Trophoblast cell surface antigen 2 (TROP2) expression predicts outcome in oral squamous cell carcinomas

Running title: Trop2 expression in oral cancer

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Abstract

Background: Trophoblast cell surface antigen 2 (TROP2) has unclear clinical role in oral squamous cell carcinomas (OSCC). Here, we investigated the association of TROP2 immunoexpression with clinicopathological parameters and survival of OSCC patients.

Subjects and Methods: Cancer-specific survival (CSS) and disease-free survival (DFS) were assessed in a cohort composed of 266 OSCC. An independent cohort with 88 OSCC samples matched with the normal oral tissue, as well as 17 metastatic lymph nodes, was used for validation.

Results: Multivariate analysis showed TROP2 as an independent marker of favorable prognosis for both CSS (HR: 0.60, 95% CI: 0.40-0.90, p = 0.01) and DFS (HR: 0.57, 95% CI: 0.36-0.89, p=0.01). Furthermore, TROP2 protein expression was significantly higher in morphologically normal tissues compared to primary tumors (p<0.0001) and lymph node metastases (p=0.001), and it was significantly associated with CSS (HR: 0.26, 95% CI: 0.09-0.74, p=0.008) in the validation cohort. A pooled mRNA analysis performed on the Oncomine™ database confirmed the underexpression in OSCC compared to normal tissues (p=0.014).

Conclusions: In summary, our results point to a favorable prognostic significance of TROP2 overexpression in a large cohort of oral cancer patients, suggesting it as an attractive clinical marker.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common tumor subtype in the head and neck region. GLOBOCAN project predicted 354.864 new cases worldwide and 177.384 deaths for 2018 (Bray et al., 2018). OSCC are often diagnosed at advanced stages, when regional metastases are present in about 40% of cases and distant metastases around 6% (Rutkowska et al., 2020). Despite the significant technical advances in OSCC treatment, the mortality rate remains elevated and around 50% of the patients eventually die in 5 years (Chi et al., 2015). Clinical parameters, based on TNM classification, are still guiding the disease management and prognosis, and surgical resection, radiation therapy, chemotherapy, and immunotherapy are the treatment options (Dourado et al., 2020; Wunschel et al., 2020). Despite the inclusion of new parameters in the TNM classification and its impact on the patient's prognosis (Moeckelmann et al., 2018), the behavior of some OSCC is still unpredictable. Therefore, the identification of markers that aids the separation of more aggressive from indolent tumors is required, especially for those patients classified within the same stage (Coletta et al., 2020).

Trophoblast cell surface antigen 2 (TROP2; also named M1S1, GA733-1, EGP-1), is a type I transmembrane surface glycoprotein encoded by the gene *TACSTD2* (McDougall et al., 2015; Shvartsur & Bonavida, 2015; Tang et al.,2018). TROP2 contains phosphorylation sites in its cytoplasmatic tail, and for that reason the protein has been involved in several intracellular signalling pathways, including calcium transportation (Shvartsur & Bonavida, 2015; Fong et al., 2008). TROP2 is relevant in early development and for the maintenance of tight junction integrity of epithelial cells (Shvartsur & Bonavida, 2015).

While TROP2 overexpression was reported to worsen prognosis in several solid (Zeng et al., 2016; Xu et al., 2017), the opposite effect or even lack of association with survival was reported in non-small cell lung carcinoma (NSCLC) (Inamura et al., 2017; Mito et al., 2020), hepatocellular carcinoma (Sin et al., 2018), and cervical cancer (Fang

et al., 2012). TROP2 overexpression has been associated with shortened overall survival (OS) in OSCC (Fong et al., 2008; Tang et al., 2018; Jia et al., 2020). In contrast, the absence of association between protein expression and survival or lymph node metastasis has been also reported (Noorlag et al., 2015). Herein we evaluated TROP2 expression in a large cohort of OSCC, as well as its association with clinicopathological parameters and survival after a long-term follow-up. TROP2 levels were also examined in morphologically normal oral tissue and metastatic lymph nodes. The mRNA levels of TROP2 in normal oral tissues and OSCC were compared using the Oncomine database

Material and Methods

This study was based on the REMARK guidelines for studying prognostic tumor markers (Sauerbrei et al., 2018) and was approved by the ethics committee of each of the participant hospitals.

Subjects and Clinicopathological data

This is a retrospective, collaborative, and multi-institutional study that combined samples from six different referral hospitals in four countries. Two different sets of human samples characterizing two independent cohorts were included, totaling 354 OSCC. Cohort 1 was composed of whole tumor sections derived from surgical specimens of 266 primary OSCC. The subjects were diagnosed and treated at CEONC and UOPECCAN Cancer Hospitals in Paraná (Brazil) between 1998 and 2013 (n=89), at the Hospital Bom Pastor in Minas Gerais (Brazil) between 2002 and 2014 (n=25), at the Hospital El Carmen in Maipú (Chile) between 1999 and 2017 (n=37), and at the University Hospital of Oulu (Finland) who were treated 1987 and 2016 (n=115). Cohort 2 was composed of 88 paired primary OSCC and matched normal oral tissues, and 17 lymph node metastases from subjects treated at the Jewish General Hospital, Montreal (Canada). The normal tissues were collected from tumor adjacent areas and were clinically and morphologically confirmed as normal, without dysplasia. These samples were mounted in a tissue

microarray (TMA) and used as an independent validation of the TROP2 findings in the experimental cohort 1.

All individuals were subjected to radical surgery and no subject received any pre-operative therapy. Complete clinical and demographic data such as age, sex, TNM clinical stage (Edge et al., 2010), type of treatment, tumor location, surgical margins status, recurrence, and survival were obtained from subjects' records. The patients were followed up regularly and, except in cases of death, all were followed up for at least 5 years in the cohorts from Brazil and Canada. In the cohorts from Chile and Finland, the patients were followed up for at least 3 or 2 years, respectively. The tumors histological grade was based in the World Health Organization (WHO) system (Gale et al., 2005). Cancer-specific survival (CSS; time from first treatment until either death due to disease or last known date alive), and disease-free survival (DFS; time from first treatment the first recurrence or last follow-up information) were the main accessed outcomes. The recurrences were histologically confirmed, and after treatment, the mean follow-up of the subjects was 48 months (ranging from 1 to 251, with a median of 32) and 64 months (1 to 197, with a median of 51) in the cohort 1 and 2, respectively.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 3 μm sections as described before (Ferreira do Carmo et al, 2020). After preparation, slides were incubated with primary antibody against TROP2 (1:400, HPA055067, Sigma-Aldrich, MO, USA) at 4°C overnight, followed by second antibody (LSAB2 System-HRP, Dako, CA, USA) at 37°C for 30 min. Incubation with DAB (Dako, CA, USA) for 1 min was followed by hematoxylin counterstaining.

Staining assessment

TROP2 expression was assessed by two observers (MRD and RAM) blinded to clinical and demographic data. The whole tissue sections were evaluated by visual inspection

and scores were obtained based on the percentage of positive tumor cells (0, 1: 1%–25%, 2: 26%–50%, 3: 51%–75%, and 4:76%–100% staining), as well as the intensity of the staining (0: negative, 1: weak, 2: moderate and 3: strong staining). The final scores were obtained by the sum of both parameters, ranging from 0 to 7 as described by Ferreira do Carmo et al. (2020). After individual evaluation, the samples were categorized in two groups: low (< 4 points) or high TROP2 expression (≥ 4 points). Before evaluating the whole set of samples from both cohorts, 20 random slides were analyzed individually by both examiners and the final score was used to access the interrater reliability by Cohen's Kappa coefficient test (k). The agreement rate was of k= 0.87, showing that both examiners reached a substantial agreement on the staining scoring.

Database analysis

The Oncomine™ Research Premium Edition platform (www.oncomine.org) is a web-based tool that allows combined analysis of gene expression in pooled datasets. For this study, we searched for TROP2 (*TACSTD2* gene) expression comparing normal tissue *versus* OSCC, excluding lips and other locations in the head and neck area, considering a threshold p-value of $1x10^{-8}$, and a minimum fold change of 2.

Statistical analysis

Chi-squared test was used to analyze the association of TROP2 expression and clinicopathological features. Kaplan-Meier method was used to generate survival curves, compared by log-rank test. Cox proportional hazard model was employed in univariate and multivariate survival analysis. The level of significance was settled at 5% ($p \leq 0.05$). Statistical analyses were conducted using MedCalc® Statistical Software, version 19.5.3 (MedCalc Software Ltd, Ostend, Belgium).

Results

The median age of subjects was 64 years old (ranging from 17 to 99) in the cohort 1 and most subjects were men (68.4%). The tumors were located mostly in the tongue (71.4%), and histologically (WHO grading system) classified as moderately differentiated in most cases (54.5%). During the follow-up, 38.3% of the subjects developed recurrence, while 45.5% died due to the tumor. The recurrences were most commonly local (72.6%), followed by regional (21.4%), and distant metastases (6%). There was no association between CSS and the Hospital of treatment (p=0.81, data not shown). The subjects from both cohorts in this study presented similar clinicopathological profiles and the details are presented in Table 1.

TROP2 expression was found as a well-defined membranous staining and less frequently in the cytoplasm. The staining was stronger in well-differentiated areas of the tumors containing keratinizing cells. Regarding TROP2 immunoreactivity, 152 (56.5%) samples were classified as low or negative expression (Fig. 1A) and 114 (43.5%) as high expression (Fig. 1B) in the cohort 1. Tumor location (p=0.04) was significantly associated with TROP2 expression, as shown in STable 1. The oral tongue tumors were more frequently classified with high TROP2 expression than tumors in other locations, but not than tumors in the floor of mouth.

Subjects with TROP2 overexpression had longer CSS (p=0.01) and DFS (p=0.04) compared with those with TROP2 down-regulation. TROP2 overexpression was associated with a CSS of 62.6% compared with 46.8% for subjects with low TROP2 expression after a 5-year follow-up (Fig. 1C). Similarly, a DFS of 65.8% was associated with TROP2 tumor overexpression (Fig. 1D). Univariate analysis showed a significant association of age (p=0.01), clinical stage (p=0.005), and TROP2 expression (p=0.01) with CSS, and of age (p=0.04), histological grade (p=0.05), and TROP2 expression (p=0.03) with DFS (Table 2). Multivariate analysis was used to further evaluate the impact of TROP2 expression on the outcomes. In this analysis, age (HR: 1.36, 95% CI: 1.03-1.80, p=0.03), clinical stage (HR: 1.61, 95% CI: 1.10-2.45, p=0.02), and TROP2

expression (HR: 0.60, 95% CI: 0.40-0.90, p=0.01) were independently associated with CCS, whereas only TROP2 expression (HR: 0.57, 95% CI: 0.36-0.89, p=0.01) was significantly associated with DFS (Table 3).

Then, these results were replicated in a second, independent cohort. TROP2 was overexpressed in 38.7% of the cases in cohort 2 (STable 2), and univariate survival analysis confirmed a significant association of TROP2 expression with CSS (p=0.04) (STable 3 and SFig 1 A, B), which remained as an independent prognostic factor in the multivariate analysis (HR: 0.26, 95% CI: 0.09-0.74, p=0.008) (STable 4). In addition to primary OSCC samples, cohort 2 contained fragments of normal oral mucosa obtained from surrounding tumor areas and cervical lymph node metastases. Although the variation among samples was high, the expression of TROP2 was significantly higher in normal tissues when compared to primary tumors (p<0.0001) and lymph node metastases (p=0.001) (SFig. 1 C).

To further support our results, a pooled mRNA expression analysis was performed on the Oncomine™ database. The search, conducted according to the aforementioned parameters, resulted in the inclusion of 8 different datasets and a total of 311 samples. The comparative analysis revealed a significant underexpression of TROP2 mRNA in OSCC compared to normal oral tissues (p=0.014).

Discussion

For decades, several potential prognostic biomarkers have been studied in OSCC (Almangush et al., 2017), but still none has proved clinical relevance. The discovery of such predictors markers is of great interest to optimize the therapeutic approach in OSCC patients, who are often diagnosed at advanced stages of the disease. Herein we studied a large cohort of subjects with long-term follow-up to validate that TROP2 protein expression is significantly associated with clinicopathological parameters, such as tumor location and it was a putative predictor of a better patient's outcome. In addition, our

group is the first one to observe a higher expression of TROP2 in normal oral tissues compared to primary OSCC and lymph node metastases.

TROP2 has a conflicting biological role in different types of cancer, acting as either a tumor suppressor or an oncogene, as a reflex of its plasticity and complex signaling network (Zimmers et al., 2018). TROP2 is differentially expressed in several tissues, and recent meta-analyses have linked its overexpression with poorer OS and DFS in various solid tumors (Zeng et al., 2016; Xu et al., 2017). Despite its overall effect, a protective function of TROP2 expression has been reported in breast (Ambrogi et al., 2014), lung SCC (Inamura et al., 2017), and prostate cancer (Remšík et al., 2018). The lack of significant association with survival in other cancer types including endometrial carcinoma (Bignotti et al., 2012) and gastric cancer (Mühlmann et al., 2009) was also reported.

To date, TROP2 expression in OSCC was linked to poor outcome in four studies (Fong et al., 2008; Tang et al., 2018; Jia et al., 2020; Zhang et al., 2020), whereas no association with survival or lymph node metastasis was found in another publication (Noorlag et al., 2015). It has been pointed out a significant intratumoral and temporal heterogeneity of TROP2 in virtually every breast and prostate tumor (Remšík et al., 2018), which might also be the case with OSCC and could partially explain these results. Another possible explanation is the sample size used in these studies and the absence of information regarding the anatomical oral subsite location. In our study, TROP2 expression was associated with tumor subsite, and the tumors were mostly located in the tongue (71.4% in cohort 1 and 63.6% in cohort 2). A previous study described OSCC clinical data per tumor subsite, which were widely distributed in the oral cavity, did not find association between TROP2 expression and tumor location (Tang et al., 2018). We observed a significantly TROP2 overexpression in morphologically normal tissues than in tumors and lymph node metastases. This finding is in agreement with previous results in literature showing that TROP2 has lower expression in the majority of the lung tumors, esophageal cancer or head and neck tumors when compared with their normal

counterpart tissues (Stepan et al., 2011).As a major player in OSCC local recurrence and lymph node metastasis, epithelial-mesenchymal transition (EMT) has been associated with a low survival rate of patients with oral cancer (Jayanthi et al., 2020). Since TROP2 was reported to be overexpressed in some epithelial tumors, Wang et al. (2011) investigated its role in epithelial carcinogenesis and showed that loss of TROP2 expression drives EMT of normal keratinocytes and increases the chances of skin carcinomas development in response to carcinogens (Wang et al., 2011). In head and neck SCC, decreased TROP2 expression was described in tumors showing EMT features, while sarcomatoid variants were completely negative (Wang et al., 2011). In consonance, a study carried out by Remšík et al. (2018) confirmed that TROP2 expression was positively correlated with E-cadherin levels, while negatively correlated with the mesenchymal gene signature in breast and prostate cell lines (Remšík et al., 2018). The authors assumed that both the EMT transcription factors and the epigenetic mechanisms act in the regulation of the cell phenotypes, mirroring the EMT state of the cell and, in turn, governing TROP2 expression in the membrane (Remšík et al., 2018).

The use of bioinformatics tools revealed that low TROP2 mRNA expression was linked to poor outcome for prostate and breast cancer patients (Remšík et al., 2018). In head and neck cancer, TROP2 expression is frequently decreased and presents tumor-suppressive functions (Zhang et al., 2014). In line with these findings, several publications evaluating NSCLC reported association TROP2 with poor outcome in adenocarcinomas, but not in SCC (Pak et al., 2012; Jiang et al., 2013; Inamura et al., 2017; Mito et al., 2020), suggesting a possible role dependent on tumor histology. Besides, the prognostic value of TROP2 has been conditioned to the clinical stage of colon cancers, showing tumor-suppressing characteristics in the initial stages of disease (Fang et al., 2012). In hepatocellular carcinoma, the loss of TROP2 expression related to its promoter hypermethylation predicts poor overall survival (Sin et al., 2018). In fact, DNA hypermethylation is suggested to be the main mechanism to regulate TROP2 (Lin et al., 2012; Zimmers et al., 2018; Remšík et al., 2018). In lung cancer, DNA

hypermethylation inhibited TROP2 expression, and its loss leads to hyperactivation of IGF-1R and subsequent oncogenic effects (Lin et al., 2012). In the breast cancer cell line resistant to Tamoxifen TMX2 28, which is significantly hypermethylated, 5-Aza-dC treatment decreased cell proliferation and increased TROP2 mRNA levels (Zimmers et al., 2018). Indeed, it is widely accepted that OSCC tumorigenesis is closely related to DNA methylation, which could be related to the conventional disease risk factors such as heavy alcohol consumption and smoking (Gasche et al., 2012).

Even though the functional role of TROP2 remains largely unknown, targeted therapies against TROP2 are already being tested in clinical trials (Remšík et al., 2018; Goldenberg & Sharkey, 2020). The drug development process, preclinical evidence and clinical trials evaluating the use of Sacituzumab Govitecan (SG), which is an antibody-drug conjugate that targets TROP2, have been recently reviewed by Goldenberg & Sharkey (2020). Mostly, SG has been tested in difficult-to-treat patients (advanced and metastatic disease) with triple-negative breast cancer (TNBC), HR+/HER2-breast cancer, urothelial cancers, small-cell lung cancer and NSCLC, who have failed at least in two prior therapies. SG presented manageable toxicity and good efficacy as a monotherapy in several solid cancers (Goldenberg & Sharkey, 2020). However, due to intratumoral heterogeneity, the monotherapy targeting TROP2-expressing cells might lead to selection of resistant cancer cells that present mesenchymal phenotype (Remšík et al., 2018). The effect of SG in OSCC has not been studied yet, and the current evidence does not suggest that such therapy would be beneficial for this particular type of cancer.

Our study has some limitations, including its observational and retrospective nature, and the intrinsic limitations of the immunohistochemical technique. Another limitation that should be noted when interpreting the results is the lack of detailed information on exposure assessment of risk factors. However, the study has several strengths. We examined tumors from several countries, the multivariate analysis included several independent parameters that are known to influence outcome, and we

have validated our results in two independent cohorts, suggesting that the magnitude of information is strong and true. As the p values of associations were borderlines and the interpretation should be with caution.

In summary, our results suggest that TROP2 is highly expressed in normal oral tissues, and its low protein expression in OSCC promotes shortened CSS and high rates of recurrence. If the protective role of TROP2 in OSCC is confirmed, assessing its levels would be an important tool to differentiate patients with increased risk for poor outcomes. Further large studies in combination with *in vitro* experiments to identify the precise mechanisms and signaling pathways related to TROP2 expression, will be of great value to understand its key role in OSCC.

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Conflicts of Interest

None to declare.

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Figure Legends

Figure 1. Immunohistochemical location of TROP2 is associated with the outcome of patients with OSCC. (A) A representative sample displaying a few TROP2-positive carcinoma cells in the center of the tumor islands (tumor classified as low expression), and in (B) a sample with tumor cells showing a broad distribution and an intense staining of TROP2 (tumor classified with high expression) (magnification x100). (C) Kaplan-Meier curves for cancer-specific survival, and (B) disease-free survival of patients with OSCC of cohort 1 based in TROP2 expression levels.

Supplementary Figure 1. TROP2 expression was evaluated in an independent OSCC cohort. (A) Kaplan-Meier survival curve revealed that higher TROP2 expression is significantly associated with increased CSS ($p = 0.04$). (B) Only a tendency towards association between TROP2 expression and disease-free survival was observed ($p = 0.31$). (C) TROP2 expression was significantly higher in normal oral mucosa (n=88) when compared to OSCC and metastatic lymph nodes (n=17). (D) Representative images of the normal oral mucosa, primary tumor, and metastatic lymph node are shown from left to right, in varying levels of TROP2 expression (magnification 100x). *$p<0.001$; **$p<0.0001$.

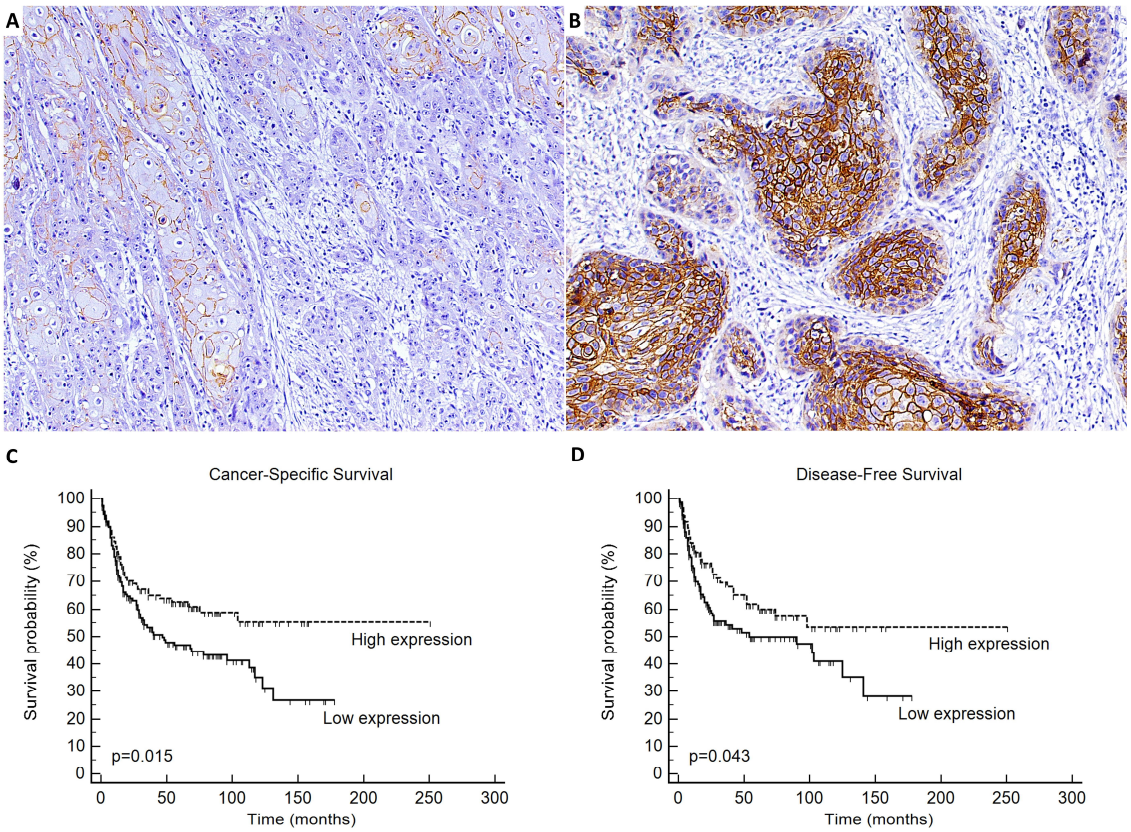

A
B
C
Cancer-Specific Survival
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
0
High expression
Low expression
p=0.015
0
50
100
150
200
250
300
Time (months)
D
Disease-Free Survival
Survival probability (%)
High expression
Low expression
p=0.043
Time (months)

Table 1. Clinicopathological features of patients with oral squamous cell carcinoma from cohort 1 (whole sections, n = 266) and cohort 2 (TMA, n = 88).

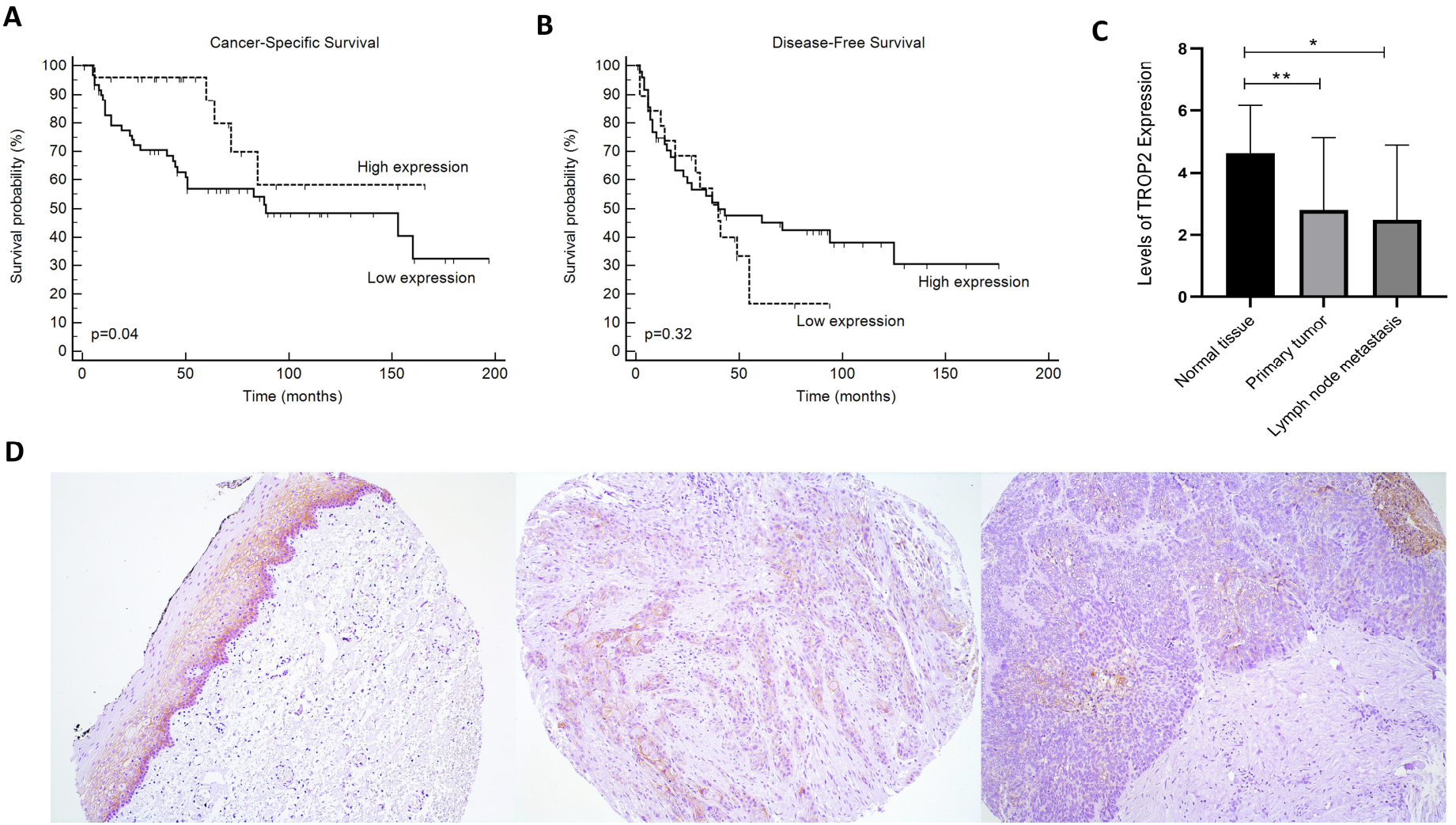
	Cohort 1	Cohort 2
Age (years)		
Mean ± SD	62.7 ± 14.1	64.1 ± 13.7
Range	17-99	32-98
Sex		
Male	182 (68.4%)	48 (54.5%)
Female	84 (31.6%)	40 (45.5%)
Clinical stage		
I	51 (19.2%)	22 (25.0%)
II	56 (21.1%)	16 (18.2%)
III	47 (17.6%)	14 (15.9%)
IV	61 (22.9%	29 (32.95%)
Missing data	51 (19.2%)	7 (7.95%)
Location		
Tongue	190 (71.4%)	56 (63.6%)
Floor of month	32 (12.1%)	7 (8.0%)
Others	42 (15.8%)	25 (28.4%)
Missing data	2 (0.7%)	-
Histological grade		
Well-differentiated	83 (31.2%)	14 (15.9%)
Moderately-differentiated	145 (54.5%)	50 (56.8%)
Poorly-differentiated	38 (14.3%)	22 (25.0%)
Missing data	-	2 (2.3%)
Treatment		
Surgery	121 (45.5%)	61 (69.3%)
Surgery + Radiotherapy	87 (32.7%)	21 (23.9%)
Surgery + Radiotherapy + Chemotherapy	51 (19.2%)	6 (6.8%)
Missing data	7 (2.6%)	-
Margen status		
≥ 5 mm	167 (62.8%)	69 (78.4%)
< 5 mm	66 (24.8%)	17 (19.3%)
Missing data	33 (12.4%)	2 (2.3%)
Recurrence		
No	147 (55.3%)	26 (29.5%)
Yes	102 (38.3%)	42 (47.7%)
Missing data	17 (6.4%)	20 (22.8%)
Status		
Alive	141 (54.5%)	51 (57.95%)
Dead	125 (45.5%)	36 (40.9%)
Missing data	-	1 (1.15%)

Table 2. Univariate analysis for cancer-specific survival and disease-free survival of 266 patients with oral squamous cell carcinoma from cohort 1.

	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
Age (years)						
≤ 64years	62.7	1		62.2	1	
> 64years	46.7	1.56 (1.10-2.23)	**0.01**	49.7	1.52 (1.02-2.25)	**0.04**
Sex						
Male	53.6	1		53.3	1	
Female	55.9	0.90 (0.61-1.32)	0.59	62.4	0.92 (0.59-1.42)	0.71
Clinical stage						
Early (I + II)	63.8	1		59.9	1	
Advanced (III + IV)	45.0	1.68 (1.17-2.41)	**0.005**	52.1	1.42 (0.95-2.12)	0.08
Tumor site						
Tongue	55.6	1		56.9	1	
Floor of mouth	60.0	0.91 (0.54-1.54)	0.74	61.4	0.81 (0.44-1.47)	0.49
Other	41.2	1.34 (0.79-2.26)	0.27	44.6	1.45 (0.81-2.61)	0.20
Histological grade						
Well-differentiated	56.9	1		62.0	1	
Moderately-differentiated	55.0	1.03 (0.67-1.57)	0.91	45.6	1.60 (0.99-2.58)	**0.05**
Poorly-differentiated	44.4	1.64 (0.85-3.16)	0.13	43.1	1.70 (0.78-3.68)	0.17
Treatment						
Surgery	58.6	1		55.5	1	
Surgery + Radiotherapy	60.6	0.95 (0.57-1.57)	0.84	61.6	0.81 (0.50-1.31)	0.40
Surgery + Radiotherapy + Chemotherapy	43.9	1.36 (0.90-2.06)	0.13	46.6	1.32 (0.80-2.19)	0.27
Margin status						
≥ 5 mm	58.2	1		56.0	1	
< 5 mm	55.8	1.00 (0.64-1.56)	0.98	52.2	1.20 (0.73-1.97)	0.45
TROP2 (*TACSTD2*)						
Low expression (score ≤ 4)	46.8	1		50.2	1	
High expression (score (> 4)	62.6	0.64 (0.44-0.91)	**0.01**	65.8	0.65 (0.43-0.97)	**0.03**

Table 3. Multivariate analysis of cancer-specific survival and disease-free survival for the 266 patients with oral squamous cell carcinoma from cohort 1.

	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Age (years)				
≤ 64 years	1			
> 64 years	1.36 (1.03-1.80)	**0.03**		
Clinical stage				
Early (I + II)	1			
Advanced (III + IV)	1.61 (1.10-2.45)	**0.02**		
TROP2 (*TACSTD2*)				
Low expression (score ≤ 4)	1		1	
High expression (score > 4)	0.60 (0.40-0.90)	**0.01**	0.57 (0.36-0.89)	**0.01**


A
Cancer-Specific Survival
Survival probability (%)
High expression
Low expression
p=0.04
Time (months)
B
Disease-Free Survival
Survival probability (%)
High expression
Low expression
p=0.32
Time (months)
C
Levels of TROP2 Expression
*
**
Normal tissue
Primary tumor
Lymph node metastasis
D

Supplementary Table 1. Association of the clinicopathological parameters of the oral squamous cell carcinoma (cohort 1, n = 266) with the immunohistochemical expression of TROP2.

Parameter	LowTROP2 n (%)	HighTROP2 n (%)	p value
Age			
≤ 64 years	81 (53.3)	60 (52.6)	
> 64 years	71 (46.7)	54 (47.4)	0.91
Gender			
Male	105 (69.1)	78 (68.4)	
Female	47 (30.9)	36 (31.6)	0.90
Clinical stage			
Early (I + II)	74 (49.0)	56 (51.4)	
Advanced (III + IV)	77 (51.0)	53 (48..6)	0.70
Tumor site			
Tongue	100 (66.2)	90 (78.9)	
Floor of mouth	20 (13.2)	13 (11.4)	
Other	31 (20.6)	11 (9.6)	**0.04**
Histological grade			
Well-differentiated	44 (28.9)	39 (34.2)	
Moderately-differentiated	83 (54.6)	62 (54.4)	
Poorly-differentiated	25 (16.5)	13 (11.4)	0.42
Treatment			
Surgery	69 (46.6)	52 (46.8)	
Surgery + Radiotherapy	51 (34.5)	36 (32.5)	
Surgery + Radiotherapy + Chemotherapy	28 (11.9)	23 (20.7)	0.91
Margin status			
≥ 5 mm	101 (74.8)	66 (67.3)	
< 5 mm	34 (25.2)	32 (32.7)	0.08
Recurrence			
No	80 (54.8)	70 (66.0)	
Yes	66 (45.2)	36 (34.)	0.07

Low expression TROP2: score ≤ 4, and High expression of TROP2: score > 4.

Supplementary Table 2. Association between immunohistochemical expression of TROP2 and clinicopathological parameters of the oral squamous cell carcinoma in the cohort 2 (n = 88).

Parameter	Low TROP2 n (%)	High TROP2 n (%)	p value
Age			
≤ 63 years	25 (40.3)	16 (61.5)	
> 63 years	37 (59.7)	10 (38.5)	0.19
Gender			
Male	36 (58.1)	12 (46.2)	
Female	26 (41.9)	14 (53.8)	0.31
Clinical stage			
Early (I + II)	26 (44.1)	13 (56.5)	
Advanced (III + IV)	33 (55.9)	10 (43.5)	0.31
Tumor site			
Tongue	42 (67.7)	15 (57.7)	
Floor of mouth	6 (9.7)	2 (7.7)	
Other	14 (22.6)	9 (34.6)	0.50
Histological grade			
Well-differentiated	11 (18.0)	3 (12.0)	
Moderately-differentiated	32 (52.5)	18 (72.0)	
Poorly-differentiated	18 (29.5)	4 (16.0)	0.24
Treatment			
Surgery	42 (67.7)	19 (73.1)	
Surgery + Radiotherapy	15 (24.2)	6 (23.1)	
Surgery + Radiotherapy + Chemotherapy	5 (8.1)	1 (3.8)	0.75
Margin status*			
≥ 5 mm	47 (78.3)	22 (84.6)	
< 5 mm	13 (21.7)	4 (15.4)	0.57
Recurrence			
No	20 (41.7)	6 (30.0)	
Yes	28 (58.3)	14 (70.0)	0.37

Low expression TROP2: score ≤ 4, and High expression of TROP2: score > 4.

*Fisher's exact test instead of chi-squard test.

Supplementary Table 3. Cancer-specific survival and disease-free survival of 88 cases of oral squamous cell carcinoma contained in the TMA (cohort 2) based on univariate analysis.

	Cancer-specific survival			Disease-free survival		
	% in 5 years	HR (95% CI)	p value	% in 5 years	HR (95% CI)	p value
Age (years)						
≤ 63 years	64.6	1		37.8	1	
> 63 years	65.3	0.93 (0.49-1.77)	0.83	60.4	0.66 (0.36-1.19)	0.17
Gender						
Male	65.8	1		49.6	1	
Female	64.1	1.08 (0.57-2.07)	0.79	27.0	1.37 (0.75-2.51)	0.29
Clinical stage						
Early (I + II)	76.7	1		48.6	1	
Advanced (III + IV)	54.0	2.14 (1.11-4.12)	**0.02**	33.7	1.60 (0.86-2.97)	0.13
Tumor site						
Tongue	61.9	1		35.1	1	
Floor of mouth	66.7	0.71 (0.25-2.01)	0.51	44.4	0.96 (0.36-2.54)	0.93
Other	71.2	0.70 (0.35-1.42)	0.33	49.0	0.69 (0.36-1.34)	0.27
Histological grade						
Well-differentiated	92.9	1		60.6	1	
Moderately-differentiated	65.2	1.61 (0.67-3.82)	0.28	41.1	1.84 (0.92-4.08)	0.09
Poorly-differentiated	41.7	3.11 (1.19-8.11)	**0.02**	38.6	2.88 (1.13-7.32)	**0.02**
Treatment						
Surgery	57.1	1		32.8	1	
Surgery + Radiotherapy	81.2	0.49 (0.24-1.01)	0.06	51.8	0.54 (0.29-1.01)	0.06
Surgery + Radiotherapy + Chemotherapy	50.0	1.20 (0.30-3.45)	0.91	38.1	0.82 (0.31-2.21)	0.70
Margin status						
≥ 5 mm	62.7	1		37.8	1	
< 5 mm	73.3	0.84 (0.41-1.73)	0.64	42.2	0.80 (0.42-1.51)	0.49
TROP2 (*TACSTD2*)						

Low expression (score ≤ 4)	56.9	1		16.6	1	
High expression (score (> 4)	87.8	0.49 (0.23-0.96)	**0.04**	47.5	0.70 (0.34-1.41)	0.31

Supplementary Table 4. Multivariate analysis of cancer-specific survival and disease-free survival for the 88 patients with oral squamous cell carcinoma in the TMA cohort.

	Cancer-specific survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Clinical stage				
Early (I + II)	1		1	
Advanced (III + IV)	5.10 (2.15-12.09)	**0.0002**	3.86 (1.73-8.57)	**0.001**
Histological grade				
Well-differentiated + Moderately-differentiated	1			
Poorly-differentiated	1.76 (1.20-2.59)	**0.004**		
TROP2 (*TACSTD2*)				
Low expression (score ≤ 4)	1			
High expression (score > 4)	0.26 (0.09-0.74)	**0.008**		