with αSMA were evaluated for the frequency of expression of CAFs. The blood vessels stained positively with αSMA acted as an internal positive control for each slide. The slides were evaluated by three independent oral pathologists with similar training and experience. The difference in scoring of CAFs was settled by consensus using a Penta-head microscope. Kellerman's scoring criteria [14] was modified and utilized to evaluate the slides based on the percentage of stained cells. The cells were evaluated at ×10 and confirmed further at ×40. The score was considered as 0 for no expression of CAFs, 1 for 1-20% CAFs, 2 for 21-40% CAFs, 3 for 41-60% CAFs and 4 for >60% CAF expression. This criterion for scoring was used to reduce the manual errors caused by subjective evaluation of staining intensity.

2.1. Statistical analysis

The data were tabulated and analyzed in the SPSS software. The data were analyzed using Kruskal–Wallis tests and Mann–Whitney test ($p \leq 0.05$ was considered significant).

3. Results

The frequency scores of αSMA positive CAFs in OSCCs ranged from 1 to 4 (Figure 1A-D). About 40% OSCCs (12/30 cases) exhibited CAF score 3, that is, 41-60% CAFs in the stroma (Figure 1C). In case of HNMOSCC, 13.3% of cases showed no CAF expression (Figure 2A) and remaining showed expression of score 1 (Figure 2B). About 10% of VCs did not show αSMA positive CAF (Figure 3A). 50% VCs (15/30 cases) had the expression of Score 1 (Figure 3B), 20% VCs expressed Score 2 (Figure 3C), 16.7% VCs had Score 3 (Figure 3D), and only 3.3% VCs expressed Score 4 CAF frequency (Figure 3E). On comparing the CAF frequency between the study groups using Kruskal-Wallis and Mann-Whitney test, we found the results to be statistically significant ($P<0.001$) (Tables 1 and 2).

Histologically, OSCCs showed CAFs with the network-like (Figure 1C) and spindled (Figure 1D) arrangement pattern. These CAFs were primarily noted in the tumor-invasive front. Whereas

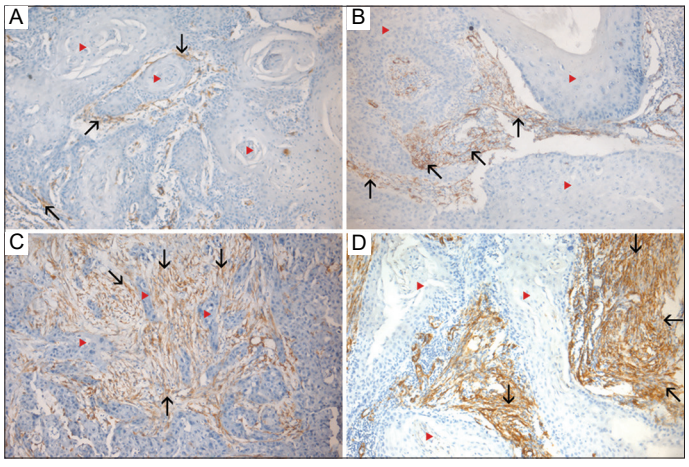

Figure 1. Photomicrographs of OSCC stained with αSMA antibody depicting different scores of expression of CAFs (**A)** Score 1, that is, 1–20% CAFs (×10), (**B)** Score 2, that is, 21–40% CAFs (×10), **(C)** Score 3, that is, 41–60% CAFs (×10) and **(D)** Score 4 for >60% CAF expression (×10). (Red arrowheads show tumor islands and black arrows indicate CAFs). OSCC: Oral squamous cell carcinoma, CAFs: Cancer-associated fibroblasts.

DOI: http://dx.doi.org/10.18053/jctres.08.202201.007

Table 1. Comparison of αSMA expression scores/Cancer-associated fibroblast frequency in all the study groups using Kruskal–Wallis test.

Groups (n)	Score 0 (Negative)	Score 1	Score 2	Score 3	Score 4	Chi square (χ^2) *P* value
OSCC (30)	0	3 (10%)	9 (30%))	12 (40%)	6 (20%)	χ^2=51.840
VC (30)	3 (10%)	15 (50%)	6 (20%)	5 (16.7%)	1 (3.3%)	*P*<0.001*
HNMOSCC (30)	4 (13.3%)	26 (86.7%)	0	0	0	

*The difference in αSMA expression was statistically significant. OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma, HNMOSCC: Histologically normal mucosa adjacent to oral squamous cell carcinoma

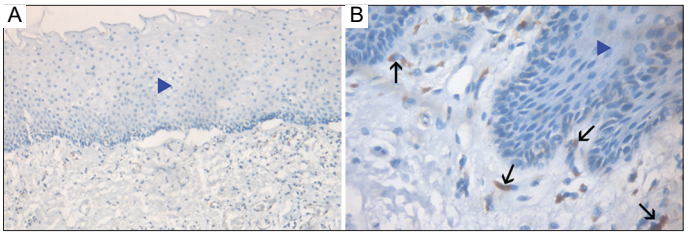

Figure 2. Photomicrograph of HNMOSCC stained with αSMA antibody. **(A)** Score 0, that is, no CAFs (×10). **(B)** Score 1 of CAFs in HNMOSCC located juxtaepithelially (×40), (blue arrowheads show normal epithelium and black arrows show CAFs). HNMOSCC: Histologically normal mucosa adjacent to oral squamous cell carcinoma, CAFs: Cancer-associated fibroblasts.

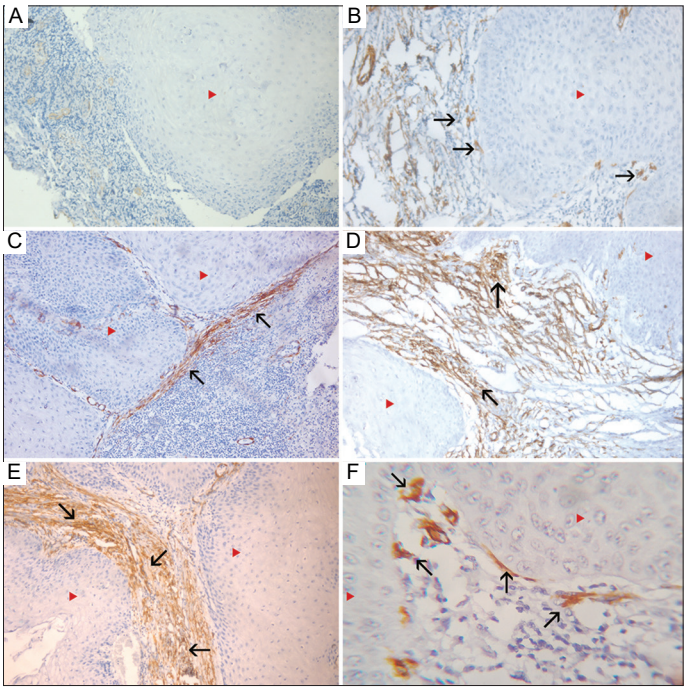

Figure 3. Photomicrograph of VCs stained with αSMA demonstrating varying score of CAFs (×10) **(A)** Score 0 with no expression of CAFs, **(B)** Score 1, that is, 1–20% CAFs (×10), **(C)** Score 2, that is, 21-40% CAFs (×10), **(D)** Score 3, that is, 41–60% CAFs (×10), **(E)** Score 4 for >60% CAF expression (×10) and **(F)** CAFs abutting the tumor rete pegs stained with αSMA (×40) (Arrows point to CAFs; red arrowheads point tumor rete ridges). VCs: Verrucous carcinomas, CAFs: Cancer-associated fibroblasts.

Table 2. Comparison of αSMA expression scores/Cancer-associated fibroblast frequency among the study groups using Mann–Whitney test.

Groups (n)	Z value	*P* value
OSCC (30)	4.063	<0.001*
VC (30)		
OSCC (30)	3.020	<0.001*
HNMOSCC (30)		
VC (30)	6.497	<0.001*
HNMOSCC (30)		

*The difference in αSMA expression was statistically significant. OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma, HNMOSCC: Histologically normal mucosa adjacent to oral squamous cell carcinoma

VCs showed the CAFs arrangement in spindled patterns adjacent to bulbous rete ridges (Figure 3F). The occurrence of CAFs in HNMOSCC was absent or scarce to patchy in distribution.

4. Discussion

Mortality rates of OSCC have remained high even with the advances in treatment modalities due to the high loco-regional recurrence and metastasis of tumors [13]. Tumor micro-environment is implicated in bringing about this progression and metastatic changes. The CAFs which are an integral part of the tumor micro-environment play a central role in facilitating tumor invasion, progression, metastasis, and therapeutic resistance [3]. It has been observed that the epithelial component of OSCC shows intra-tumor and inter-tumor heterogeneity, but the stroma of the OSCC shows a consistent signature [1]. This is also reflected through our present study wherein αSMA positive CAFs were noted in all the cases of OSCC. These results are concurrent with those of Vered *et al.* [15] (100%), Angadi *et al.* (94%) [17], and Chaudhary *et al.* [18].

The frequency of CAFs in OSCCs ranged from Score 1 to Score 4. In OSCCs, the CAFs were found surrounding the tumor islands and cords in the stroma, and often in the deep invasive front of OSCCs (Figure 1). The arrangement pattern in some cases was in the form of delicate rows surrounding and abutting the tumor islands, while others showed a marked syncytium-like arrangement of the CAF in the stroma. These arrangement patterns could reflect the biological dynamic interactions between the components of tumor tissues. Kellerman *et al.* [14] and Vered *et al.* [15] have put forth that increasing frequency of CAF expression is associated with poor prognosis. This could be attributed to the fact that the CAFs serve as "factories" producing a wide range of proteins, signaling

DOI: http://dx.doi.org/10.18053/jctres.08.202201.007

molecules, cytokines, and growth factors. They boost tumor angiogenesis, drive tumor invasion, and thereby help in tumor progression and metastasis [8,11]. Hence, the carcinomas with higher expression of CAF may result in loco-regional recurrence.

Furthermore, in HNMOSCC, we found the presence of αSMA positive CAFs in 86.7% of cases. These CAFs were scanty and showed juxtaepithelial localization (Figure 2B). The expression of CAFs in HNMOSCC was significantly lower than that in OSCC (P<0.001). Since CAFs were not present in normal mucosa, as demonstrated by Chaudhary *et al.* [18] and Angadi *et al.*, [22] their presence in HNMOSCC suggests that HNMOSCC harbors tumor-induced changes. Clinically, even if the epithelial malignancy is removed with safe margins, yet histologically, CAFs can be left behind in the tumor-associated stroma undiagnosed. These CAFs can undergo a mesenchymal-epithelial transformation, or they may be instrumental in modifying the epithelium to produce second primary tumors [22]. Increased frequency of CAFs is significantly associated with disease recurrence and poor patient survival [15]. Analyzing the "molecular status" of the surgical margins to predict the outcome and plan adjuvant therapy for OSCC is a more pragmatic approach [30]. CAFs mediate microenvironment changes and possible mesenchymal-epithelial interactions. Their evaluation can aid in understanding the biological behavior of OSCCs and surgical margins. Hence, αSMA alone or with other markers should be employed to screen the surgical margins to predict treatment outcomes.

In the case of VCs, we found that the CAFs were expressed in 96.7% of cases localizing predominantly around the rete pegs or juxtaepithelially (Figure 3B-F). This result was in concordance with the findings of Chaudhary *et al.* [18], who observed 86.67% of cases showing expression of the CAF in VC. In our study, the frequency of CAF in VCs was significantly lower than the frequency in OSCCs (P<0.001) and higher than that in HNMOSCC (P<0.001). The expression of CAFs in HNMOSCC, VCs, and OSCCs can be in a continuum, with HNMOSCC having the minimum frequency of CAFs while the cases of OSCCs having the maximum frequency.

In HNMOSCC and VCs, CAFs were localized juxtaepithelially with no breach in the continuity of the basement membrane. This could be because secreted factors such as TGF-β1 or microRNAs produced by tumor cells may pass through the basal lamina to reach the connective tissue thereby bringing about the differentiation of CAFs [3,6]. A previous study has found an increased expression of TGF-β1 in VCs and OSCCs [27]. CAFs through matrix metalloproteinases breakdown the basement and mediate digestion to generate tracts for carcinoma cells. Alternatively, they can increase permeability and remodel the matrix by applying contractile force, thus creating a breach in basement membrane fibers to allow the passage of carcinoma cells. Hence, VCs with CAFs may be hypothesized to harbor areas of basement membrane destruction like that of OSCC. This premise could potentially explain the fact reported by Johnson *et al.* [21] that 1/5th of VCs are hybrid tumors that demonstrate traditional OSCC like areas and show a tendency to recurrence. Immunohistochemical evaluation of CAFs in the stroma of VC can help predict molecular micro-environmental changes in these indolent lesions. This can enable the surgeons to maintain stern follow-up of VCs with a high frequency of CAFs. Chaudhary *et al.* [18] have reported CAFs in VC but, in the present study, we have proposed the possible hypothesis for transformation of CAFs even in lesions with intact basement membrane. Moreover, we also hypothesize that lesions with more CAFs may have poor prognosis.

Theoretically, we could associate the CAF frequency to the differentiation of epithelial cells. However, being a preliminary research, the present study is limited by the lack of follow-up data. Further inclusion of histological parameters such as tumor thickness, depth of invasion, and neurovascular invasion is needed to elaborate on the role and interactions of CAFs with the tumor microenvironment. Molecular studies for testing the association of high frequency of CAFs with extracellular matrix gene signature, immune profiles of inflammatory cells such as macrophages or tumor-infiltrating lymphocytes, role of reactive oxygen species, and their cross-talks can aid to elicit the dynamics that regulate progression and loco-regional recurrence of oral malignancies.

5. Conclusion

The presence of CAFs in VCs highlights the possibility that VCs could have an aggressive biological course with increased predilection toward post-surgical recurrence. Expression of CAF in HNMOSCC supports the theory of the role of CAF in field cancerization and development of second primary tumors. Hence, instead of a treatment plan solely dependent on clinical or histopathological features, screening the tumor and surgical margins for CAFs will be of prognostic significance. The present report distinctly discusses the role of CAF screening in OSCCs and VCs for predicting patient prognosis and highlights the need for the development of a CAF-based targeted therapy. Targeted therapy for CAFs can aid in minimizing immune response failure, recurrence, and distant metastasis.

Acknowledgment

The authors would like to thank Dr Sidramesh Shivanand Muttagi, Department of Oral and Maxillofacial Surgery KLE VK Institute of Dental Sciences for his support in conducting the study.

Conflict of Interest

The authors declare no affiliations and/or personal relations which could have influenced the work reported in the paper.

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DOI: http://dx.doi.org/10.18053/jctres.08.202201.007