Quantitative determination of c-myc facilitates the assessment of prognosis of OSCC patients

M. PÉREZ-SAYÁNS[1*], J.M. SUÁREZ-PEÑARANDA[2*], E. PADÍN-IRUEGAS[3], P. GAYOSO-DIZ[4],
M. REIS-DE ALMEIDA[1], F. BARROS-ANGUEIRA[5], P. GÁNDARA-VILA[1],
A. BLANCO-CARRIÓN[1] and A. GARCÍA-GARCÍA[1]

[1]Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela; [2]Department of Pathology and Forensic Sciences, University Hospital and School of Medicine of Santiago de Compostela; [3]Laboratorio Oncología Traslacional, Fundación Idichus, Hospital Clínico Universitario de Santiago de Compostela; [4]Clinical Epidemiology and Biostatistics Unit, Hospital Clínico Universitario de Santiago de Compostela, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela; [5]Unidad de Medicina Molecular, Fundación Pública Galega de Medicina Xenómica, Edificio de Consultas Planta-2, Hospital Clinico Universitario, Santiago de Compostela, Spain

Abstract. Myc genes are a family of proto-oncogenes whose proteins are implicated in the regulation of cell proliferation, differentiation and apoptosis, and in regulating the activity of genes involved in cell division. The aim of the present study was to establish a quantitative description of the expression of c-myc and evaluate its relationship with other clinical and prognostic factors, as well as to establish a multivariate survival prediction model. This is a retrospective study of 68 patients diagnosed with oral squamous cell carcinoma (OSCC). We constructed a tissue microarray for investigating the expression of c-myc by immunohistochemistry. Statistical analyses were carried out, and a multivariate model that predicts survival was established. The average expression of c-myc was 50.32 (SD, 26.05) with a range from 6.60 to 99.48; similar for initial and advanced tumor stages. Non-smoking patients had higher levels of c-myc, showing statistically significant differences (Kruskal-Wallis χ^2=5.975; P=0.05). We found no statistically significant relationship between the quantitative expression of c-myc and any other clinical or pathological parameters. For each unit of increase of c-myc, the risk increased by 1.15 (P<0.001; HR, 1.150; 95% CI, 1062-1245). Further study of this protein, which may have a significant diagnostic, prognostic and therapeutic value is warranted. Its determination can be valuable when used together with other markers to assess the prognosis of OSCC patients.

Introduction

An oral squamous cell carcinoma (OSCC) is a tumor characterized by multiple multistep genetic alterations, that lead to genomic instability and disordered cell growth due to oncogene overexpression, subexpression of tumor-suppressor genes and other genetic, epigenetic and microRNA alterations (1-5). The two most studied oncogenes (dominant) in human solid tumors are HER-2/neu and c-myc; while p53 is a tumor-suppressor gene involved in almost all human malignancies (6).

Myc genes are a family of proto-oncogenes comprised of several members (L-myc, N-myc and c-myc). Myc proteins are involved in the regulation of cell proliferation, differentiation and apoptosis, and in regulating the activity of genes involved in cell division (7). Contrary to classical theory, c-myc is also implicated in the control of apoptotic phenomena, possibly leading to tumor regression depending on cell type, cell interactions, extracellular matrix and neighboring cells (3,8,9). The c-myc protein acts as a transcription factor (10), and ectopic expression of this protein is sufficient to induce the progression of the cell cycle. c-myc is also related to a poor tumor prognosis (11-14) as well as the self-renewal of tumor stem cells (15-18).

The aim of the present study was to establish a quantitative description of the expression of c-myc and to evaluate its relationship with clinical and prognostic factors of OSCC, as well as to establish a multivariate survival prediction model.

Materials and methods

Patients. We performed an observational study on a cohort of 68 patients diagnosed with OSCC who were surgically treated

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Correspondence to: Dr Mario Pérez-Sayáns, Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Entrerríos s/n, C.P. 15782, Santiago de Compostela, Spain
E-mail: perezsayans@gmail.com

*Contributed equally

at the Maxillofacial Surgical Unit of the Santiago Teaching Hospital (Hospital Clinico Universitario de Santiago), Galicia, Spain, between January 2001 and March 2010. The inclusion criteria was established according to the surgical treatment in compliance with standard procedures, including resection of the primary tumor; radical, selective ipsilateral or bilateral removal of the regional lymph glands; clinical and pathological data and the availability of sufficient paraffin-embedded material to construct matrices. The clinical and pathological variables of each case included age, gender, tumor location, tumor stage, smoking habits, drinking habits, recurrence, dysplasia in the adjacent margin and vital status (death, by any cause) until February 2011.

The sample consisted of 35 men (51.5%) and 33 women (48.5%), with an age range from 41 to 96 years (average age, 67±13.08 years). Tumors were classified according to tumor stage at the time of diagnosis in accordance with the 7th Edition of the AJCC Cancer Staging Manual by the American Joint Committee on Cancer (19). Informed consent for use of the samples and data analysis were obtained from each patient or caretaker.

Tissue microarray generation. Hematoxylin and eosin-stained (H&E) slides were available for review in all cases. Paraffin blocks were selected on the basis of the availability of suitable formalin-fixed, paraffin-embedded tissue (at least 1-mm thick). For characterization of immunohistochemical protein expression, we constructed five different tissue arrays containing representative areas of every tumor. After microscopic evaluation, two areas of each tumor were selected, avoiding necrosis and keratin pools. Each tissue array was assembled as previously described (20). Briefly, two 1.5-mm-diameter cylinders of tissue were obtained from representative areas of each archival paraffin block and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD, USA). These tissues were fixed in 4% buffered formalin and were paraffin-embedded according to routine procedures. Areas chosen for the cylinder core were representative of the tumors. In addition, normal tonsil samples were placed adjacent to the tumoral tissues to serve as internal controls and to ensure the quality of staining in the slides. Initial sections were stained with hematoxylin and eosin to verify histopathologic findings.

Immunohistochemistry. Tissue sections (3-μm) from the TMA blocks were sectioned and applied to special immunohistochemistry coated slides (Dako, Glostrup, Denmark). Immunohistochemical analysis for c-myc was performed using a monoclonal antibody (clone 9E11; Novocastra, Newcastle, UK) with a concentration 1:100, according to the manufacturer's instructions. In brief, antigen retrieval was performed for 10 min at 95-99°C in a water bath, with a citrate buffer, pH 9.0. After blocking endogenous peroxidase activity, the slides were incubated for 30 min with the c-myc antibody. A secondary antibody reagent (polymer-based goat anti-mouse antibody fragment conjugated to horseradish peroxidase) was applied for 30 min. After applying the chromogenic visualization step using the 3,3'-diaminobenzidine chromogen, slides were counterstained with hematoxylin. Negative controls were performed using the negative reagent control (isotype control antibody). Dysplasia was graded as mild, moderate, severe and carcinoma '*in situ*', according to the criteria of the WHO (21).

Evaluation of immunohistochemical results, image digitizing and semiquantitative analysis. Cases showing cytoplasmic or nuclear positivity for c-myc were considered as positive. Slides were digitized using an automated slide scanner to produce high-resolution images for visual quantitative analysis using an ACIS® III automatic image analysis system (Dako). The digital images of the slides were captured by the ACIS scanner at low power magnification, and the entire slide images were viewed on a monitor. The regions with the highest immune percentage of positive cells were selected for further automatic scoring. A minimum of three of these areas containing only tumor cells was selected manually for quantitative evaluation. The final score was the average result of the different areas measured. To confirm the accuracy of the measurements, selected areas from 10 of the specimens were measured three times.

Statistical analysis. Qualitative variables are expressed as frequencies and percentages; quantitative variables are expressed as means (standard deviation) and ranges. The χ^2 test or Fisher's exact test was used as required to compare the qualitative variables. ANOVA or the Kruskal-Wallis test was used to contrast quantitative and qualitative variables. An association was considered statistically significant when the P-value was ≤0.05. The Kaplan-Meier method/estimator and the Cox regression model were used for evaluating survival in the study sample.

Initially, we carried out a univariate analysis in which we studied the Kaplan-Meier curves of each of the categorical/ qualitative variables to assess if we detected differences in the survival between the different groups or levels of these variables. Subsequently, we adjusted the Cox regression univariate models to assess the influence of each of the analyzed variables on the survival prognosis.

A time-dependent multivariate Cox regression mode was fitted to jointly evaluate the possible risk factors in terms of survival. The recurrence of the patients was considered as the time-dependent covariate. The predictive multivariate analysis/model was performed using a stepwise procedure in terms of the best (the lowest) Akaike Information Criterion (AIC) value. The magnitude of the association between covariates and survival was evaluated through hazard ratios (HR), together with their corresponding 95% confidence intervals (CI). Taking censored survival times into account, the log hazard ratios of the Cox model were used as criterion variables Y, to construct time-dependent ROC curves (22).

All statistical analyses were conducted using SPSS Statistics 17.0 and R 2.15.0 (R Development Core Team, 2012), using the survival package (for fitting parametric Cox regression models), the survivalROC package (for computing time-dependent ROC curves) and the censboot function (from the bootstrap package) for bootstrapping survival models with censored observations. The function stepAIC (from the MASS package) was also used for obtaining the multivariate model with the best AIC. All these packages are freely available at http://www.R-project.org.

Table I. Clinical characteristics of the OSCC patients, c-myc expression and association of variables.

| Characteristics	Patients n (%)	N=53 c-myc mean (SD)	P-value
Gender			
Male	35 (51.5)	44.40 (25.56)	0.092
Female	33 (48.5)	56.47 (25.58)	
Exposure to tobacco			
Non-smoker (never)	32 (47.1)	56.15 (5.00)	**0.05**[a]
Former smoker (>10 years)	24 (35.3)	52.43 (5.98)	
Current smoker (≥5 years)	12 (17.6)	32.69 (7.47)	
Exposure to alcohol			
Non-drinker	33 (48.5)	56.28 (25.32)	0.206
Not current drinker	13 (19.1)	52.18 (26.06)	
Current drinker	22 (32.4)	42.03 (26.05)	
Primary location			
Buccal mucosa	6 (9)	40.05 (11.45)	0.208
Gums	17 (25.4)	40.99 (26.53)	
Retromolar trigone	6 (9)	57.80 (32.77)	
Tongue	22 (32.8)	57.13 (24.58)	
Floor of mouth	8 (11.9)	39.77 (24.46)	
Soft palate	8 (11.9)	65.53 (25.38)	
T			
T1	21 (30.9)	56.00 (24.26)	0.286
T2	21 (30.9)	41.87 (25.06)	
T3	4 (5.9)	68.27 (11.00)	
T4	22 (32.4)	50.11 (28.66)	
N			
N0	55 (80.9)	49.22 (27.21)	0.462
N1	7 (10.3)	65.98 (12.44)	
N2	6 (8.8)	47.52 (21.80)	
Clinical stage			
I	20 (29.4)	56.01 (24.26)	0.450
II	12 (17.6)	42.06 (27.70)	
III	9 (13.2)	60.94 (20.13)	
IV	27 (39.7)	47.65 (27.54)	
Differentiation			
Good	30 (44.1)	51.06 (5.49)	0.970
Moderate	30 (44.1)	49.55 (5.24)	
Poor	8 (11.8)	50.45 (13.09)	
Recurrence			
Yes	29 (42.6)	50.39 (25.87)	0.979
No	39 (57.4)	50.19 (1.56)	
Dysplasia in margin			
No	38 (55.9)	51.46 (1.29)	0.822
Low	8 (11.8)	40.55 (2.94)	
Moderate	4 (5.9)	49.35 (20.29)	
Severe	1 (1.5)	68.64	
Carcinoma *in situ* (CIS)	17 (25)	51.91 (27.91)	

[a]Statistically significant.
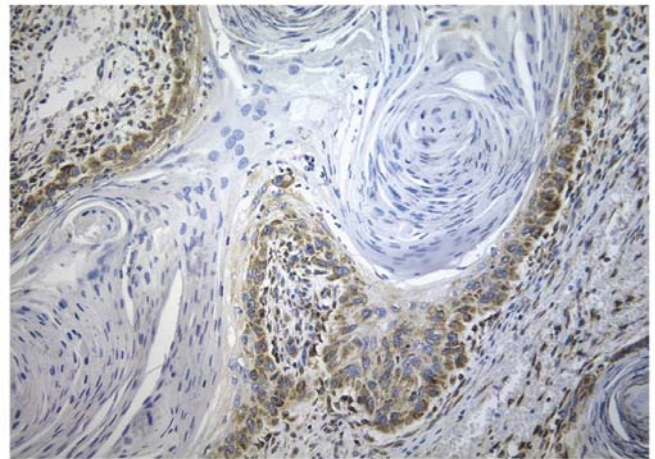
Figure 1. A case of oral squamous cell carcinoma showing moderate cytoplasmic staining while nuclei are only faintly positive. In this case, expression of the antibody was restricted to the lower layers of the tumor nests.

Results

Clinical, anatomical and pathological characteristics of the sample. The main clinical characteristics of the 68 patients selected for the present study are documented in Table I. Tumors were classified into initial stage (stages I and II), which accounted for 32 cases (47.8%), and advanced stage (stages III and IV), which amounted to 36 cases (52.9%). More men were diagnosed in early tumor stages [21 (65.6%)], whereas women were found more frequently in advanced stages at the time of initial diagnosis [22 (61.1%)] (χ^2=4.848, P<0.05).

In terms of the relationship with exposure to tobacco, no statistically significant differences in terms of staging at the time of diagnosis (χ^2=1.227, P=0.541) were noted. In regards to alcohol, of the 22 cases exposed to the carcinogen, 16 (72.7%) were diagnosed in early stages (χ^2=11.338, P<0.01).

The existence of dysplasia in the adjacent margin was positive in 42.6% of cases; 34.4% of the tumors in initial stages and 50% of the tumors in advanced stages (χ^2=2.176, P=0.337). The existence of CIS was slightly higher in advanced stage tumors [9 (25%)] in contrast to 21.9% found in initial cases, although the differences were not statistically significant.

c-myc expression in OSCC. Immunohistochemistry showed both nuclear and cytoplasmic staining in neoplastic cells, while in many cases, the intensity was higher in the cytoplasm than that in the nuclei (Fig. 1).

The average expression of c-myc (N=53) was 50.32 (SD, 26.05) with a range from 6.60 to 99.48; similar for the initial stages [mean 50.77 (25.94)] and advanced stages [49.94 (26.59)] (F=0.013; P=0.909). Women showed higher levels [56.47 (25.58)] than men [44.40 (25.56)] although there were no statistically significant differences (F=2.951; P=0.877). The patients showed virtually similar values of c-myc in terms of the degree of tumor differentiation (Kruskal-Wallis χ^2=0.062; P=0.970). Non-smoking patients had higher levels of c-myc [56.15 (5.00)], showing statistically significant differences (Kruskal-Wallis χ^2=5.975; P=0.05). We found no statistically significant relationship between the quantitative expression
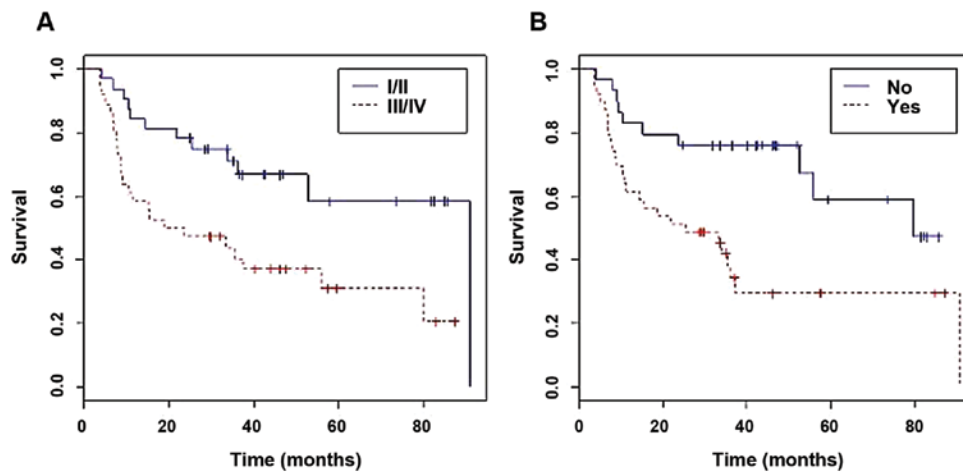
Figure 2. Kaplan-Meier survival curves. (A) The patients at initial stages exhibited prolonged survival (40.72 months) when compared with the patients in advanced stages (28.6 months). (B) The survival of the recurrence group (Yes) was reduced when compared with the survival of the non-recurrence group (No).
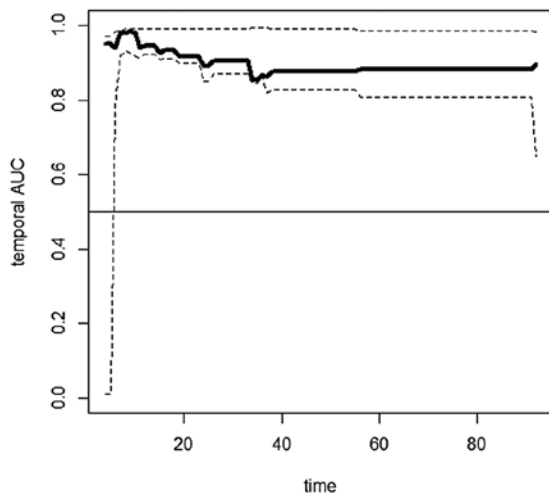

Figure 3. Temporary AUC shows an approximate mean value of 0.9 for the follow-up between 35 months and the end of the follow-up period, which indicates a good discrimination capacity of the Cox model.

of c-myc and any other clinical or pathological parameters (Table I).

Survival and follow-up analysis. The average follow-up was 33.5 months (CI, 28.23-40.38). Of the 36 (52.9%) patients who died, 24 (66.7%) were in the advanced stage group at the time of diagnosis (χ^2=5.785, P<0.05). The average survival of the sample was 50.52 months (95% CI, 41.07-59.96); according to the Kaplan-Meier curve, the cases at the initial stages had a higher survival rate (40.72 months) than those in advanced stages (28.6 months) and the differences were statistically significant (P<0.01) (Fig. 2A). Recurrence occurred in 39 cases (57.4%) during the follow-up period, regardless of the tumor stage at the time of diagnosis (χ^2=0.30, P=1.000). We detected statistically significant differences in the survival of patients with recurrence in contrast with those who did not relapse, showing a lower overall survival rate in the recurrence group (P<0.01) (Fig. 2B). Survival was also statistically significantly reduced in patients with any degree of dysplasia

in the adjacent margin, in contrast with those who did not have dysplasia (P<0.05).

The Cox univariate regression analysis verified that the effect of the value of c-myc alone was not statistically significant (P=0.735). In a multivariate Cox analysis, however, the multivariate with the lower ACI obtained included the covariates: recurrence, c-myc, gender, location, differentiation, stage, size, alcohol consumption, dysplasia in the adjacent margin, interaction between c-myc and recurrence, and the interaction between gender and stage (ACI=155.0655). All of the variables were statistically significant, except for gender, stage and dysplasia in the adjacent margin. Gender and stage were not significant, but their relevant interactions were (Table II). Using this model, we observed that patients with recurrence had a 8303.37-fold higher risk than those without recurrence (P<0.01; HR, 8303.37; 95% CI, 34.30-2010000). For each unit of increase of c-myc, the risk increased by 1.15 (P<0.001; HR, 1.150; 95% CI, 1062-1245). Patients with tumors located in the gum presented a 278.86-fold higher risk (P<0.001; HR, 278.86; 95% CI, 13.76-5653), followed by tumors in the tongue, with a risk of 16.24 (P<0.05; HR, 16.24; 95% CI, 1.37-193) when compared to tumors located in the buccal mucosa. Patients with moderate differentiation presented a risk 8.87-fold higher (P<0.01; HR, 8.87; 95% CI, 2.01-39.13) than those with well-differentiated tumors. We found the same results for poorly differentiated tumors, which showed a risk 7.48-fold higher (P<0.05; HR, 7.48; 95% CI, 1.06-52.73). In patients who were former drinkers, the risk decreased by 0.02 (P<0.001; HR, 0.02; 95% CI, 0.02-0.19).

Temporary AUC showed an approximate mean value of 0.9 for the follow-up between 35 months and the end of the follow-up period, which indicates a good discrimination capacity of the Cox model (Fig. 3).

Discussion

The influence of c-myc in the carcinogenic process, in general, has been previously described in many tumors. In the specific case of oral tumors, Goessel *et al* (23) developed a cellular model of oral-esophageal carginogenesis, in which cyclin D1

Table II. Final multivariate time-dependent Cox model including the studied variables.

Variables	HR	95% CI	P-value
Recurrence			
No	1		
Yes	8303.372	34.30-2010000	<0.01[b]
c-myc	1.150	1.062-1.245	<0.001[c]
Gender			
Male	1		
Female	0.4011	0.4416-3.643	<0.01[b]
Alcohol			
No	1		
Currently	1.90617	0.3871-9.386	0.428
Not currently	0.02146	0.024-0.1927	<0.001[c]
Tumor location			
Buccal mucosa	1		
Gums	278.8612	13.76-5653	<0.001[c]
Trigone	1.3314	0.0581-30.51	0.858
Tongue	16.2401	1.367-193.0	<0.05[a]
Floor of mouth	21.1731	0.5043-14.62	0.103
Soft palate	2.7970	0.1563-50.04	0.485
Tumor size			
T1	1		
T2	27.517	1.865-406.1	<0.05[a]
T3	0.013	0.0002-0.6301	<0.05[a]
T4	0.027	0.0121-0.6142	<0.05[a]
Differentiation			
Good	1		
Moderate	8.8741	0.1563-50.04	<0.01[b]
Poor	7.4756	1.060-52.73	<0.05[a]
Tumor stage			
I/II	1		
III/IV	0.8587	0.5043-14.62	0.916
Dysplasia in adjacent margin			
Without dysplasia	1		
Dysplasia	2.6953	0.6047-12.01	0.194
CIS	0.3277	0.0542-1.981	0.224
c-myc and recurrence			
No	1		
Yes	0.89792	0.8377-2221	<0.01[b]

Risk from c-myc linear modeling. HR, hazard ratio. [a]Statistically significant at level 0.05; [b]statistically significant at level 0.01; [c]statistically significant at level 0.001. CIS, carcinoma *in situ*.

overexpression and inactivation of p53 lead to the immortalization of keratinocytes. Additionally, overexpression of ectopic epidermal growth factor receptor (EGFR) and c-myc, and the resulting malignant reactivation of telomerase induced by EGFR, are sufficient for the malignant transformation of oral epithelial cells. Thus, this demonstrated the importance of the overexpression of this gene in OSCC (24,25).

Several studies have analyzed c-myc expression in this type of tumor showing different results, with an average positivity of 41.28% (2.4-75%) between the different studies (6,26-34). The results are extremely contradictory, as well as the variation in the quantification methods. In the present study we effectively developed a quantitative evaluation.

Shah *et al* (6) found a significantly higher expression in T3/T4 tumors in comparison with T1/T2 tumors, although we were unable to verify this fact in our research. On the other hand, c-myc was correlated with tumor stage and lymphatic permeation, while in our study the expression was practically the same. Rodrigo *et al* (35) found no relationship between c-myc overexpression and tumor prognosis, similar to Hayry *et al* (33). Baral *et al* (27) found that tumors with positive p53 and c-myc were in the advanced stages of the disease (poorly differentiated, stage III), while OSCCs in early stages did not show positive immonoreactivity for p53 and c-myc.

Eversole and Sapp (36) studied c-myc levels in precancerous lesions and early cancerous lesions, and found that, in cases of dysplasia, *in situ* carcinoma and carcinoma, c-myc nuclear tinting was dominant in all the strata harboring atypical cells and the degree of tinting increased as the levels of atypia were higher. Shah *et al* (30) described an odds ratio (OR) of 6 in the transformation from hyperplasia to dysplasia, for c-myc (+) lesions and an OR of 3 for progressions beyond early stage carcinoma.

Vora *et al* (28), in a multivariate analysis, found that c-myc multiexpression is a clear indicator of poor prognosis, which can be used to evaluate specimens, paired with clinical staging, to determine locally advanced tumors. However, Tsuzuki *et al* (37), found no correlation between c-myc and 5-year survival of patients with oral and oropharangeal carcinomas. In our multivariate model, the increase in c-myc levels was positively statistically correlated with the risk of death.

The role of c-myc in carcinogenesis has been well described; however, its relationship with OSCC and clinical and pathological variables of tumors is not entirely clear. Thus, further study of this protein, which may have significant diagnostic, prognostic and therapeutic value is warranted. The determination of c-myc can be valuable when used together with other markers to assess the prognosis of OSCC patients.

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