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Article type   : Original Article

Title: An immunohistochemical score to predict the outcome for oral squamous cell carcinoma

Running title: Prognostic markers in OSCC

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jop.12682

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Abstract

Background: Oral cancer is a major public health problem worldwide, with a poor survival. Our aim was to evaluate several protein markers in oral squamous cell carcinomas (OSCC) and analyse their prognostic value on patient´s survival.

Methods: We analysed the expression of EGFR, p53, p27, p16, cyclin D1, cyclin A2, COX-2, Ki-67, Bcl-2, VEGFR-1, and VEGFR-2, by immunohistochemistry on 67 primary OSCC. Cancer-specific survival (CSS) analysis was evaluated by the Cox regression model.

Results: Markers showed variable expression between 27.9% to 95.2%. In univariate analysis for CSS we found that four of the tested markers namely, high expression of p53 (P=0.001), EGFR (P=0.003), cyclin A2 (P=0.005) and low expression of p16 (P=0.019), along with clinical stage (P<0.001), tumour size (P<0.001), presence of nodal metastasis (P<0.001), and perineural permeation (P=0.039) were related with decreased survival. Based on these results, we constructed an immunohistochemical score hinging on the possibility that any tumour could express none of these four markers (score 0), one or two markers (score 1), and three or more markers (score 2). In multivariable analysis, this immunohistochemical score revealed an independent prognostic value on cancer-specific survival (P=0.001; HR: 3.7: 95%CI 1.7-7.9). Moreover, we confirmed that in early stage tumours (stage I or II) this score maintained its independent prognostic value (P=0.025; HR: 7.9, 95%CI 1.3-49.1) on CSS.

Conclusion: The expression of the markers p53, p16, EGFR and cyclin A in OSCC, combined to give an immunohistochemical score, may identify high-risk subgroups for decreased survival and to further guide therapeutic decisions.

Keywords: Oral squamous cell carcinoma; prognosis; p53; p16; EGFR; cyclin A

Introduction

Globocan 2012 on world cancer data reported an estimated incidence (ASR) of 7.0 per 100,000 habitants for lip, oral cavity and pharynx with 529,451 new cases.[1] In the oral cavity squamous cell carcinomas account for almost 90% of malignant neoplasms.[2] They result in a high morbidity and mortality where in most countries only 50% of the patients survive for 5 years from the time of detection.[1,2]

In the past two decades, biomarkers based on proteins linked to the molecular pathways of oral carcinogenesis have been studied for the prediction of outcomes. During carcinogenesis several pathways may be altered that modify tumour proliferation and apoptosis and induce a transformed cell to a clonal expansion of tumour cells. Some aberrant genes or proteins of this tumour expansion could serve as biomarkers of tumour progression and prognosis.[3-5]

Aberrant expression of EGFR, p53, p16, cyclin D1 have been proposed as having a prognostic influence on oral cancer.[5] For several decades researchers have speculated that ~6 mutations in key genes were both necessary and sufficient to cause cancer. Califano's group working on head and neck cancer have described models that involve a sequence of genetic changes that are involved in the evolution of cancer.[6] Most authors have studied these molecular changes as individual proteins and their significance described as single markers has not been clear.[5] We therefore hypothesised that evaluation of several biomarkers studied all together in a single cohort of oral cancers may provide a better characterisation of markers which have a predictive influence on patients with OSCC.

Based on our previous review on biomarkers for oral squamous cell carcinomas[7] that have been reported in earlier studies we selected a range of protein markers that have been previously studied by us and other authors. Our objective was to evaluate eleven protein markers (EGFR, p53, p27, p16, cyclin D1, cyclin A2, COX-2, Ki-67, Bcl-2, VEGFR-1, and VEGFR-2) in oral squamous cell carcinomas (OSCC) and to analyse their prognostic value on cancer-specific survival. Using these markers we aimed also to determine a potential score of protein biomarkers that could predict the prognosis of the patients with this tumour.

Material and methods

Patients

A descriptive and transversal study was performed on 67 patients with OSCC diagnosed at the *Clinical University Hospital of Santiago de Compostela (Spain)*, in the period between 1995 and 2003, and treated by the Maxillofacial Surgery team of the same hospital. The study was undertaken following approval of the institutional ethical board of the hospital and performed in accordance with the Declaration of Helsinki. Only primary OSCC, without any history of radiotherapy or chemotherapy prior to surgery, and with clinical information and follow-up were considered. The final sample included 67 patients, 52 men (77.6%) and 15 women (22.4%) (ratio 3.5:1), with a median age of 60 years (range from 31 to 85 years). Some of clinical and demographic data were published in our previous paper.[8] Briefly, the most frequently affected site was the tongue (n=28; 41.8%) followed by the floor of the mouth (n=20; 29.9%), alveolar bridge (n=7; 10.4%); hard palate (n=5; 7.5%), retromolar trigone (n=4; 6%), and buccal mucosa (n=3; 4.5%). Stage classification was made according to the 7th edition of the classification of malignant tumours of American Joint Committee on Cancer. Stage-I corresponded to 21(31.3%) cases, stage-II to 20 (29.9%), stage-III to 11 (16.4%), and stage-IV (A+B) to 15 (22.4%) cases. Twenty-one (31.3%) patients presented nodal metastasis at diagnosis. New haematoxylin-eosin (HE) slides were performed to confirm the diagnosis and tumour differentiation grade was reclassified according to the WHO classification (2005). Tumours were graded as well-differentiated in 34 (50.7%) and moderately or poorly differentiated in 33 (47.3%) cases. Of the 63 patients with report of surgical margin status, 9 cases (14.3%) had margins with tumour. Perineural permeation was observed in 11(16.4%) cases, and vascular permeation in 5 (7.5%) cases.

Patients were observed for a minimum of 3 years after treatment or until their death. During follow-up, 36 (53.7%) had died of oral cancer.

Immunohistochemical technique

For immunohistochemical analysis we used 2 tissue microarray (TMA) blocks of the 67 cases of OSCC constructed according to our previously reported data.[8] Briefly, we selected two representative tumoral areas of interest (specially of the front of the tumour invasion) on the HE slides of tumour blocks of each patient, avoiding areas of necrosis or keratin pools. Then, two tissue cores from previously selected areas, each one with a diameter of 1.5 mm, were inserted into a recipient paraffin block using a semi-automatic microarray instrument (Beecher Instruments, Silver Spring, MD, USA).

Immunohistochemical staining was performed on consecutive series of 4-µm sections using the dextran-polymer system (EnVisionTM Detection Kit, Dako, Glostrup, Demark) to detect EGFR, p53, p27, p16, cyclin D1, cyclin A2, COX-2, Ki-67, Bcl-2, VEGFR-1, and VEGFR-2 proteins (data on EGFR, p53, p27 have been previous report by our group).[8] Antigen retrieval, clones and dilutions are presented in Table 1. Visualization was performed with diaminobenzidine tetrahydrochloride (Dako, Glostrup, Demark) for 5 min; and counterstaining with Harris hematoxylin for 2 min. Negative controls (omitting the primary antibodies) and positive controls slides were added in each marker staining run. As positive controls, following the antibodies manufacture's indications, we included human tonsil mucosa tissue (for EGFR, Ki-67, p53, p27, p16, cyclin A2, Cyclin D1, and bcl-2), human colon carcinoma (Cox-2), human esophageal mucosa for VEGFR-2 and human lung tissue for VEGFR-1. Moreover, cores with tonsil (including also oral mucosa) were included in each TMA for internal positive controls.

Evaluation of immunohistochemical expression

Two authors (LSM and MF) independently performed the staining evaluation on TMAs blinded to the prognostic and clinical variables. Markers were evaluated on an Olympus BX41 microscope. Any discrepancies in scoring were discussed, reviewed together and were given an agreed score.

EGFR was evaluated considering membrane or both membrane and cytoplasm expression and VEGFR-1, VEGFR-2 when presenting both membrane and cytoplasm expression. p16 was evaluated considering nucleous and cytoplasm expression. The rest of the markers were evaluated considering either nuclear (for Ki-67, Cyclin A2, Cyclin D1, p53, p63, and p27) or cytoplasmic (Cox-2, Bcl-2) expression (Table 1).

EGFR was semi-quantitatively classified following a four-point score: 0 (no labelling, or labelling in <10% of tumour cells); 1+ (weak labelling, homogeneous or patchy, in >10% of tumour cells); 2+ (moderate labelling, homogeneous or patchy, in >10% of tumour cells); 3+ (intense labelling, homogeneous or patchy, in >10% of tumour cells). EGFR was also classified according EGFR cell location as EGFR expression in membrane and cytoplasm or only in membrane.

All other markers were semi-quantitatively classified as: 0: no labelling or labelling in <10% of tumour cells; 1+: labelling in 10% to 24% of tumour cells; 2+: labelling in 25% to 49% of tumour cells; 3+: labelling in 50% or more of tumour cells. Intensity of the marker was expressed in low, moderate and strong expression. Cut-off for high expression established on basis of the expression on normal oral mucosa for each marker is given in Table 1.

Statistical analysis

For statistical analysis an IBM SPSS Statistics™ version 20.0 software (IBM Corporation, NY, US) was used. The relations between categorical variables were analysed by chi-square test.

Cancer-specific survival (CSS) was defined as the time interval (months) between effective treatment or death by oral cancer or last follow-up. Univariate analysis of the influence of markers on CSS was performed using the Kaplan-Meier and the log-rank test. Factors that were significant in the univariate analysis were then analysed by multivariate analysis using the Cox proportional hazards model. The location of the tumour categorized in tongue, floor of the mouth and others were included in the multivariate analysis to discard any influence of location on the results. The level of statistical significance was considered at $P<0.05$.

Results

The semi-quantitative patterns of expression of various markers observed in our study are listed in Table 2. EGFR was expressed by most cancers (95.2%). Over 80% of the cancers expressed p27, cyclin A2, cyclin D1 and VEGFR2 and other markers were found to be expressed in variable levels (Table 2). With reference to cell pattern of immunostaining of markers, EGFR was found in both plasma membrane and cytoplasm in 29 cases (35%), Cox-2 was cytoplasmic and the remainder of markers were predominantly nuclear.

Prognostic significance of clinicopathological and biological parameters

We evaluated the relationships between clinical, pathological and biomarkers on cancer specific survival (CSS) of our patients. Using Kaplan-Meir curves and log-rank test several variables were related with patient’s survival, including T status (p<0.001), N+ status (p<0.001), clinical stage (p<0.001), perineural permeation (P=0.039), and also the biomarkers EGFR (p=0.003), p53 (p=0.001), p16 (p=0.019), and Cyclin A2 (p=0.005) (Table 3). Using the results of these four proteins with significance on the univariate analysis, we constructed an immunohistochemical score based on the possibility that a given tumour could have none of these protein markers (score 0; low aggressiveness tumour), one or two markers (score 1; moderate aggressiveness tumour), or three or more significant markers (score 2; high aggressiveness tumour). The Kaplan-Meir curves showed a CSS of 100% for score 0, dropping to 60.2% for score of 2 and to 5.9% for score 3 in univariate analysis (p<0.001) (Figure 2).

In multivariable analysis, this immunohistochemical score revealed an independent prognostic value on cancer-specific survival (P=0.001; HR: 3.7: 95%CI 1.7-7.9) additional to the advanced clinical stage (*P* = 0.004; HR:2.7, 95%CI 1.4-5.4) (Table 4). Moreover, when we restricted our evaluation to early stage tumours (stage I or II) this protein score maintained an independent prognostic value (P=0.025; HR: 7.9, 95%CI 1.3-49.1).

Discussion

OSCC is a common and aggressive tumour associated with a high mortality and morbidity rates. The existent scoring systems to determine prognosis in routine use are outdated and limited mainly to the size, presence of metastasis and some pathological characteristics such as histological grade, vascular or perineural permeation.[9] The grading systems based on morphological features of tumours proposed in the latter part of the last century by Bryne *et al*[10] and Annoreth *et al*[11] have not been utilised widely in routine use. We aimed to determine immunohistochemical markers that can predict the OSCC prognosis to help in the treatment plan for an individual patient with this tumour. We selected this panel of biomarkers as many of them have been used in earlier studies as single markers to predict outcome.[8,12-14]

Our data confirm the importance of clinical and pathological variables as previously reported in OSCC. Clinical stage and related variables such as tumour size and presence of nodal metastasis remain significant prognostic variables in OSCC. The prognostic value on survival of TNM classification and clinical stage has been clearly reported in oral cancer.[9,15] Perineural permeation has been reported to be present in 6 to 30% of head and neck carcinomas and related with decreased patients' survivals.[9,16]

Four of the eleven biomarkers studied here (EGFR, p16, p53, and cyclin A) showed significant associations with CSS. Earlier studies have previously described the relation of these markers with survival. EGFR high expression has been associated with a decreased survival rate of OSCC.[12,17-19] In particular, EGFR cell sublocalization could be an important aspect and related with decreased survival. We have previously reported that the simultaneous EGFR expression both on the membrane and in the cytoplasm are reliable indicators of EGFR high expression in OSCC and are related with decreased patient's survival.[8] Nuclear localization of the EGFR was also related with decreased survival in oropharyngeal squamous cell carcinoma[20] and correlated with increased cell cycle progression and proliferation.[21-23] Low or loss of p16 staining has been documented in OSCC and related with decreased survival.[24,25] Bova *et al*[25] reported the prognostic value of p16 protein with significant value on multivariate analysis where patients with tongue carcinomas having low p16 expression had shorter overall survival than patients that presented normal p16 expression. P53 is one of the most studied markers in OSCC. A strong association between p53 high expression and survival has been reported where patients with OSCC having p53 high expression had decreased overall survival.[26-28] The prognostic value on CSS of cyclin A observed in the present paper is in accordance with other reports.[13,14] Chen *et al*[14] in a study with patients with OSCC reported an association of cyclin A and survival where high expression of cyclin A protein was associated with tumour progression and decreased survival in OSCC.

Having undertaken the assessment of a panel of markers, our data led us to propose that a combined expression of these four significant markers could reflect an increasing aggressiveness of OSCC. To evaluate this possibility, we constructed an immunohistochemical score based on protein results (EGFR, p53, p16, and Cyclin A2) with a three scale score. Patients with a score 0 had no protein alterations and patients with score 2 presented alterations in all of the biomarkers examined. We observed that this immunohistochemical score had a significant effect on CSS. No deaths were found in patients with tumours with score 0 contrasting with patients with tumours with a score 2 where only 5% of cases survived up to three years following detection of their cancers. More importantly when we analyse the data from early stage tumours this immunohistochemical score maintained a significant and independent value on multivariate analysis. We propose that patients with OSCC may benefit by this immunohistochemical score to determine a better categorization of the aggressiveness of the tumour and to make an appropriate treatment plan choice for these patients.

We wish to highlight few limitations of the study. Due to the small number of cases, our panel of oral cancers included several anatomical locations of the oral cavity. Nevertheless, we analyse possible influence of subgroups of localizations on univariate and multivariate analysis. We acknowledge also that patients were treated by more than one surgeon over the period of the study, however always from the same unit team. We only have studied cases with oral cancers. It would be interesting in future research to introduce a group of patients without oral lesions to functioning as control group.

A range of biomarkers expressed in OSCC with some potential value for targeted therapy have recently been discussed.[7] In the published literature the combined expression of few of these markers have shown to be superior with a higher prognostic value in OSCC.[25,27,29] Baschnagel *et al*[19] and Warnakulasuriya *et al*[28] described similar findings on head and neck cancers. These studies have reported on combining 2 or 3 markers to assess the prognosis while in our study we used 11 prognostic markers that had previously been reported in OSCC with some individual prognostic value on CSS. To our knowledge this is the first instance such an array of markers has been utilized on a single cohort of cases.

In conclusion, the evaluation of several protein markers (high expression of p53, EGFR and cyclin A2 and low expression of p16) in OSCC, combined to give an immunohistochemical score, may identify a high-risk subgroup of patients with decreased survival and to personalise the therapy.

Acknowledgements

Part of this study was supported by a grant from the Fundación de Investigación Médica Mutua Madrileña (Spain) and Instituto de Salud Carlos III (FIS PI 061902).

Conflicts of interest: There are no potential conflicts of interest.

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FIGURES Legends

Figure 1 – Images corresponding to Immunohistochemical technique performed in OSCC: EGFR immunostaining: (a) membrane expression, (magnification ×200); (a') membrane and cytoplasmic expression, (magnification ×200). VEGFR-1 immunostaining: (b) low expression, (magnification ×200); (b´) high expression, (magnification ×200). VEGFR-2 immunostaining: (c) low expression, (magnification ×200); (c´) high expression, (magnification ×200). p16 immunostaining: (d) low expression, (magnification ×200); (d´) high expression, (magnification ×200). p27 immunostaining: (e) low expression, (magnification ×200); (e´) high expression, (magnification ×200). p53 immunostaining: (f) low expression, (magnification ×200); (f´) high expression, (magnification ×200). Ki-67 immunostaining: (g) low proliferative tumour, (magnification ×200); (g´) high proliferative tumour, (magnification ×200). Cyclin D1 immunostaining: (h) low expression, (magnification ×200); (h´) high expression, (magnification ×200). Cyclin A2 immunostaining: (i) low expression, (magnification ×200); (i´) high expression, (magnification ×200). Cox-2 immunostaining: (j) low expression, (magnification ×200); (j´) high expression, (magnification ×200). Bcl-2 immunostaining: (k) low expression, (magnification ×200); (k´) high expression, (magnification ×200).

Figure 2 - Kaplan-Meier curves according to immunohistochemical score demonstrating a worse cancer-specific survival in the OSCC patients with the increasing grade of the score.

Table 1 – Main characteristics of the biomarkers used in the study

Antibody	Pretreatment	Clone	Dilution	Manufacturer	Cell location expression	Cut-off for high expression for extent (1) and intensity (2)
EGFR	Proteinase K	2-18C9	Prediluted	DakoCytomation, Glostrup, Denmark	Membrane or Membrane + Cytoplasm	- ≥10% tumour cells with moderate or strong intensity - Presence of cytoplasm staining
VEGFR-1	HIER (1)	SC-316	1/1000	Santa Cruz Biotech Santa Cruz, CA, USA	Membrane + Cytoplasm	1) ≥25% tumour cells 2) moderate or strong intensity
VEGFR-2	HIER (1)	SC-6251	1/2000	Santa Cruz Biotech, Santa Cruz, CA, USA	Membrane + Cytoplasm	1) ≥25% tumour cells 2) moderate or strong intensity
Cyclin D1	HIER (1)	SP4	Prediluted	Master Diagnostica, Spain	nucleus	1) ≥25% tumour cells 2) moderate or strong intensity
Cyclin A2	HIER (1)	6E6	1/10	Novocastra Leica Biosystems, Newcastle upon Tyne, UK	nucleus	1) ≥25% tumour cells 2) moderate or strong intensity
P53	HIER (1)	DO7	1/20	Novocastra Leica Biosystems, Newcastle upon Tyne, UK	nucleus	1) ≥50% tumour cells 2) moderate or strong intensity
P27	HIER (1)	1B4	1/20	Novocastra Leica Biosystems, Newcastle upon Tyne, UK	nucleus	1) ≥25% tumour cells 2) moderate or strong intensity
P16	HIER (1)	OA315	Prediluted	MTM, Heidelberg, Germany	nucleus+ cytoplasm	1) ≥10% tumour cells 2) moderate or strong intensity
Cox-2	HIER (1)	SP21	Prediluted	Master Diagnostica, Spain	cytoplam	1) ≥25% tumour cells 2) moderate or strong intensity
Bcl-2	HIER (1)	124	1/20	DakoCytomation, Glostrup, Denmark	cytoplam	1) ≥10% tumour cells 2) moderate or strong intensity
Ki-67	HIER (1)	MIB-1	1/200	DakoCytomation, Glostrup, Denmark	nucleus	1) ≥50% tumour cells

HIER – heat-induced epitope retrieval

Table 2 - Patterns of expression of various markers by extent and intensity in OSCC cases

	Extension				*Intensity*			
Marker*	**0**	**1+**	**2+**	**3+**	***0***	***low***	***moderate***	***strong***
EGFR	3	3	4	53	3	10	28	22
p53	25	10	5	25	18	13	15	19
p16	44	8	3	6	42	5	9	5
p27	6	3	18	30	6	11	34	6
Cyclin A	11	29	15	10	3	16	23	23
Cyclin D	7	6	13	39	1	9	30	25
Cox-2	21	9	7	26	19	11	23	10
Bcl-2	32	5	4	3	30	9	1	4
VEGFR-1	24	4	10	24	22	23	12	5
VEGFR-2	5	4	7	44	5	21	22	12
Ki-67 **	3	9	23	29	-	-	-	-

*Some cases were lost due to TMA cores lost; ** evaluated only by percentage of stained cells

Table 3 – Cancer-specific survival by various markers at 3 years (univariate analysis) of the 67 OSCC

Factor	**Group** (Extension) (Intensity)	**N**	**death**	**CCS**	***P-value***
EGFR	0-10% or ≥10% low intensity	13	5	69.2	
	≥10% moderate or strong	50	27	47	0.116
	membrane	39	15	63.1	
	membrane+cytoplasm	21	15	26.5	***0.003***
p53	0-49%	40	17	69.6	
	50-100%	25	17	29.5	***0.001***
	Absent/low	31	14	67.4	
	Moderate/strong	34	20	43.1	0.113
p16	0-9%	44	24	49.6	
	10-100%	17	7	63.7	0.155
	Absent/low	47	27	46.0	
	Moderate/strong	14	4	77.9	***0.019***
p27	0-24%	9	4	66.7	
	25-100%	48	25	51.3	0.233
	Absent/low	17	11	41.2	
	Moderate/strong	40	18	59.2	0.512
Cyclin A	0-24%	40	17	69.4	
	25-100%	25	17	30.8	***0.005***
	Absent/low	19	12	51	
	Moderate/strong	46	22	55.9	0.703
Cyclin D	0-24%	13	6	67.7	
	25-100%	52	28	51.2	0.219
	Absent/low	10	5	58.3	
	Moderate/strong	55	29	53.9	0.572
Cox-2	0-24%	30	17	49.1	
	25-100%	33	15	56.8	0.409
	Absent/low	30	17	49.3	
	Moderate/strong	33	15	56.5	0.335

Bcl-2	0-9%	32	15	52.9	
	10-100%	12	9	48.6	0.658
	Absent/low	39	20	53	
	Moderate/strong	5	4	40	0.375
VEGFR-1	0-24%	28	14	59.9	
	25-100%	34	17	52.1	0.568
	Absent/low	45	24	52.8	
	Moderate/strong	17	7	64.2	0.534
VEGFR-2	0-24%	9	4	55.6	
-	25-100%	51	27	51.8	0.635
	Absent/low	26	14	50	
	Moderate/strong	34	17	54.3	0.939
Ki-67	0-49%	35	16	65.5	
	50-100%	29	17	44.0	0.111

CCS – cancer-specif survival

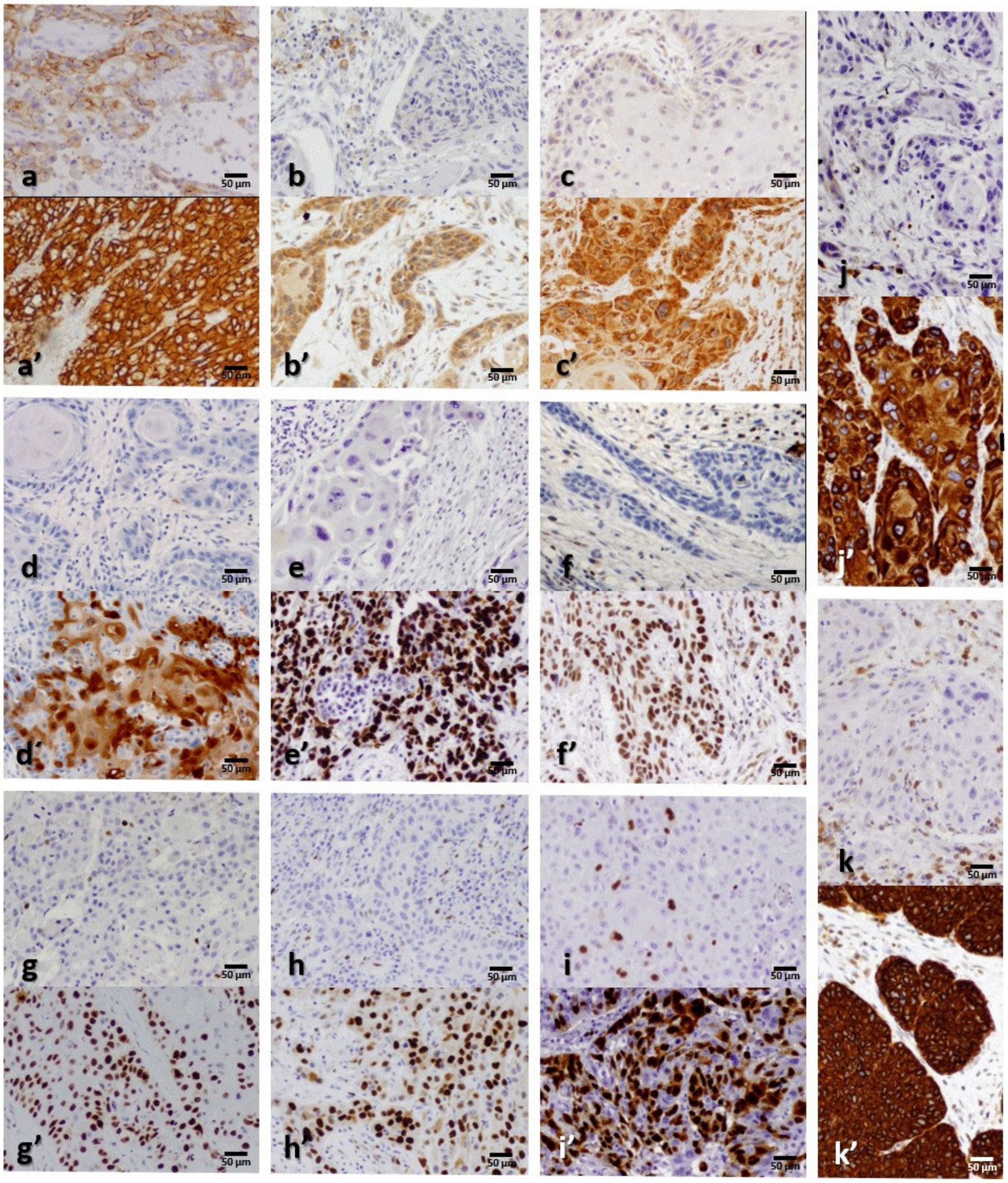
Table 4 – Cancer-specific survival at 3 years of the 67 OSCC (multivariate analysis)

Cancer-specific survival (all cases)			
	Exp (β)	***P***	**95% CI of Exp (β)**
Clinical stage	2.769	0.003	1.416-5.417
T category	1.057	0.914	0.391-3.238
N category	1.125	0.827	0.391-3.238
Perineural permeation	1.720	0.267	0.661-4.476
Tumour location (T/FOM/others)	1.149	0.460	0.795-1.662
Immunohistochemical score	3.696	0.001	1.733-7.884

Cancer-specific survival (stage I+II)

	Exp (β)	***P***	**95% CI of Exp (β)**
Clinical stage (I vs II)	1.468	0.679	0.239-9.030
Perineural permeation	5.139	0.105	0.710-37.172
Tumour location (T/FOM/others)	0.889	0.775	0.398-1.988
Immunohistochemical score	7.987	0.025	1.300-49.071

CI – confidence interval; **T** – tongue; **FOM** – floor of the mouth


a
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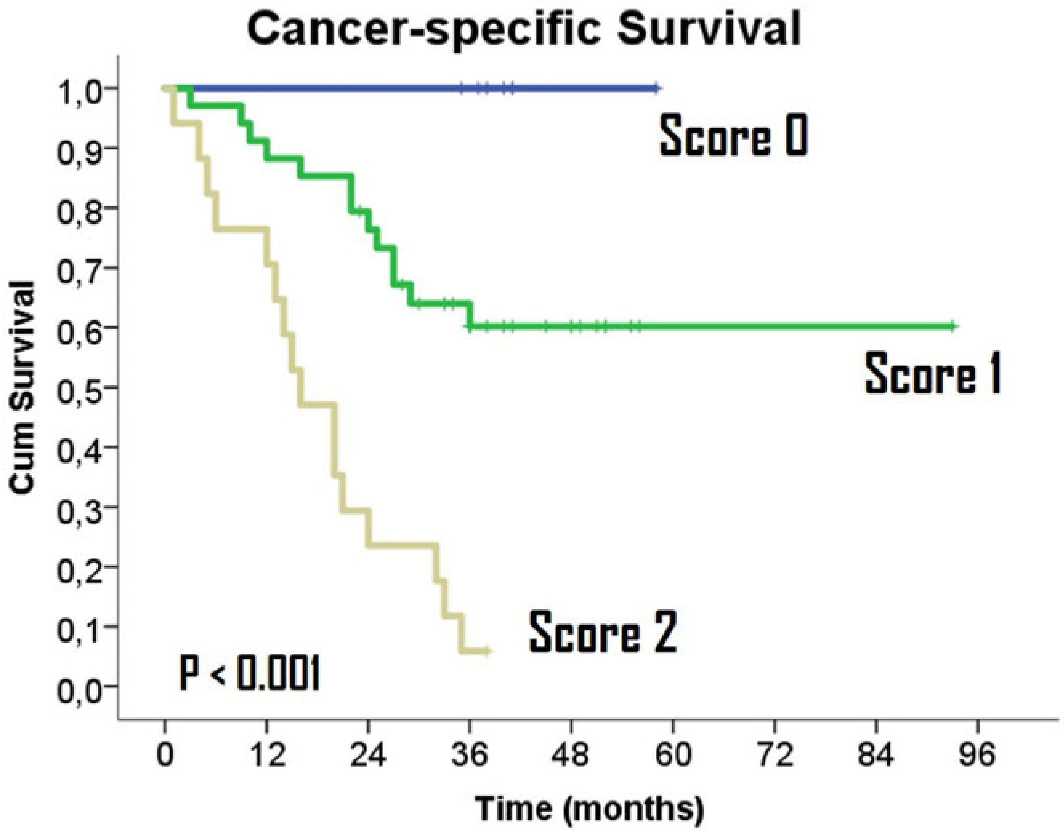
Cancer-specific Survival
Cum Survival
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Score 0
Score 1
Score 2
P < 0.001
0
12
24
36
48
60
72
84
96
Time (months)