

## **Cancer-associated fibroblasts in the tumor microenvironment of tongue carcinoma is a heterogeneous cell population**

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### **Keywords:**

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### **Abbreviations:**

$\alpha$ SMA – alpha smooth muscle actin

CAFs - cancer-associated fibroblasts

MMT - macrophage-to-myofibroblast transition

MSC – mesenchymal stem cells

OSCC – oral squamous cell carcinoma

SCC – squamous cell carcinoma

TSCC – tongue squamous cell carcinoma

**Abstract**

**Objectives:** To examine different immunophenotypes of cancer-associated fibroblasts (CAFs) in tongue squamous cell carcinoma (TSCC) and to investigate how they related to clinical outcomes.

**Methods:** Serial sections from 54 cases of TSCC were immunohistochemically stained with  $\alpha$ -smooth muscle actin ( $\alpha$ SMA, CAF marker) to determine CAF density, and double-immunostained with  $\alpha$ SMA combined with CD80 and CD86 (myeloid/monocytic-derived cell markers), Nanog (mesenchymal stem cell marker) and CD133 (hematopoietic/endothelial stem cell marker). Density of cells co-expressing these marker combinations was semi-quantitatively assessed in 5 randomly selected high power fields within the tumor area and scored as 1 – one-to-five stained cells in each field, 2 – more than 5 stained cells in each field; any finding less than score 1, was allocated a score of 0.

**Results:** There were 26 CAF-poor, 16 CAF-rich and 12 CAF-intermediated cases. CD86<sup>+</sup> $\alpha$ SMA<sup>+</sup> cells were the most frequent (80.4%) followed by CD80<sup>+</sup> $\alpha$ SMA<sup>+</sup> (72%) and Nanog<sup>+</sup> $\alpha$ SMA<sup>+</sup> cells (56%). The CD133<sup>+</sup> $\alpha$ SMA<sup>+</sup> phenotype was found only in association with blood vessels. High density of  $\alpha$ SMA<sup>+</sup> CAFs was associated with disease recurrence and poor survival ( $p < 0.05$ ). Increased density of CD86<sup>+</sup> $\alpha$ SMA<sup>+</sup> cells was significantly associated with CAF-rich tumors and with poor survival ( $p < 0.05$ ).

**Conclusion:** in TSCC, CAFs demonstrate heterogeneous and overlapping phenotypes with the myeloid/monocytic type being the most frequent and having an impact on the clinical outcomes. Further studies are needed in order to further characterize CAF phenotypes in carcinomas of various oral sites, as this may open new frontiers for personalized medicine.

## Introduction

The most common cancer involving the oral cavity originates from the lining squamous epithelium - oral squamous cell carcinoma (OSCC). There is a 5.5:2.5 male-to-female ratio, with most patients aged 50-70 years (Sloan et al., 2017). However, recent reports from several regions in the world show an inexplicit shifting trend toward women and/or younger patients, especially regarding tongue cancer (Ng et al., 2017; Patel et al., 2011). The mainstay treatment strategy consists of surgery and radiotherapy aimed to eradicate the malignant epithelial cells. Unfortunately, this approach has yielded almost no progress over the past 4 decades and patients face poor survival rates (only ~50% five-year survival) (Hiemer et al., 2015).

In OSCC, similar to other types of solid cancers, malignant cells interact with cells from the tumor micro-environment (TME), such as fibroblasts, endothelial cells, inflammatory cells, and others (Augsten 2014, Kalluri 2016; Kalluri and Zeisberg, 2006). The molecular cross-talk among all these cellular components gives rise to phenotypical modifications with corresponding functional alternations that are collectively aimed to facilitate tumor proliferation, invasion and metastatic spread. Cancer-associated fibroblasts (CAFs), a characteristic sequela of this cross-talk, are the most abundant cell type within the TME (Augsten 2014). In principal, CAFs are phenotypically similar to myofibroblasts that are elicited during wound healing, but in contrast to those cells, CAFs do not disappear and continue to co-exist with the tumor and play a key role in its progression (Cirri and Chiarugi, 2011).

It is now believed that CAFs represent a heterogeneous population of cells of distinct subtypes. Based on the notion that they express an array of markers, such as alpha smooth muscle actin ( $\alpha$ SMA), fibroblast specific factor 1 (FSP1, also known as S100A4), platelet-derived growth factor receptor beta (PDGFR $\beta$ ), fibroblast activating protein (FAP) and NG2, a number of potential cellular origins have been proposed, including resident fibroblasts, mesenchymal stem cells, endothelial cells, pericytes, adipocytes and carcinoma cells that undergo epithelial-mesenchymal transition (EMT) process (Augsten 2014, Kalluri 2016; Kalluri and Zeisberg, 2006). Furthermore, the inconsistency of CAF markers that reflects the plasticity of the tumor and its microenvironment, led investigators to use the term "cell state" for the definition of CAFs rather than the more restricting term of cell origin (Madar et al.,

2013). In *in vivo* models it has been shown that different types of cancers are characterized by CAFs of selective cellular origins/cell states according to the expression of specific markers (Augsten 2014).

In our previous analysis of the TME in tongue SCC (TSCC), we found an inflammatory response rich in T lymphocytes and macrophages and that the presence of this response was inversely related to the presence of CAFs (Dayan et al., 2012). In the present study we aimed to investigate the possibility of CAFs being a product of macrophage "cell state" using CD80 and CD86 as markers of hematopoietic-derived immune cells (Tsushima et al., 2006). In addition, we also investigated whether CAFs co-express Nanog, a marker of cellular pluripotency and self-renewal (Das et al., 2011; Jeter 2009), and CD133, a hematopoietic stem cell marker (Lee et al., 2017; Ranji et al., 2016), as feasible other "cell states" that would add to the phenotypic heterogeneity of the CAF population in TSCC. Finally, associations between CAF phenotypes and the clinical outcomes of disease recurrence and patients' survival, we analyzed.

## **Material and Methods**

### **1. Study group**

Serial sections from resection specimens of 54 patients with TSCC were used, as previously described (Dayan et al., 2012). The study was approved by the IRB of the Chaim Sheba Medical Center, Tel Hashomer. The clinical outcomes were measured by two endpoints: locoregional disease control expressed by locoregional recurrence and overall survival. Time to recurrence was calculated as the interval between the date of diagnosis and the first sign of treatment failure at the primary tumor site, at the site of cervical metastases, or both. The calculation for overall survival included patients alive and free of disease and those alive with disease at the last follow-up visit.

### **2. Cancer-associated fibroblasts (CAF): staining and immunomorphometry**

CAFs were immunohistochemically stained with  $\alpha$ SMA mouse monoclonal antibody (Clone 1A4, 1:100, Zytomed, Berlin, Germany). Positive control tissue was appendix; negative control included section from which the primary antibody was omitted.

Immunoreaction assessment relied on our previously described 5-scale scoring system (Dayan et al., 2012): 0 = absent, 0.5 = a few spindle-shaped CAFs at the periphery of the tumor, 1 = CAFs surrounding the tumor in a few concentric layers in several foci, 2 = CAFs with both spindle-shaped and plump morphology in many areas of the tumor, and 3 = similar to “2” but CAFs exceptionally abundant throughout the section, occasionally exceeding the carcinomatous component. For statistical survival analysis, stained cases were divided into CAF-poor (scores of 0, 0.5, 1), CAF-intermediate (score of 2), and CAF-rich (score of 3) groups.

### **3. Double immunoreactions: staining and immunomorphometry**

These stains included  $\alpha$ SMA coupled with CD80, CD86, CD133 and Nanog. For this part of the study we used the above mentioned antibody against  $\alpha$ SMA that was co-stained with CD80 (rabbit monoclonal, clone EPR1157(2), Abcam, Cambridge, UK; 1:200) and CD133 (rabbit polyclonal, Abnova, Walnut, CA, USA; 1:100). CD86 (goat monoclonal, clone AF-141, R&D Systems, Minneapolis, MN, USA; 1:20) and Nanog (mouse monoclonal, clone 5A10, Acris Antibodies, San Diego, CA, USA; 1:100) were co-stained with  $\alpha$ SMA (rabbit polyclonal, Zytomed, Berlin, Germany; 1:100). Prior to the double immunostaining procedure, the immunoreaction of each antibody was examined separately on positive controls: Nanog on seminoma; CD133 on hepatocellular carcinoma, and CD80 and CD86 on lymph nodes. Negative controls included sections from which the primary antibodies were omitted. For the double immunostains of CD80 and CD133 with  $\alpha$ SMA the primary antibodies were mixed and incubated overnight at 4°C. Secondary antibody was HRP rabbit and mouse (Innovex Biosciences, Richmond, CA, USA). The color reaction of  $\alpha$ SMA was performed using di-amino benzidine (DAB; Invitrogen, Carlsbad, CA, USA) while the reactions of the other antibodies was performed using permanent AP-red chromagen (Thermo Scientific, San José, CA, USA). In regard to the double immunostains of CD86 and Nanog, the slides were first stained with  $\alpha$ SMA and color reaction was performed with DAB (Invitrogen); Meyer's hematoxylin nuclear staining was not performed. Then slides were put in water at 90°C for 5 minutes, cooled to room temperature and incubated with the other primary antibody (i.e., CD86 or Nanog) overnight at 4°C. The CD86 set of slides were further incubated with an anti-goat secondary antibody (Bethyl Laboratories, Montgomery, TX, USA) and the Nanog set with HRP-AP rabbit (Innovex Biosciences, Richmond,

CA, USA). Finally, color reaction was performed with permanent AP-red chromagen (Thermo Scientific, San José, CA, USA).

Density of cells co-expressing these marker combinations was semi-quantitatively assessed in 5 randomly selected high power fields within the tumor area and scored as 1 – one-to-five stained cells in each field, 2 – more than 5 stained cells in each field; any finding less than score 1, was allocated a score of 0. Results are presented as the mean scores for each marker combination per CAF-related group (i.e., poor, intermediate and rich). The association between the density of each of the double immunostain type with clinical outcome of disease recurrence and patients' survival was investigated.

#### **4. Statistical analysis**

The differences in the mean scores for the double immunostains among the CAF-related groups were calculated by One-way ANOVA. Survival analysis was performed using the Kaplan–Meier method, and significance was confirmed by the log-rank test. All statistical analyses were done using SPSS, version 22 (SPSS Inc., Chicago, IL), and the significance level was set at  $p < 0.05$ .

### **Results**

#### **1. CAF density and association with clinical outcomes**

Classification of cases according to CAF density yielded 26 (48%) CAF-poor, 12 (22.2%) intermediate and 16 (29.6%) CAF-rich cases. Examples for each class of CAF are illustrated in Fig. 1. High CAF density (score 3) had a negative impact on disease recurrence ( $p < 0.05$ ) and was associated with poor survival ( $p < 0.05$ ) (Fig. 2).

#### **2. Double immunohistochemical stains and association with clinical outcomes**

Stromal cells showed co-expression of  $\alpha$ -SMA with CD80, CD86, Nanog and CD133. The TME was rich in  $CD86^+ \alpha SMA^+$  stained cells: 19 (35.2%) cases were scored as 2, 22 (40.7%) as 1, and 10 (18.5%) cases were allocated a score of 0. Stromal cells co-expressing  $CD80^+ \alpha SMA^+$  were found to a lesser extent: 10 (18.5%) cases were given a score of 2, 29 (53.7%) a score of 1 and 15 cases were considered as negative (score 0).  $Nanog^+ \alpha SMA^+$  stained cells were the less frequent: 22 (44%) were negative (score of 0), 27 (54%) were weakly immunoreactive (score 1) and only one (2%) was scored as 2.  $CD133^+ \alpha SMA^+$  cells were found only in association with blood vessels (Fig. 3).

In regard to the associations with the clinical outcomes, only CD86<sup>+</sup>αSMA<sup>+</sup> was found to be a negative prognostic factor for survival ( $p < 0.05$ ) (Fig. 4).

The mean score of the CD86<sup>+</sup>αSMA<sup>+</sup> cells in the CAF-rich tumors was significantly higher than in CAF-poor tumors ( $p < 0.001$ ; Fig. 5). The mean score of the CD80<sup>+</sup>αSMA<sup>+</sup> cells did not differ among the CAF-related groups of tumors. Nanog<sup>+</sup>αSMA<sup>+</sup> increased only slightly as the density of CAFs increased but it did not reach any statistical significance.

## Discussion

We have examined αSMA<sup>+</sup>CAF cells in terms of co-expression of a series of markers that represent various cell origins/cell states in order to assess the phenotypic heterogeneity of CAF cell population in TSCC. We also investigated the associations between CAF phenotypes and the clinical outcomes of disease recurrence and patients' survival. We found that high density of αSMA<sup>+</sup>CAF was associated with disease recurrence and had a negative impact on patients' survival. Furthermore, we found that the CAF cell population comprised heterogenic phenotypes, with the CD86<sup>+</sup>αSMA<sup>+</sup> being the predominant variety. Furthermore, we showed that CD86<sup>+</sup>αSMA<sup>+</sup>CAFs were associated with poor survival.

Although CD80 and CD86 are generally considered to play a similar co-stimulatory effect for T lymphocyte response, the CD80<sup>+</sup>αSMA<sup>+</sup> phenotype was less common than CD86<sup>+</sup>αSMA<sup>+</sup> among the CAF cell population. CD80<sup>+</sup>αSMA<sup>+</sup> CAFs were not found to be associated with clinical outcomes. The Nanog<sup>+</sup>αSMA<sup>+</sup> phenotype, assumedly associated with pluripotent and self-renewal cellular functions, was the least common among the CAFs. CD133<sup>+</sup>αSMA<sup>+</sup> phenotype was found only in blood vessel walls and was not associated with the stromal spindle-shaped CAFs. This suggests that cells of a hematopoietic origin are not involved in generation of the CAF population in TSCC.

Our findings regarding the negative impact that TSCC CAFs have on the clinical outcomes are supported by previous studies (Bello et al., 2011; Dayan et al., 2012; Ding et al., 2014; Vered et al., 2010), however this is the first study that attempted to investigate the diversity of phenotypes within the CAF population. It should be noted that the sum of percentages of the various combined phenotypes was higher than 100% as there were cells that expressed markers characteristic to more than one cell

phenotype. This highlights the dynamic state of CAFs that may express overlapping markers at each time point, thus increasing their phenotypical heterogeneity (Augsten, 2014; Kalluri, 2016).

In the present study we found that among the stromal, spindle-shaped cells, the most frequent phenotype was CD86<sup>+</sup>αSMA<sup>+</sup>, closely followed by CD80<sup>+</sup>αSMA<sup>+</sup>. CD86 and CD80, co-stimulatory molecules for inducing T cell response, are characteristically expressed by "professional" antigen presenting cells, such as B lymphocytes, dendritic cells and macrophages (Saada et al., 2006). Acquisition of αSMA<sup>+</sup> expression in cells of the myeloid/monocytic lineage, such as macrophages, has been already reported. For example, in human and experimental kidney disease, macrophages were shown to directly transdifferentiate into collagen-producing αSMA<sup>+</sup> myofibroblasts, the counterpart of CAFs in non-malignant conditions (Meng et al., 2016). The macrophage-to-myofibroblast transition (MMT) process was specifically demonstrated in bone marrow-derived macrophages that contributed to the process of tissue fibrosis (Wang et al., 2015). Furthermore, in that study, it was shown that the MMT occurred in the M2-type macrophages. In our previous study (Dayan et al., 2012), which serves as the base for the present investigation, we showed that a considerable part of the macrophages in the TME of TSCC were of M2-phenotype, thus, it may be assumed that these cells were preferred candidates for the acquisition of αSMA<sup>+</sup> CAF "cell state". In another study, it has been shown that when the MMT process occurs within the bone marrow, the αSMA<sup>+</sup> macrophages became resistant to radiation-induced cell death (Ludin et al., 2012). In addition, the αSMA<sup>+</sup> state in these macrophages was associated with upregulated expression of cyclooxygenase (COX)-2 under stress conditions. The COX-2-mediated production of prostaglandin (PG)-E<sub>2</sub> led, in turn, to a reduced production of reactive oxygen species within the adjacent cells, especially in progenitor cells that preserved their primitive stem phenotype. Future investigation of these sequelae in the context of TSCC is of great importance, since both effects of resistance to radiation of the CD86<sup>+</sup>αSMA<sup>+</sup>/CD80<sup>+</sup>αSMA<sup>+</sup> TME cells and maintenance of stemness within the cancer cells could explain, at least in part, the biological aggressiveness of TSCC and the poor clinical outcomes. Although it would be difficult to specify the origin of the TSCC CD86<sup>+</sup>αSMA<sup>+</sup>/CD80<sup>+</sup>αSMA<sup>+</sup> cells, either from local and/or bone marrow, it is interesting to mention that bone-marrow-derived infiltrating macrophages were found



to express mainly CD80, while resident macrophages within the TME of the cancer express mainly CD86 (Hallam et al., 2009).

The molecular pathway for the up-regulation of  $\alpha$ SMA mRNA and its protein levels in cells of the monocyte/macrophage lineage depends not only on transforming growth factor beta (Wang et al., 2016) but also on nutlin-3, a small molecule with the ability to inhibit the mouse double minute (MDM)-2 - p53 interactions (Secchiero et al., 2012). The "converted" monocytes/macrophages exhibited characteristic elongated, spindle-like morphology of CAFs and produced fibronectin and collagen type I, both markers of fibrocyte differentiation. If this will be supported by additional studies in TSCC, then nutlin-3 may serve in the future as a new therapeutic target in TSCC.

The dynamics and plasticity of cells within the  $\alpha$ SMA<sup>+</sup> CAF population can also be reflected by the acquisition of CD86<sup>+</sup>/CD80<sup>+</sup> phenotypes (Saada et al., 2006). It has been shown that colonic  $\alpha$ SMA<sup>+</sup> fibroblasts (i.e., myofibroblasts) express major histocompatibility type II molecules together with CD80/86 costimulatory molecules and, as such, they can present antigens to T cell lymphocytes and thus play an important role in regulating the local immune response. In the context of cancer, CD80 was shown to function as an inducer of regulatory T-cells (Zheng et al., 2004), which are known as pro-tumorigenic components of the cancer-related immune response and, therefore, can drive the tumor towards escape from the immune attack.

It is now speculated that normal tissue fibroblasts in a resting state (i.e., negligible metabolic and transcriptomic activity) may share many features with mesenchymal stem cell (MSC) precursors or monocyte precursor-derived mesenchymal cells (Kalluri 2016). These cells will be activated by appropriate stimuli and become MSCs with functional properties of  $\alpha$ SMA<sup>+</sup> myofibroblasts (wound healing and inflammatory response), or of their counterparts in TME of cancer -  $\alpha$ SMA<sup>+</sup> CAFs. Nanog together with additional markers, such as SOX2, OCT3, KLF4 and LIN28, support a stem cell-like phenotype (Herrera et al., 2013). Expression of Nanog and related markers has been reported in CAFs from human colorectal cancer, with most of these cells having a pro-migratory effect on the cancer cells. In our study, expression of Nanog was less than that of CD86 and CD80, yet it can be assumed that since its expression overlapped with the expression of CD86<sup>+</sup>/CD80<sup>+</sup>, it hints toward a stem-like state of these cells, irrespective of their origin.

We have shown that CAFs in TSCC, although sharing the  $\alpha$ SMA<sup>+</sup> phenotype, are actually a heterogeneous cell population representing phenotypes of different cell origins or cell states. Stromal cells with an  $\alpha$ SMA<sup>+</sup> phenotype and spindle-shaped morphology could represent cells of monocyte/macrophage lineage that acquired expression of  $\alpha$ SMA<sup>+</sup>, or, alternatively, activated fibroblasts that acquired expression of CD80<sup>+</sup>/CD86<sup>+</sup>, emphasizing that irrespective of the origin of the cells, their phenotype and functionality (i.e., "cell state") can be modulated according to given temporal and spatial conditions. Furthermore, selective CAF phenotypes have a significant impact on the clinical outcomes. This is the first study of its kind and should be extended to further characterize the composition of the CAFs in TSCC as well as in cancers of other oral sites. Recently, it has been shown that CAFs were infrequent in cancers of the buccal mucosa and gingivae (Akrish et al., 2017) with these patients having a better prognosis than patients with TSCC. Therefore, it would be interesting to investigate how CAF phenotypes vary in SCCs at different sites of the oral cavity and how these phenotypes relate to the clinical outcomes.

In conclusion, the novelty of the study lies in showing the heterogeneity of the CAF population and the negative impact that specific phenotypes might have on the clinical outcomes. It seems that generation of CAFs is a dynamic, inter-related and complex process resulting in "cell states" that acquire diverse, and possibly opposing, functions in the context of specific TMEs. Studies are needed in order to further characterize CAF phenotypes in carcinomas of various oral sites, as this may open new frontiers for personalized medicine.

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**Legend to Figures**

**Fig. 1.** Immunohistochemical stain of tongue squamous cell carcinoma with alpha smooth muscle actin antibody reveal spindle-shaped, positively stained cells in the tumor stroma that closely related to the tumor islands. These cells are consistent with cancer associated fibroblasts (CAFs). A – an example of CAF-poor tumor (CAFs are shown by arrows). B – an example of CAF-intermediate tumor, and C – CAF-rich tumor (scale bars 100  $\mu$ )

**Fig. 2.** Kaplan–Meier analysis for overall survival (A) locoregional recurrence (B) by CAF scores

**Fig. 3.** Stromal spindle cells double immuno-stained with alpha smooth muscle actin (brown color) and CD86 (A), CD80 (B) and Nanog (C) (purple color; arrows). Co-expression of alpha smooth muscle actin and CD133 was found only in walls of blood vessels. (scale bars 25 $\mu$ ).

**Fig. 4.** Kaplan–Meier analysis for overall survival by alpha-smooth muscle actin<sup>+</sup>CD86<sup>+</sup> scores

**Fig. 5.** Distribution of the mean scores of the double immunostains (alpha-smooth muscle actin<sup>+</sup>CD86<sup>+</sup>/CD80<sup>+</sup>/Nanog<sup>+</sup>) as a factor of the density of the cancer-associated fibroblasts, poor, intermediate and rich