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Cancer-associated fibroblasts are an infrequent finding in the microenvironment of proliferative verrucous leukoplakia-associated squamous cell carcinoma

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BACKGROUND: Cancer-associated fibroblasts (CAFs) are generally associated with negative prognostic factors. This study compares the clinicopathologic impact of CAFs in oral squamous cell carcinoma in patients with a history of proliferative verrucous leukoplakia (p-scca) and patients with conventional squamous cell carcinoma of the buccal mucosa, gingiva, and palate (c-scca).

METHODS: A retrospective clinicopathologic and immunohistochemical analysis of 97 tumor specimens from 78 patients (13 patients with proliferative verrucous leukoplakia-associated squamous cell carcinoma (n = 32) and conventional squamous cell carcinoma from the buccal mucosa, gingiva, and palate (n = 65) was conducted. Immunostaining with anti-alpha-smooth muscle actin (α-SMA) antibody was used to evaluate the presence of CAFs.

RESULTS: α-SMA expression was an infrequent finding in p-scca and seen in only 6% of p-scca compared to 40% of c-scca (P < 0.0004). In the c-scca subgroup, α-SMA significantly correlated with tumor size (T) (P = 0.009), tumor thickness (P < 0.0009), perineural invasion (P = 0.009), and microscopic grade (P = 0.018).

CONCLUSIONS: The presence of CAFs was an infrequent finding in our p-scca cohort which may contribute to its seemingly slower growing and less invasive growth pattern. In the cohort of c-scca patients, higher levels of CAFs correlated with microscopic invasiveness, tumor size, and perineural invasion. Practically, these are important observations as targeting strategies are being developed to combat carcinoma types where CAFs significance has been validated.

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Introduction

Oral cancer (OSCC) represents 2.8% of all new cancer cases in the United States with an estimated 5-year survival of 83% for localized disease, 47% for OSCCA that presents with lymph node metastasis, and 18% for OSCCA that presents with a distant metastasis (1). Increasing evidence seems to indicate that OSCCA is a heterogeneous disease rather than a single entity with clinically relevant subtypes (2, 3).

Within the oral cancer community, there is a unique subgroup of patients who develop OSCCA with a prior history of proliferative verrucous leukoplakia (PVL). PVL, a 'potentially malignant disorder' is a unique type of oral leukoplakia that presents in multiple sites within the oral cavity and is highly difficult to treat, resulting in multiple recurrences over a time span of many years (4–6). PVL presents clinically in a progressive series of stages. Initially, the lesions appear innocuous and are indistinguishable clinically from leukoplakia. The second developmental stage is clinically exophytic lesions, diagnosed microscopically as verrucous hyperplasia and/or verrucous carcinoma (4–6). In the later stages, over 74% of patients with PVL will experience multiple and multifocal incidences of OSCCA, particularly on the gingiva, palate, and buccal mucosa (5, 6). The diagnosis is retrospective because the early stages lack identifiable microscopic or clinical features that can distinguish PVL from other clinically similar lesions. In a previous study, we compared the clinicopathological features of OSCCA arising in PVL patients (p-scca) with conventional buccal mucosa, gingival, and palatal OSCCA (c-scca) and discovered that p-scca patients seem to present with more favorable prognostic features and longer duration of illness (DOI). Based on the results of this earlier study, we suggested that p-scca should be considered a separate clinical entity (6).

Current treatment strategies for treating OSCCA include wide tumor resection with supplement radiotherapy and/or chemotherapy for a subset of patients with negative clinical features such as lymph node metastasis and/or positive surgical margins (7). Designing new treatment strategies

such as targeted and molecular-based treatments may improve patient outcome. In the last two decades, we have discovered that cancer is a complex and heterogeneous disease that involves a complex network of communication between the malignant cancer cells and non-malignant cells found within the supportive microenvironment surrounding the tumor. Heterotypic signals originating within the stroma activate a variety of growth factors that stimulate the resident fibroblasts into becoming 'cancer-associated fibroblasts' (CAFs) (8). CAFs, a subpopulation of cells of various origins with diverse functions, are implicated in a variety of negative properties associated with cancer behavior (9–11). Unlike quiescent fibroblasts, activated fibroblasts (myofibroblasts) may be identified by their wide-spread expression of alpha-smooth muscle actin (α-SMA) (8). Myofibroblasts are a rich source of chemokines, cytokines, inflammatory mediators, extracellular proteins, and extracellular matrix degrading proteases [matrix metalloproteinases (MMPs)] (8). In malignancies, these myofibroblasts, also known as CAFs, are capable of providing the necessary tools for inducing angiogenesis, local invasiveness, and metastasis (12–14). Practically, recent investigations have shown that (CAF)-specific proteins serve as both prognostic markers and targets for anticancer drugs (15).

This study was initiated to explore the presence of CAFs in two subgroups of oral cancer patients (c-scca and p-scca) and correlate its expression with various clinicopathological characteristics to try and determine the role of the tumor microenvironment in driving PVL and non-PVL-related carcinomas.

Materials and methods

Data collection

The patient cohort consisted of two oral cancer subgroups treated at Rambam Medical Center of Haifa, Israel from 1990 to 2015: (i) c-scca from the buccal mucosa, gingiva, and palate and (ii) patients retrospectively diagnosed with PVL who developed squamous cell carcinoma (p-scca). The inclusion criteria were patients treated with tumor resection and confirmation of lymph node status by way of a neck dissection or PET scan and available for immunohistochemical analysis. This represents an expansion and update of a previously existing cohort including patients with the same criteria from 1990 through 2012 (6) and includes follow-up data as of July 1, 2016. The study was approved by the IRB of Rambam Medical Center. Medical records following a process of anonymization for data collection were retrieved from the department archives. There were 13 PVL patients that presented with 52 p-scca of which 32 fulfilled the inclusion criteria and were included in the study and 65 cases of c-scca. The overall cohort consisted of 97 tumors. PVL was diagnosed retrospectively by at least two experienced oral pathologists according to the criteria set forth by Batsakis, et al. (16): a history of multiple, white homogenous lesions (hyperkeratosis with/without lichenoid features) that recurred and progressed to an exophytic lesion (papillary hyperplasia/verrucous carcinoma) that eventually dedifferentiated to a squamous cell carcinoma. The included patients were staged according to the 1997 International Union against Cancer classification (TNM). Stages I and II were considered early stage and stages III and IV, late-stage patients. Clinical outcomes were measured by the following endpoints: (i) loco regional disease control expressed by loco regional recurrence, second primary tumors and/or cervical and distant metastasis. (ii) overall survival. Data regarding cause of death were grouped as follows: (i) dead of disease (DOD) which represented the patients with whom their oral malignancy was the major component of the cause of death. DOD was indicated whether the patients' time of death occurred during or within 3 months of active treatment for their tumor. (ii) censored patients were those lost to follow-up or those who died of unrelated causes. Treatment options included surgery and neck dissection, with or without adjuvant treatment (radiotherapy, radiochemotherapy, or chemotherapy alone). Twenty-nine of 97 (36%) patients received adjuvant therapy. Follow-up time was a minimum of 6 months and up to 15 years with an average 30.9 ± 32.8 months.

Microscopic data collection and immunohistochemical analysis

Microscopic analysis was conducted retrospectively. Paraffin-embedded tumor blocks of all 97 p-scca and c-scca cases that were resected between 1990 and 2015 were retrieved from the archives of the department of pathology at Rambam Medical Center. The paraffin-embedded blocks were sectioned in 4-μm-thick slides and stained with H&E. For immunohistochemical analysis of CAFs (identified by α-SMA), 4 μm-thick sections were deparaffinized and immunostained with anti-alpha-smooth muscle actin (α-SMA) antibody (Dako A/S, Glostrup, Denmark) at a dilution of 1:100, in a Ventana Benchmark automatic stainer. The streptavidin–biotin peroxidase complex (SABC) method was used by means of an automatic stainer (Ventana, benchmark system). The rich vasculature network within the examined tumor cases served as an internal control.

Scoring of immunohistochemistry

The quantity and pattern of CAFs expression for each stained section was evaluated blindly by two experienced oral pathologists and used a four-scale scoring system based on the method proposed by Bello et al. (17) with some modest modifications. (iv) Score 4: dense overlapping of myofibroblasts distributed throughout the tumor predominately of epithelioid morphology, with essentially no distinct border with the scca. (iii) Score 3: similar to grade 4, somewhat less dense, or myofibroblasts not distributed throughout the entire tumor. (ii) Score 2: predominately spindle, less dense with a clear border between the myofibroblasts and the scca and not distributed throughout the entire tumor. (i) Score 1: no staining or staining with either a spindle or epithelioid morphology in a focal pattern. Scores 1 and 2 were considered negative/weak staining, score 3 moderate staining, score 4 strong staining. Blood vessel walls served as positive internal controls for α-SMA staining in each case. Cells were considered positive for α-SMA if there was intracytoplasmic staining, regardless of its intensity. CAFs were distinguished from microvessels based on the CAFs

spindle-shaped morphology. The evaluation of α-SMA expression was conducted at the invasive front of the SCCA. CAFs, noted by α-SMA expression, was calculated and analyzed for its association with various microscopic characteristics including tumor thickness, tumor grade, and perineural invasion. Tumor thickness was defined as the measurable distance from the surface epithelium to the invasive tumor front. Measuring tumor thickness for exophytic tumors was performed according to the methods recommended by Woolgar and Scott (18) and defined by the surface line of the surrounding healthy mucosa. CAFs, noted by α-SMA expression, was calculated and analyzed for its association with various clinical characteristics including TNM stage (tumor size, lymph node metastasis, and distant metastasis) and overall survival.

Statistical analysis

Variables measured included incidence rate, clinical stage (TNM; tumor size, lymph node metastasis and distant metastasis), survival and microscopic features such as microscopic grade, perineural invasion, and depth of invasion (tumor thickness). Associations between the levels of α-SMA staining and discrete clinicopathological variables were tested using the chi-square test or the Fisher exact test as needed with the Benjamini–Hochberg correction for multiple hypotheses. Two-tailed *P*-values of 0.05 or less were considered statistically significant.

Results

Patient cohort

Refer to Table 1 for details on the clinicopathologic variables and their correlation to survival. In the c-scca subgroup ($n = 65$), there were 34 males (mean age 65 ± 11.9 years) and 31 females (mean age 71 ± 11.8 years) and the specific location included gingiva and palate ($n = 38$) and buccal mucosa ($n = 27$). Twenty-one (32%) patients had a history of smoking. In the p-scca subgroup ($n = 13$ patients with $n = 32$ tumors), there were five males (mean age 58 ± 13.7 years) and eight females (mean age 68 ± 19 years) and the specific location included gingiva/palate ($n = 15$), buccal mucosa ($n = 15$), and dorsal tongue ($n = 2$). Four (31%) patients had a history of smoking.

α-SMA expression

Statistically significant differences in α-SMA expression was noted between the two groups as its expression was an infrequent finding in the p-scca subgroup ($P < 0.0002$). P-scca α-SMA expression is as follows: negative, weak expression ($n = 30/32$; 94%), and moderate-strong

Table 1 Correlation between SMA expression and clinicopathologic features in the total patient cohort

			C-scca n = *65*					*P-scca* n = *32* *13 patients*				*5 year dead (total cohort)* n = *28*	
			Scores 1-2		*Scores 3-4*			*Scores 1-2*		*Scores 3-4*			
			N	%	N	%	*P-value	N	%	N	%	N	%
Epidemiology	Gender	Male	18	28	13	20	NA	4	30	1	8	13	46
		Female	23	35	11	17		7	54	1	8	15	53
	Age	<65	20	31	8	12	NA	6	46	1	8	11	39
		>65	21	32	16	25		5	39	1	8	17	45
	Smoker	Yes	11	17	10	15	NA	2	15	2	15	10	36
		No	30	46	14	22		9	70	0	0	18	64
Clinical	TNM stage	1	16	25	2	3	0.03	20	62	0	0	2	7
		2	19	29	4	7		3	9	1	3	6	21
		3	5	8	7	5		7	21	1	3	9	32
		4	11	17	10	15		0	0	0	0	11	39
	Tumors size	1	19	29	3	5	0.009	19	59	1	3	2	7
		2	15	23	6	9		5	16	0	0	8	29
		3	5	2	9	14		6	19	1	3	8	29
		4	2	3	6	9		0	0	0	0	10	36
	Lymph node metastasis	Yes	14	22	10		0.4	0	0	0	0	14	50
		No	28	43	13			30	94	2	6	14	50
	Distant metastasis	Yes	4	2	6	9	0.09	0	0	0	0	8	29
		No	38	58	17	26		30	94	2	6	20	71
Microscopic	Tumor grade	Poor	2	3	7	11	0.018	1	3	0	0	7	25
		Moderate	12	18	7	11		5	14	0	0	7	25
		Well	28	43	9	14		24	75	2	6	14	50
	Perineural invasion	Yes	8	12	9	14	0.009	1	3	0	0	10	36
		No	34	52	14	22		29	91	2	6	18	64
	Tumor thickness (cm)	<0.5	14	22	2	3	0.0009	23	71	1	3	9	32
		0.5–0.7	22	34	5	8		5	16	1	3	10	36
		>0.7	6	9	16	25		2	6	0	0	9	32
SMA and 5-year dead (c-scca)			16	57	12	43	>0.06	5	83	1	17		

*P value with the Benjamini–Hochberg correction for multiple hypotheses.
**Statistically significant differences in α-SMA expression was noted between the two subgroups ($P < 0.0004$).

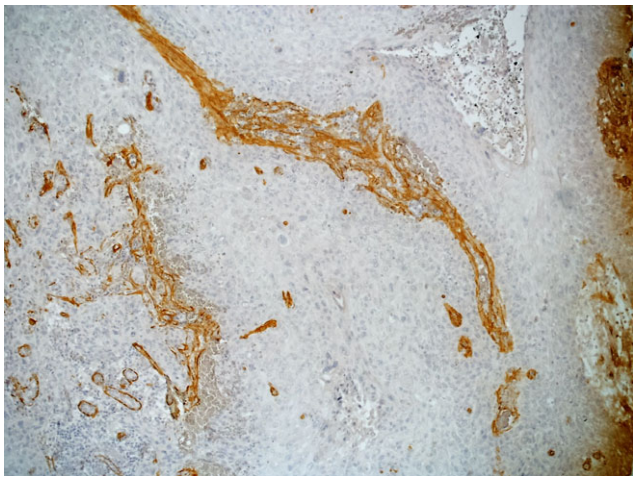

Figure 1 An example of an invasive SCCA with score 3 SMA expression of the myofibroblasts showing overlapping of myofibroblasts with essentially no distinct border with the SCCA.

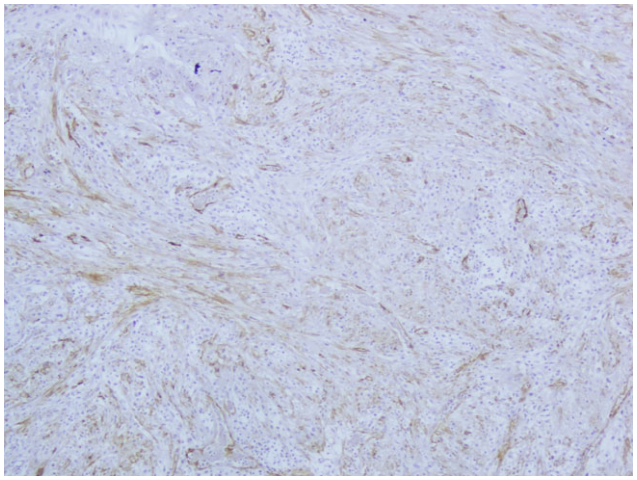

Figure 2 An example of SCCA with score 2 SMA expression of the myofibroblasts showing a predominately spindle, less dense staining pattern.

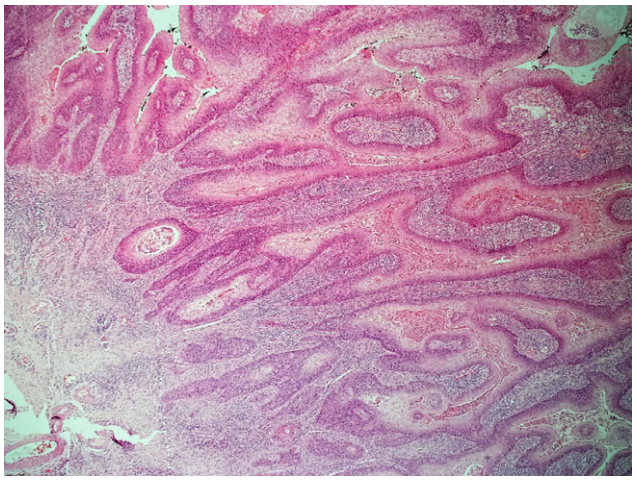

Figure 3 An example of an exophytic, superficially invasive buccal mucosa SCCA (hematoxylin–eosin stain).

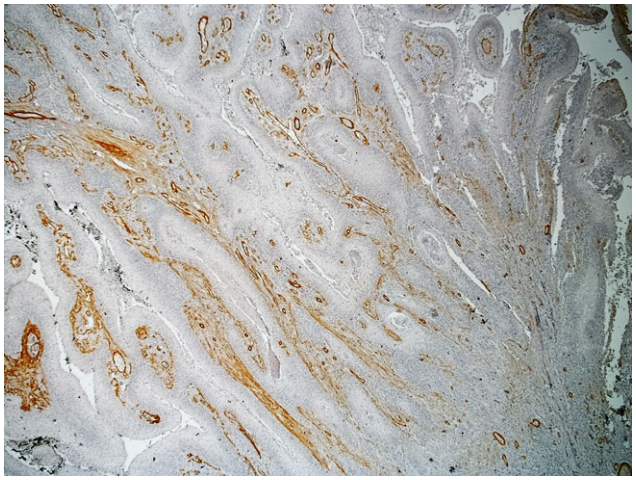

Figure 4 SMA staining of the same superficially invasive SCCA with score 3 SMA expression of the myofibroblasts.

expression (n = 2/32; 6%). C-scca α-SMA expression is as follows: negative, weak expression (n = 39/65; 60%) and moderate/strong expression (n = 26/65; 40%). Neither subgroup in the total patient cohort strongly (score 4) expressed α-SMA (Figs 1 and 2).

α-SMA expression and clinicopathological variables

SMA expression was an infrequent finding in the p-scca subgroup, and its correlation with clinicopathologic parameters was not statistically significant.

Microscopic analysis (c-scca subgroup)

Deeply invasive tumors (tumor thickness), tumor grade, and perineural invasion were significantly associated with moderate α-SMA expression: tumor thickness ($P < 0.0009$): >0.7 cm (n = 16/22; 73%), 0.5–0.7 cm (n = 5/27; 19%), and <0.5 cm (n = 2/16; 12%); the presence of perineural invasion ($P = 0.009$): (n = 11/17; 65%); and microscopic grade ($P = 0.018$): well differentiated (n = 9/37; 24%), moderately differentiated (n = 7/12; 59%), and poorly differentiated (n = 7/9; 78%) (Figs 3 and 4).

Clinical outcome and survival (c-scca subgroup)

Tumor stage (TNM) was significantly associated with moderate α-SMA expression ($P = 0.03$): early stage (n = 7/32; 22%) and late stage (16/33; 48%). Tumor size (T) was significantly associated with moderate α-SMA expression ($P = 0.009$): T1 (n = 2/18; 11%), T2 (n = 4/14; 29%), T3 (n = 9/14; 64%), T4 (n = 6/8; 75%). Association of α-SMA expression with survival, lymph node metastasis, and distant metastasis was not statistically significant ($P > 0.05$).

Discussion

Increasing evidence seems to indicate that head and neck squamous cell carcinoma is a heterogeneous disease rather than a single entity, with clinically relevant subtypes (2, 3).

One subgroup of oral cancer patients are those previously diagnosed with PVL who subsequently develop OSCCA (p-scca). The majority of patients in this distinctive subgroup experience unremitting recurrent and multiple malignant tumors, regardless of the treatment method, over many years. In a previous 15-year retrospective study, our data seemed to establish that patients with p-scca, compared to c-scca, presented with more favorable clinical and histologic features and unique epidemiological characteristics. It was proposed that PVL-induced OSCCA may represent a less aggressive subgroup of oral cancer (6). Recent investigations indicate that the microenvironment is capable of stimulating tumor growth and may have prognostic significance. Practically, the presence of CAF-specific proteins may be used as a prognostic marker and a target for anticancer drugs so detecting CAFs in the stroma of oral SCCA may potentially provide an additional therapeutic method with improved clinical outcome (15).

In the current study, we analyzed and compared the presence of CAFs in the two subgroups. Most p-scca tumors presented on the gingiva, buccal mucosa, and palate so only tumors from these locations were included in our study (4–6). Myofibroblasts play important regulatory roles in normal biologic processes such as biological wound healing and embryogenesis (10). During wound healing, keratinocytes at the border undergo a process known as 'epithelial-mesenchymal transition' (EMT) where they transform into myofibroblasts which provide the migratory and invasive properties required for re-epithelialization of the wound (10).

The cancer microenvironment behaves with striking resemblance to the biological wound site. Heterotypic signals originating in the reactive stroma of carcinomas impinge on neoplastic cells located at the outer edges and by way of the EMT process, transform these cells into myofibroblasts (CAFs) which provide the proliferative and survival skills that favor tumorigenesis such as angiogenesis, local invasiveness, and metastasis (12–14).

Previous studies on CAFs and oral cancer found incidence rates ranging from 60 to 84% (17, 19–22). In our cohort of patients, only 6% of p-scca and 40% of c-scca presented with moderate levels of stromal CAFs. Of the total cohort, none presented with strong staining as described by Bello et al. (17). In the p-scca cohort, CAF levels were very low in both the superficially invasive (75%) and deeply invasive tumors (25%). It can be argued that only a minority of tumors were deeply invasive because the patient was on close follow-up or it can be said that low levels of stromal CAFs were partially responsible. Evidence for the latter belief comes from samples in our study of strong SMA expression in early invasive c-scca as well as from previous studies that noted myofibroblasts the precursors of malignant tumors which emphasizes the CAFs role in the initiation of invasion (23). In fact according to some studies, pre-invasion or early scca is the time myofibroblasts are induced and their numbers increase with the clinical stage (24). Previous studies on verrucous carcinoma and intra-epithelial lesions from the breast, cervix, and oral mucosa, malignancies that have not yet breached the basement membrane, are reported to present with stromal CAFs (25). Our findings seem to indicate that targeting CAFs may have potential for c-scca but little benefit for p-scca patients.

There was a lower quantity and intensity of α-SMA staining in our c-scca cohort compared to other studies which may be explained by the fact that the majority of reported studies focused on tongue cancer. Luksic et al. (20) noted a similar finding and claimed that 'tumors from the gingiva and buccal mucosa were less likely than tongue tumors to harbor CAFs'. It seems that CAFs interact with tumor cells and other players in the tumor microenvironment and affect the clinical outcomes in differential ways according to the context, and tissue of origin.

CAFs and clinicopathologic features

Due to the low levels of CAFs in the p-scca cohort, our clinicopathologic analysis was limited to the c-scca subgroup only. Although the defined role of CAFs is poorly understood, a myofibroblastic stroma has been reported to correlate with adverse clinical outcome in several tumor types including breast and colorectal cancer (26, 27). Our findings, in agreement with others who analyzed CAFs in oral cancer, seem to concur with this assertion (19, 20, 28). Higher levels of stromal CAFs were a more common finding in tumors with expanded growth such as greater clinical tumor size (T stage) and microscopic tumor thickness, as well as perineural invasion which may indicate that CAFs are involved in this process.

The acquisition of local invasiveness and tumor growth requires malignant epithelial cells to drastically alter their characteristic morphology and gene expression pattern and assume the shape and transcriptional characteristic of mesenchymal cells because the organization of the epithelial cell layers in normal tissues is incompatible with the motility and the invasiveness displayed by malignant carcinoma cells. Expression of cytokeratin and E-cadherin, hallmarks of epithelial cell protein expression, is repressed and replaced with the mesenchymal cell markers such as α-SMA (11). CAFs also secrete proteolytic enzymes such as MMPs that participate in the matrix degeneration process which is required for cancer cell invasion (12, 25). Sobral, et al. (28) used ELISA and enzymography to analyze the production and activity of MMPs in OSCCA and found that myofibroblasts promote OSCCA invasion through MMP elevation. CAFs also influence invasiveness by secreting pro-invasive molecules into adjacent cancer cells such as cytokines, inflammatory mediators, and growth factors such as TGF-beta and hepatocyte growth factor (HGF) (29).

Previous studies on tongue squamous cell carcinoma claim that high CAFs levels are associated with decreased survival (17, 19, 22, 28). In our cohort, higher CAFs levels and a decreased survival rate were noted but could not be confirmed statistically. In agreement with some yet not others, our study failed to make an association of CAFs with lymph node or distant metastasis (19, 20, 22, 30). Microscopically, in disagreement with some, high CAFs' levels were associated with less differentiated tumors (20–22, 30). These disparagies with other studies may be explained by the lack of strong staining in our cohort which may be location dependent.

Conclusion

The presence of CAFs was an infrequent finding in our p-scca cohort which may contribute to its seemingly slower growing and less invasive growth pattern. In the cohort of c-scca patients, higher levels of CAFs correlated with microscopic invasiveness, tumor size, and perineural invasion. Practically, these are important observations as targeting strategies are being developed to combat carcinoma types where CAFs significance has been validated.

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Conflict of interest

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