Eukaryotic translation elongation factor 1 delta, N-terminal propeptide of type I collagen and cancer-associated fibroblasts are prognostic markers in oral squamous cell carcinoma

Abstract

Objectives: Identifying markers that influence oral squamous cell carcinoma (OSCC) prognosis is a fundamental strategy to improve the overall survival of patients. Markers such as eukaryotic translation elongation factor 1 delta (EEF1D), fascin, N-terminal propeptide of type I collagen (PINP) and cancer-associated fibroblasts (CAFs) have been noticed in OSCCs, whose levels are closely related to the prognosis of tumors. Our aim was to confirm the role of those markers in OSCC prognosis.

Study Design: Immunohistochemistry was performed in 90 OSCC specimens. The associations between clinicopathologic features and expression of markers were assessed by chi-square test. The Kaplan-Meier curves, univariate and multivariate Cox regression models were used for survival analysis. Markers were analyzed individually and in combination.

Results: High expression of EEF1D (p=0.017) and PINP (p=0.02) and abundant density of CAFs in tumor stroma (p=0.005) predicted significantly poor survival in OSCC patients. Multivariate analysis revealed that all 3 parameters are individually independent prognostic factors of OSCC patients, and their combination improved the discrimination of patients at high-risk for poor survival.

Conclusions: Our results suggested that the expression of EEF1D and PINP and the density of CAFs might influence the survival of patients with OSCC.

Keywords: oral cancer, eukaryotic translation elongation factor 1 delta, fascin, N-terminal propeptide of type I collagen, cancer-associated fibroblast, prognosis.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common tumor in the head and neck region, with a global incidence of more than 300,000 new cases and 177,000 deaths every year[1]. OSCC is highly aggressive, with 5-year survival rates around 50%, which have remained unchanged over recent decades[2]. Clinical features, mainly based on the TNM clinical stage, are the most consistent prognostic factors for OSCC, but it frequently shows unpredictable prognosis. Among recent advances on OSCC prognosis, the new edition of the clinical staging manual of the American Joint Cancer Committee incorporated depth of invasion into T stage classification and extranodal extension in a metastatic lymph node in N category[3]. This revised version has shown better survival prediction than the previous one[4, 5]. Although our understanding of the clinical and pathological parameters associated with more aggressive tumors is evolving, better prognostic markers for early diagnosis, post-therapeutic monitoring and the development of novel therapeutic approaches are still required.

Several prognostic markers expressed by tumor cells, including eukaryotic translation elongation factor 1 delta (EEF1D)[6] and fascin[7], and by microenvironment cells, including N-terminal propeptide of type I collagen (PINP) by cancer-associated fibroblasts (CAFs)[8], have been identified to have independent prognostic potential in OSCC. Indeed, the presence of CAFs, as assessed by α-SMA-positive fibroblasts in the tumor microenvironment, is recognized as a poor prognosis for patients with OSCC[9]. Apart from its well-characterized function in translation elongation, EEF1D has been implicated in the tumorigenesis of several cancers[10]. In medulloblastoma, EEF1D overexpression was associated with worse overall and disease-free survival[11], and in osteosarcomas, EEF1D was clinically associated with recurrence, and in vitro assays revealed that EEF1D knockdown in cell lines inhibits proliferation via AKT-mTOR-BAD signaling[12]. In OSCC, EEF1D has been linked in mediating

proliferation and epithelial-mesenchymal transition (EMT)[6]. Fascin is essential for the cytoskeleton organization by controlling actin filaments into bundles and networks with other cytoskeleton proteins[13]. In cancers, fascin expression is frequently dysregulated and associated with EMT, invasion, and metastatic potential of the tumor cells[14]. In OSCCs, fascin expression level was associated with aggressiveness[15, 16] and in vitro studies confirmed its role regulating EMT and invasion of OSCC cells[7, 17]. The purpose of this study was therefore to determine the prognostic significance of expression of EEF1D, fascin, PINP, and CAF density in a cohort composed of 90 OSCCs. The discriminatory ability of the combination of those markers in determining OSCC prognosis was also assessed.

Material and Methods

Samples

We collected retrospectively 90 surgical specimens of patients treated with curative intent for OSCC at the Hospital Bom Pastor of Varginha, Brazil, between 1998 and 2014. The inclusion criteria included tumors in tongue or floor of the mouth, complete demographic and clinical data, treatment based on radical surgery with or without postoperative radiotherapy and chemotherapy, availability of paraffin-embedded blocks, and follow-up information of at least 5 years for survivors. The number of available blocks of the primary tumor for each case ranged from 5 to 12, and the most representative block of each case, which contained large areas with both tumor and invasive tumor front, was selected for immunohistochemical staining.

Demographic and clinical data, including gender, age, habits such as smoking and alcohol consumption, TNM clinical stage (7th edition), treatment, recurrence, and survival, were obtained from patient's records. OSCCs were histologically classified according to the

World Health Organization (WHO) grading system, and histopathological parameters including depth of invasion, tumor budding and turmor-stroma ratio were previously determined on the postoperative surgical specimens stained with HE[18]. All recurrences were histologically confirmed. The outcomes were categorized as disease-specific survival (DSS), time from treatment initiation until death due to cancer or last known date alive, and disease-free survival (DFS), the time from treatment initiation until the diagnosis of the first recurrence (local, regional or distant) or last follow up information for those without recurrence. The clinicopathological features of the tumors are depicted in Supplementary Table 1. The overall survival ranged from 1 to 116 months, with a mean of 84.5 months. The study was carried out in accordance with the Declaration of Helsinki and approved by the Human Research Ethics Committee of the Federal University of Alfenas (protocol number: 1.775.304).

Immunohistochemistry

The 3 µm sections were treated with 3% hydrogen peroxide followed by antigen retrieval with 10 mM citrate buffer pH 6.0 in a pressure cooker for 15 min. After washing with phosphate-buffered saline (PBS), the sections were incubated with primary antibodies followed by avidin-biotin complex (LSAB2 System-HRP kit, Dako, USA). The primary antibodies were rabbit anti-EEF1D polyclonal antibody (Sigma-Aldrich, USA) diluted 1:10,000[6], mouse anti-fascin monoclonal antibody (clone IM20; Abcam Inc, USA), diluted 1:700[7], rabbit anti-PINP polyclonal antibody, diluted 1:5000[8], and mouse anti-α-SMA monoclonal antibody (clone 1A4, Dako, USA), diluted 1:400[9]. Reactions were developed with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAKO, USA) and counterstained with hematoxylin. Control reactions were performed by omission of the primary antibodies.

Immunohistochemical semiquantitative analysis and carried out by two trained examiners (CBD and WGA for EEF1D, fascin and PINP; CBD and LPR for α-SMA) at the same time, unaware of the clinical outcome at the time of the analysis. Immunoexpression of EEF1D and fascin was assessed in tumor cells, whereas PINP was quantified in the stromal cells (fibroblast-like cells) of the tumor microenvironment. The number of positive cells was graded in quartiles (0: negative, 1: 1%-25% staining, 2: 26%-50% staining, 3: 51%-75% staining, and 4: 76%-100% staining), and the intensity of staining was scored in 0: negative, 1: weak staining, 2: moderate staining and 3: strong staining. Both grades were added together, producing scores from 0 to 7 that were classified as low (0-4 scores) and high (5-7 scores) expression for comparative analysis, as previously described by us[9]. The α-SMA-positive cells (CAFs) were assessed as described by Kellermann et al. (2007)[19]. Tumors lacking α-SMA-positive cells were classified as negative, scanty if more than 1% and less than 50% of the stromal cells were α-SMA-positives, and abundant if more than 50% of the stromal cells were α-SMA-positive cells. For statistical purposes, samples classified as negative or scanty density of CAFs were grouped and compared with samples with abundant presence of CAFs.

Statistical analysis

Chi-square test was used to evaluate the associations of immunohistochemical expression and clinicopathological parameters of the tumors. The Kaplan-Meier method and univariate and multivariate Cox regression models were used for survival analysis. Spearman's rank test was used to determine the correlation between markers. $P \leq 0.05$ was considered statistically significant.

Results

EEF1D (Fig. 1A) and fascin (Fig. 1B) were observed as cytoplasmic stain with variable distribution and intensity in the tumor cells. Positivity for EEF1D was also observed in some stromal cells with fibroblast features. PINP was also observed as a cytoplasmic stain in the stromal cells (fibroblasts), but immunopositivity was found in scattered tumor cells (Fig. 1C). CAFs, represented by α-SMA-positive fibroblasts, were located in close contact with neoplastic islands, and areas of tumor-free stroma demonstrated a complete lack of CAFs (Fig. 1D). Interestingly, many CAFs were reactive for the antibody anti-PINP. Spearman's coefficient was measured to determine the degree of association between markers. The only significant correlation, though modest, was between PINP and CAF (rho=0.24, p=0.03).

The associations of the immunoexpression of EEF1D, fascin, and PINP and the density of CAFs with demographic and clinicopathological features of the tumors are shown in Supplementary Table 2. The only significant associations were between CAF density and tumor-stroma ratio (p=0.005) and between PINP and smoking habit (p=0.05). Then, we assessed the association with the prognosis of OSCC patients (Table 1). Although associations with disease-free survival were not detected, disease-specific survival rates were significantly different between low and high levels of EEF1D and PINP and between CAF densities. Five-years survival was 83.5% for patients with low expression of EEF1D and 50.6% for patients with high expression, yielding an HR of 3.09 (95% CI: 1.22-7.83, p=0.017). Patients with high PINP expression had significantly poorer disease-specific survival rates than those with low PINP expression (73.7% vs. 55.0%), with an HR of 3.06 (95% CI: 1.20-7.78, p=0.02). The presence of CAFs in the tumor revealed an HR of 3.94 (95% CI: 1.50-10.3, p=0.005), with survival in 5-years of 84.2% for patients with negative/scanty density of CAFs and 51.5% for patients with tumor classified as abundant presence of CAFs. Disease-specific survival was also significantly influenced by tumor

grade (p=0.0001), but the number of cases classified as poorly-differentiated was very low, biased the result. The adjusted multivariate analysis identified that EEF1D, PINP and CAF were significantly and independently associated with disease-specific survival (Table 2).

In order to strengthen the prognostic information provided by these independent factors, the expression of EEF1D and PINP and CAF density were combined and subjected to survival analysis. For the combination of 2 factors, groups were formed as follows: Low risk, tumors with low expressions of EEF1D and PINP, low EEF1D expression and negative/scanty presence of CAFs, or low expression of PINP and negative/scanty presence of CAFs; High risk, tumors with high expressions of EEF1D and PINP, high expression of EEF1D and abundant presence of CAFs, or high expression of PINP and abundant presence of CAFs; and Intermediate risk, tumors showing mixed expressions of EEF1D and PINP or mixed density of CAFs. The 3-factor combination generated also 3 groups (low risk: low EEF1D, low PINP and negative/scanty CAF density, high risk: high EEF1D, high PINP and abundant CAF density, and intermediate-risk: other combinations). In all combinations, the discriminatory ability to predict survival of OSCC patients was largely improved, with a clear better survival for patients classified at low risk compared to patients at high risk (Fig. 2). As ideally expected of a survival score system, patients classified at intermediate risk showed a distinct outcome than patients at low or high risk, with exception of combination between EEF1D and PINP (Fig. 2A).

Discussion

Surgery remains the preferred treatment for OSCCs, with adjuvant radiotherapy with or without chemotherapy in cases at advanced stage[20]. Despite remarkable advances in the field, such as innovative techniques on surgery and radiotherapy, novel chemotherapeutic

agents and the advance of immunotherapy, the mortality associated with OSCC is still a major concern. The use of the TNM clinical stage, established over the years, is a valid tool for the therapeutic and prognostic purposes, but the high mortality associated with OSCC indicates that more accurate biomarkers with predictive ability to determine response to specific therapies, post-therapeutic surveillance and patient's prognosis are needed. In the last decades, an intense activity towards identifying novel biomarkers has been performed and many proteins/cell features are described as potential biomarkers for OSCC. However, most biomarkers have not been validated[21]. Here we confirmed that EEF1D, PINP, and CAFs have a relevant prognostic role for OSCC, and the combination of them improved the discrimination of patients at high-risk for worse outcome.

Translation elongation is dependent on eukaryotic translation elongation factor 1 (EEF1) complex subunits, a family composed by 6 members including EEF1D, which delivers aminoacylated tRNAs to the ribosome to lengthen nascent proteins[22]. On their canonical function, those factors bind with each other forming a complex anchored on tubulin and the endoplasmic reticulum to perform translation elongation[23]. However, distinct non-canonical functions are found for each member of EEF1 complex. Dysregulated expression of EEF1D has been described in hepatocarcinomas[24], esophageal carcinomas[25], non-small-cell lung cancers[26], medulloblastomas[11], breast cancers[10], and osteosarcomas[12]. In esophageal carcinomas, the upregulation of EEF1D was associated with lymph node metastasis, advanced stage and reduced disease-specific survival[25]. EEF1D overexpression was also associated with poor overall and progression-free survival in medulloblastomas[11] and with high rates of relapse in breast cancers[10] and osteosarcomas[12]. In a comparative mass spectrometry analysis using OSCC tumor cells and the normal counterpart isolated by laser-capture microdissection, EEF1D was one of the most upregulated proteins identified in the tumor cells[6]. Further analysis revealed that EEF1D

levels control OSCC cell proliferation, via regulation of cyclin D1 and RB phosphorylation, and acquisition of EMT phenotypes in a SNAIL1-, ZEB1- and ZEB2-dependent manner. Although associated with parameters that influence prognosis, the clinical impact of EEF1D has never been investigated in OSCCs. EEF1D levels were not associated with clinicopathological features of tumors or with disease-free survival, but patients with a higher EEF1D expression had a worse outcome than patients with low levels (survival rate of 50.6% vs. 83.5%). In the multivariate analysis, EEF1D emerged as an independent predictor factor of disease-specific survival, confirming that EEF1D is an unfavorable prognostic factor for survival in OSCCs.

Alterations in the extracellular matrix (ECM) composition and structure are related to tumor phenotypes, including proliferation, survival, migration, and invasion, besides the effects in the angiogenesis and immune function in the tumor microenvironment[27]. The synthesis of collagen, the major component of ECM, is frequently up-regulated in tumors, as well as a higher rate of remodeling and turnover by ECM-degrading proteases such as the matrix metalloproteinases (MMPs)[28]. During collagen maturation, both amino-terminal (PINP) and carboxyterminal propeptides are cleaved off by specific proteases to form type I collagen, and those cleaved peptides were shown to have important effects on cancer progression by inducing an invasion-permissive and proangiogenic stroma[29, 30]. PINP serum levels were associated with bone metastasis in patients with breast and prostate cancers[31, 32], and more recently serum levels of PINP were significantly higher in patients with monoclonal gammopathy of undetermined significance progressing to myeloma multiple than in patients with stable disease[33]. An immunohistochemical study demonstrated that PINP is expressed by both stromal and OSCC cells and increased PINP expression by both carcinoma and stromal cells at the invasive area of tumor is associated with worse prognosis[29]. In our previous study, PINP immunoexpression was correlated with CAF

density and was significantly associated with shortened survival of OSCC patients[8]. Interestingly, high levels of PINP is reflex of elevated expression of type I collagen, and the fibrotic response (desmoplasia) in the tumor microenvironment of OSCCs is described as a prognostic indicator of occult cervical lymph node metastasis[34] and poor survival[35]. Furthermore, type I collagen is in the expression signature that distinct OSCCs from normal tissues[36], and high levels of type I collagen mRNA is detected in the expression profile associated with invasive phenotype in oral cancer[37]. Taken altogether, our results proved that high PINP expression may be useful as a prognostic marker for OSCC patient's survival.

Another potential biomarker confirmed in this study is CAF, in which at high density in the tumor stroma was associated with shortened disease-specific survival. CAFs are well-known players in tumor progression, promoting many aspects of tumorigenesis such as proliferation, migration, and invasion[38, 39]. The motility-promoting effects of CAFs are, at least in part, from their potential of ECM synthesis, including collagenous proteins[8]. Although CAFs are one of the most common components in OSCC stroma, they are not frequently found in tumors at early-stage with low depth of invasion[40] and neither in the subjacent stroma of oral potentially malignant disorders such as leukoplakia and erythroplakia[41]. Moreover, studies have demonstrated that factors released by OSCC cells, including transforming growth factor-beta (TGF-β), induce CAF activation[42], suggesting that the emergence of CAF within tumor microenvironment is influenced by tumor cell invasion. The impact of the presence of CAFs in the stroma of OSCCs has been investigated in many studies, and a recent systematic review with meta-analysis revealed CAF density is consistently associated with overall decrease in survival[9].

Several studies with clinical samples have pointed fascin as a novel candidate biomarker for aggressive solid tumors, including OSCC[7, 16, 43]. In the current study, most samples (84%) were classified as high fascin expression, unbalancing the groups and the

associations. Interestingly, increasing the cut off of fascin score to 5 improved the discrimination of patients with low- and high-expression in terms of specific survival, but still not reaching a significant p-value. Though the study has produced very interesting results, it had few limitations. The study cohort had a small sample size, only patients treated with radical resection, combined or no with postoperative radio and/or chemotherapy, were included, and the number of patients with recurrence was limited. Prognostic models containing several biomarkers have the potential of higher performance and accuracy compared to single markers. New models, such as the described here, will be required for improved prognostic performance in OSCC patients.

In conclusion, EEF1D, PINP, and CAFs were significantly associated with the outcome of patients with OSCC, and the combination of those independent biomarkers improved the stratification of OSCCs into low- and high-risk groups with distinct prognosis. Further studies are needed to confirm these findings and determine the role of combination of EEF1D, PINP and CAFs as reliable clinical predictors of OSCC outcome.

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Figure legends

Fig. 1. Immunodetection of EEF1D, fascin, PINP and α-SMA in OSCCs. Representative images with low expression of EEF1D (A), fascin (C) and PINP (E), and high expression of EEF1D (B), fascin (D) and PINP (F) are shown. Representative samples classified as scanty or abundant presence of CAFs are depicted on panels G and H, respectively. The internal control of α-SMA in the blood vessels can be observed. EEF1D and fascin were detected in the cytoplasm of the tumor cells with variable distribution and intensity. PINP was observed as a cytoplasmic stain in the stromal cells (fibroblasts), but immunopositivity was also found in scattered tumor cells. α-SMA, representing CAFs, was exclusively found in stromal cells close to tumor cells. (x200).

Fig. 2. Kaplan-Meier curves for disease-specific survival of patients with OSCC based in the combination of expressions of EEF1D and PINP and density of CAFs.

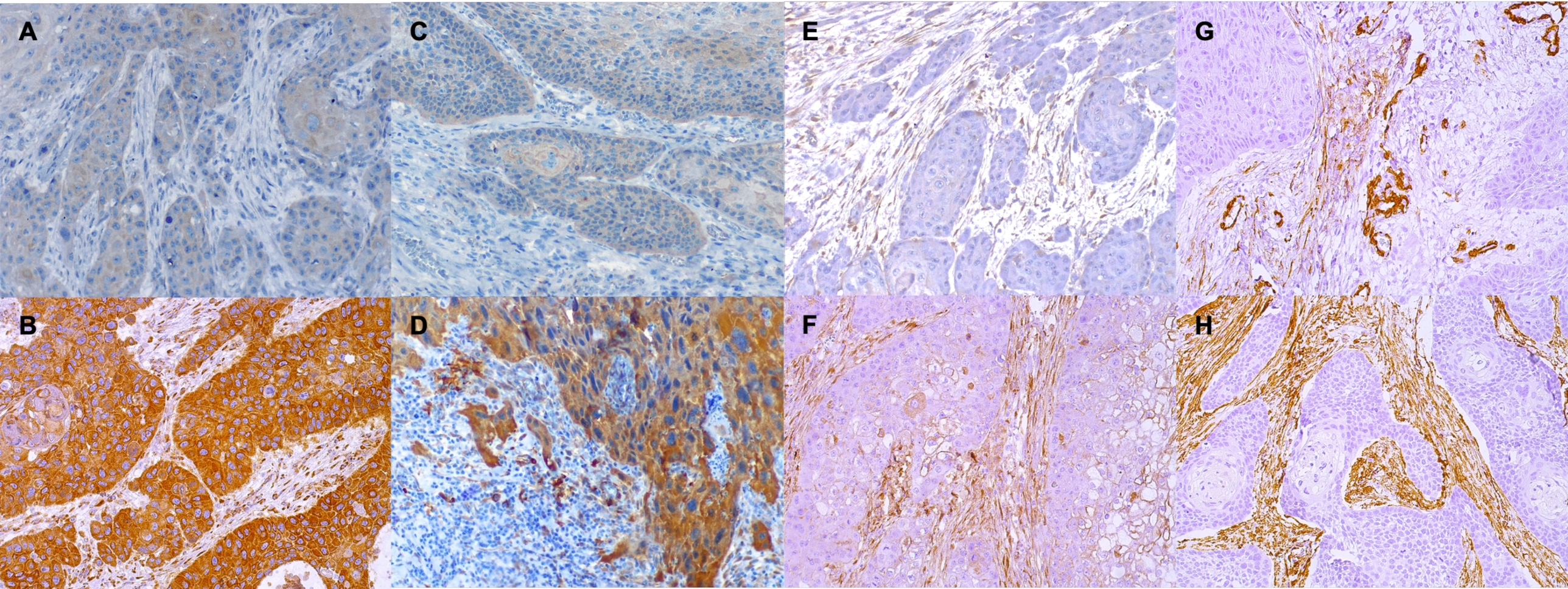
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Table 1. Univariate analysis for disease specific survival and disease-free survival of patients with oral squamous cell carcinoma.

Parameter	Disease Specific Survival			Disease-Free Survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
Age						
<61 years	80.8	1		75.1	1	
≥61 years	71.8	1.25 (0.71-2.83)	0.56	80.2	0.76 (0.26-2.23)	0.62
Gender						
Male	72.1	1		78.8	1	
Female	78.1	0.90 (0.28-2.87)	0.86	76.9	1.08 (0.28-4.07)	0.90
Clinical stage						
Early (I + II)	83.5	1		82.9	1	
Advanced (III + IV)	75.2	1.87 (0.71-4.90)	0.19	66.0	1.73 (0.61-4.82)	0.29
Treatment						
Surgery	100	1		87.4	1	
Surgery + Radiotherapy	75.9	-	0.29	71.0	4.57 (0.18-47.7)	0.36
Surgery + Radiotherapy + Chemotherapy	55.1	-	0.20	63.9	1.43 (0.23-8.98)	0.69
Histological grade						
Well-differentiated / moderately-differentiated	73.1	1		78.5	1	
Poorly-differentiated	0	326.5 (16.5-632.9)	0.0001	100	-	0.63
Depth of invasion						
< 4 mm	71.8	1		83.1	1	
≥ 4 mm	65.2	1.04 (0.28-3.70)	0.95	74.2	1.16 (0.32-5.16)	0.94
Tumor budding						
< 5 buds	71.2	1		75.0	1	
≥ 5 buds	56.9	1.38 (0.52-3.68)	0.51	66.9	1.36 (0.45-3.83)	0.80
Tumor-stroma ratio						
< 50% (stroma-poor)	75.4	1		85.5	1	
≥ 50% (stroma-rich)	66.9	1.69 (0.47-4.69)	0.49	56.7	3.46 (1.18-15.48)	0.06
EEF1D expression						
Low	83.5	1		82.3	1	
High	50.6	3.09 (1.22-7.83)	0.017	66.9	1.14 (0.40-3.19)	0.80
Fascin expression						
Low	62.3	1		100	1	

High	73.0	0.49 (0.16-1.54)	0.22	73.3	1.56 (0.43-5.65)	0.49
PINP expression						
Low	73.7	1		82.0	1	
High	55.0	3.06 (1.20-7.78)	0.02	75.0	1.26 (0.46-3.43)	0.64
CAF density						
Negative/Scanty	84.2	1		79.5	1	
Abundant	51.5	3.94 (1.50-10.3)	0.005	71.6	1.55 (0.52-4.58)	0.42

Table 2. Cox multivariate analysis for the risk of death and recurrence.

Parameter	Disease specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Age	1.49 (0.28-7.83)	0.63	1.60 (0.59-3.72)	0.58
Gender	0.97 (0.27-3.50)	0.96	1.85 (0.22-5.85)	0.87
Clinical stage	4.67 (0.98-22.2)	0.06	0.63 (0.14-2.82)	0.54
Treatment	1.73 (0.48-6.30)	0.40	0.24 (0.54-65.7)	0.46
Histological grade	3.30 (0.78-13.9)	0.10	0.89 (0.32-23.1)	0.83
Depth of invasion	1.38 (0.04-45.6)	0.85	3.13 (0.58-6.82)	0.48
Tumor budding	1.97 (0.40-9.63)	0.71	1.25 (0.42-3.73)	0.68
Tumor-stroma ratio	1.59 (0.35-7.05)	0.54	1.27 (0.83-4.83)	0.34
EEF1D expression	3.75 (1.11-12.7)	0.03	4.84 (0.79-29.5)	0.09
Fascin expression	1.14 (0.38-3.77)	0.62	1.14 (0.17-7.92)	0.88
PINP expression	2.37 (1.36-8.37)	0.02	2.52 (0.62-10.1)	0.19
CAF density	5.64 (1.03-30.9)	0.05	0.55 (0.15-1.57)	0.14

Supplementary Table 1. Clinicopathological features of patients with oral squamous cell carcinoma included in this study.

Parameter	n	%
Age		
Mean: 61.8 ± 9.7 years		
Range: 45-88 years		
Gender		
Male	67	74.4
Female	23	25.6
Smoking habit		
No	9	12.3
Yes	64	87.7
Drinking habit		
No	19	28.8
Yes	47	71.2
Clinical stage		
I	12	13.8
II	24	27.6
III	20	23.0
IV	31	35.6
Treatment		
Surgery	10	11.4
Surgery + Radiotherapy	37	42.0
Surgery + Radiotherapy + Chemotherapy	41	46.6
Histological grade (WHO)		
Well-differentiated	15	16.7
Moderately-differentiated	68	75.5
Poorly-differentiated	7	7.8
Depth of invasion		
< 4 mm	18	20.0
≥ 4 mm	72	80.0
Tumor budding		
< 5 buds	36	40.0
≥ 5 buds	54	60.0
Tumor-stroma ratio		
< 50% (stroma-poor)	67	74.4
≥ 50% (stroma-rich)	23	25.6
Recurrence		
No	69	80.2
Local	11	12.8
Regional	5	5.8
Distant	1	1.2
Status		
Alive	72	80.0
Dead	18	20.0

Supplementary Table 2. Association of the clinicopathological parameters of oral squamous cell carcinomas with the immunohistochemical expression of EEF1D, fascin and PINP and the density of CAFs (α-SMA-positive cells).

Parameter	EEF1D expression			Fascin expression			PINP expression			CAF density		
	Low n (%)	High n (%)	p value	Low n (%)	High n (%)	p value	Low n (%)	High n (%)	p value	Negative/ Scanty n (%)	Abundant n (%)	p value
Age												
<61 years	25 (52.4)	23 (47.9)		10 (66.7)	35 (46.7)		29 (52.7)	16 (47.1)		18 (45)	24 (53.3)	
≥61 years	20 (47.6)	25 (52.1	0.67	5 (33.3)	40 (53.3)	0.16	26 (47.3)	18 (52.9)	0.60	22 (55)	21 (46.7)	0.44
Gender												
Male	31 (73.8)	36 (75)		13 (86.7)	54 (72)		37 (67.3)	29 (85.3)		30 (75)	34 (75.6)	
Female	11 (26.2)	12 (25)	0.89	2 (13.3)	21 (28)	0.23	18 (32.7)	5 (14.7)	0.06	10 (25)	11 (24.4)	0.95
Smoking habit												
No	4 (11.8)	5 (12.8)		2 (15.4)	7 (11.7)		8 (19)	1 (3.3)		4 (12.5)	4 (11.1)	
Yes	30 (88.2)	34 (87.2)	0.89	11 (84.6)	53 (88.3)	0.71	34 (81)	29 (96.7)	0.05	28 (87.5)	32 (88.9)	0.86
Drinking habit												
No	8 (26.7)	11 (30.6)		2 (22.2)	17 (29.8)		14 (35)	5 (19.2)		10 (34.5)	8 (24.2)	
Yes	22 (73.3)	25 (69.4)	0.73	7 (77.8)	40 (70.2)	0.64	26 (65)	21 (80.8)	0.17	19 (65.5)	25 (75.8)	0.38
Clinical stage												
Early (I + II)	17 (41.5)	19 (41.3)		6 (42.9)	30 (41.1)		24 (45.3)	11 (33.3)		18 (47.4)	15 (34.1)	
Advanced (III + IV)	24 (58.5)	27 (58.7)	0.98	8 (57.1)	43 (58.9)	0.90	29 (54.7)	22 (66.7)	0.27	20 (52.6)	29 (65.9)	0.22
Treatment												
Surgery	5 (11.9)	5 (10.9)		2 (13.3)	8 (11)		8 (15.1)	2 (6)		5 (13.2)	3 (6.7)	
Surgery + RTX	23 (54.8)	16 (34.8)		6 (40)	33 (42.4)		23 (43.4)	16 (47)		15 (39.5)	21 (46.7)	
Surgery + RTX + CTX	14 (33.3)	25 (54.3)	0.12	7 (46.7)	32 (46.6)	0.92	22 (41.5)	16 (47)	0.99	18 (47.3)	21 (46.7)	0.56
Histological grade												
WD/MD	41 (97.6)	42 (87.5)		14 (93.3)	69 (92)		50 (90.9)	32 (94.1)		35 (87.5)	43 (95.6)	
PD	1 (2.4)	6 (12.5)	0.07	1 (6.7)	6 (8)	0.86	5 (9.1)	2 (5.9)	0.58	5 (12.5)	2 (4.4)	0.18
Depth of invasion												
< 4 mm	8 (19)	10 (20.8)		1 (6.7)	17 (22.7)		13 (23.6)	5 (14.7)		12 (30)	3 (6.7)	
≥ 4 mm	34 (81)	38 (79.2)	0.83	14 (93.3)	58 (77.3)	0.16	42 (76.4)	29 (85.3)	0.31	28 (70)	42 (93.8)	0.005
Tumor budding												
< 5 buds	17 (40.5)	19 (39.6)		8 (53.3)	28 (37.3)		22 (40)	14 (41.2)		16 (40)	18 (40)	

≥ 5 buds	25 (59.5)	29 (60.4)	0.93	7 (46.7)	47 (62.7)	0.25	33 (60)	20 (58.8)	0.91	24 (60)	27 (60)	0.99
Tumor-stroma ratio												
< 50% (stroma-poor)	30 (71.4)	37 (77.1)		13 (86.7)	54 (72)		41 (74.5)	25 (73.5)		31 (77.5)	32 (71.1)	
≥ 50% (stroma-rich)	12 (28.6)	11 (22.9)	0.54	2 (13.3)	21 (28	0.24	14 (25.5)	9 (26.5)	0.92	9 (22.5)	13 (28.9)	0.50

RTX: radiotherapy, CTX: chemotherapy, WD: well-differentiated; MD: moderately-differentiated; PD: poorly-differentiated.