

## TGF- $\beta$ 1 AND ITS ASSOCIATION WITH CLINICOPATHOLOGICAL FEATURES, PROLIFERATIVE ACTIVITY AND PROGNOSIS IN ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY

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### ABSTRACT

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**Introduction:** The prognostic value of transforming growth factor beta-1 (TGF- $\beta$ 1) in oral cancer remains unclear. Therefore, the aim of this study was to evaluate TGF- $\beta$ 1 expression in oral squamous cell carcinoma (OSCC) samples and its association with clinicopathological data, tumor proliferative activity, and patients' prognosis.

**Methods:** A total of 68 patients with histopathological diagnosis of OSCC were included, as well as 9 cases of normal oral mucosa for comparison purposes. The OSCC sample was categorized according to patients' outcomes in favorable prognosis (n=30) or unfavorable prognosis (n=38). Immunohistochemical staining for TGF- $\beta$ 1 and Ki-67 were performed. The slides were semi-quantitatively and quantitatively evaluated for TGF- $\beta$ 1 and Ki-67, respectively.

**Results:** TGF- $\beta$ 1 was significantly increased in OSCC compared to normal oral mucosa (<0.01). An inverse correlation was found between TGF- $\beta$ 1 and Ki67 staining in OSCC (p=0.01). No association was found between TGF- $\beta$ 1 expression and OSCC clinicopathological features, prognosis or survival.

**Conclusions:** TGF- $\beta$ 1 had no prognostic value and appears to maintain its suppressive role concerning cell proliferation.

**Keywords:** *Head and neck neoplasms; prognosis; transforming growth factors*

Oral squamous cell carcinoma (OSCC) represents around 95% of all malignant tumors that affect the mouth<sup>1</sup>. This malignancy still demonstrates poor prognosis, with five-year survival rates of less than 50%<sup>2,3</sup>. Different aspects have been evaluated as OSCC prognostic factors, including clinical features, such as tumor location, TNM classification and type of treatment, and histological aspects, such as the histological grade. Nevertheless, in many cases tumors with similar clinical or histological aspects present different response to therapy leading to different outcomes. Thereby, the search of biomarkers, such as protein involved in carcinogenesis, can increase the ability to predict OSCC aggressiveness and prognosis. The identification of such proteins might reveal, for example, a subset of patients that require more aggressive/additional therapies, such as radiotherapy, chemotherapy, and targeted therapies. In this scenario, the analysis of growth factors, such as transforming growth factor beta-1 (TGF- $\beta$ 1), its receptors and biochemical events activated by them might improve prognostic information regarding OSCC patients.

The TGF-beta superfamily includes more than 100 different proteins, from which more than 40 have been described in mammals. Three TGF-beta isoforms in mammals ( $\beta$ 1,  $\beta$ 2 and  $\beta$ 3) induce a number of outcomes in physiological

events<sup>4</sup>, regulating cell proliferation, migration, differentiation and reparative processes<sup>5</sup>. Although TGF- $\beta$ 1 acts by inhibiting epithelial cell proliferation, paradoxically this protein is found in high concentrations in tumour cells, contributing to cancer progression and facilitating the migration and metastasis in later stages of carcinogenesis<sup>6</sup>. High concentrations of this growth factor were found in colorectal<sup>7</sup>, gastric<sup>8</sup>, renal cell<sup>9</sup> and head and neck carcinomas<sup>10</sup>. Moreover, data from clinical studies demonstrated that higher TGF- $\beta$ 1 immunohistochemical expression in tumor samples is associated with disease progression in breast cancer<sup>11</sup>, and poor prognosis in pancreatic<sup>12</sup> and hepatocellular carcinomas<sup>13</sup>. The tumour-promoting profile of TGF- $\beta$ 1 in malignant cells could indicate that this protein loses its ability to inhibit cell proliferation and starts to promote it instead. In tissue samples, Ki67 is considered a gold standard for assessing proliferation once it is expressed through all active phases of the cell cycle (G1, S G2 and mitosis) and is absent in resting cells (G0)<sup>14</sup>.

Our group has recently evaluated the pattern of TGF- $\beta$ 1 immunohistochemical expression during lip<sup>15</sup> and oral carcinogenesis<sup>16</sup> and has observed that this growth factor plays an important role during the progression from normal mucosa through potentially malignant disorders to cancer. Concerning the prognostic value of this marker in oral cancer, some conflicting results can be observed in the literature. While some authors describe no association of TGF- $\beta$ 1 with patients' survival<sup>17</sup>, others have demonstrated that this protein is capable of predicting poor survival rates during follow up<sup>18</sup>. Therefore, the aim of the present study was to identify the role of TGF- $\beta$ 1 immunostaining as a prognostic marker of OSCC. Moreover, we investigated the correlation of TGF- $\beta$ 1 immunostaining with important clinical and demographic characteristics and with the tumor's proliferative index.

## METHODS

This transverse observational study was conducted according to ethical criteria of the Declaration of Helsinki and was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (HCPA) - CAAE: 02083012.2.0000.5327).

### Sample

Patients diagnosed with primary OSCC (CID.C02) from January 2001 to December 2009, in the Pathology Service of Hospital de Clínicas de Porto Alegre were included in this study. Information regarding demographic data, risk factors, clinical aspects of the tumour, type of treatment, and outcomes such

as recurrence and survival were collected from the medical records. The clinical stage was also collected from medical records. This classification was made based on the 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual, Head and Neck Section<sup>19</sup>. The follow-up period was defined from the date of diagnosis until the last visit to the hospital, or the date of death. Patients were categorized in favourable or unfavourable prognosis according to the outcomes in a 5-year analysis. Patients who experienced recurrence or deceased because of the tumour during this period were classified as presenting unfavourable prognosis.

The inclusion criteria consisted in a minimum of 75% of the information available in the medical records associated with paraffin blocks available for study. The exclusion criteria included very extensive cases of OSCC in which the site of origin was not possible to identify or cases occurring in the oropharynx or lip.

Nine cases of normal oral mucosa were included for comparison purposes.

### Histopathological Analysis

The OSCC histological sections were stained with hematoxylin and eosin (H&E) and further classified according to the criteria described by Bryne et al.<sup>20</sup> and validated by our group<sup>21</sup>. The histopathological grade was held by a consensus between two experienced pathologists blinded to all clinical data and prognosis.

### Immunohistochemistry Analysis

Histological sections of 3 $\mu$ m on silanized slides were deparaffinised in xylene, rehydrated in alcohol and immersed in 0.3% hydrogen peroxide solution in methanol to block endogenous peroxidase. After antigen retrieval, slides were incubated with Ki67 (MIB-1; Dako, 1:50) and TGF- $\beta$ 1 (Santa Cruz, sc-146, 1:100) for 1 and 18 hours, respectively. The detection system used was the polymeric type (Envision + Dako, Carpinteria, CA, USA). The reaction was revealed using the chromogen solution containing 0.03% 3-31-diaminobenzidine (DAB, Dako Cytomation, USA) and counter stained with Mayer's hematoxylin solution. All reactions were accompanied by positive controls according to the manufacturer's instructions. For negative controls, primary antibodies were replaced by bovine serum albumin (BSA) 1% diluted in Tris-HCl buffer, pH7.4.

All analyzes were performed by investigators blinded to the clinical aspects and prognosis. Only brown cytoplasmic staining for TGF- $\beta$ 1 and nuclear staining for Ki-67, regardless of staining intensity, were considered positive. A semi-quantitative analysis was performed on histological sections immunostained with TGF- $\beta$ 1 by the consensus of two pathologists

at a magnification of 400x using scores based on the percentage of positive tumor cells. Each case was classified in TGF- $\beta$ 1 low expression (0 to 50% of positive cells) or TGF- $\beta$ 1 high expression (>50% positive cells) according to Salvadori et al., 2014 modified classification<sup>15</sup>. The tumor proliferative labeling index (PLI) was determined by Ki-67 positivity. A quantitative analysis of histological sections was conducted according to Martinez et al., 2005<sup>22</sup>. Representative images from the invasive front of the tumor were captured using a conventional light microscopy CX41RF model (Olympus Latin America, Inc., Miami, Florida, USA) containing a QColor camera 5, Coolet, RTV (Olympus Latin America, Inc., Miami, Florida, USA) coupled and connected to a Dimension 5150 computer (Dell, Porto Alegre, RS, Brazil). The images were obtained using the QCapture software version 2.81 (Quantitative Imaging Corporation, Inc.; Surrey, DC, Canada) and analyzed with ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). The number of positive cells was evaluated in each case. A total of 1000 cells were counted in a 400x magnification and the result was presented as percentage of positive cells (mean and standard deviation).

### Statistical Analysis

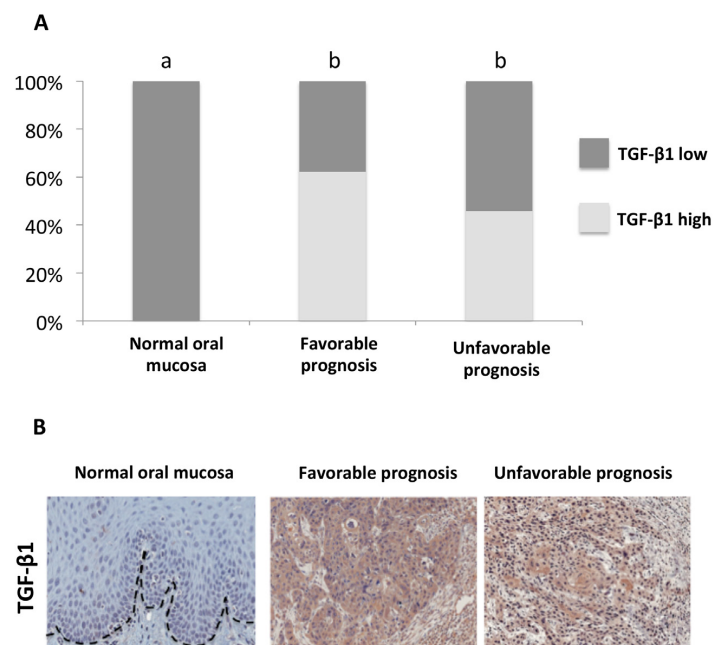
A descriptive analysis of the variables was initially performed, considering the mean, standard deviation, maximum, minimum for quantitative

variables, and frequency and percentage for qualitative variables. The association between independent variables and outcome was assessed using the chi-square test with SPSS 19. Spearman's correlation test was applied to investigate the correlation between Ki-67 and TGF- $\beta$ 1. Survival analysis was performed using the Kaplan-Meier method and survival curves were compared using the log-rank test. For all tests, statistical significance was established at  $p < 0.05$ .

## RESULTS

### Clinical and Demographic Characteristics of OSCC According to TGF- $\beta$ 1 Expression

All cases of normal oral mucosa were classified as low TGF- $\beta$ 1 expression. Among OSCC cases, 36 (53%) were classified as low expression of TGF- $\beta$ 1 and 32 (47.0%) as high expression of TGF- $\beta$ 1. There was a significant higher TGF- $\beta$ 1 expression in OSCC compared to normal mucosa ( $p < 0.01$ ). The proportion of patient with high TGF- $\beta$ 1 immunorexpression was greater in the unfavorable prognosis group, however no significant difference was observed between OSCC cases with different prognoses ( $p=0.22$ ) (Figure 1 A and B). TGF- $\beta$ 1 immunostaining was also not associated with any demographic, clinical or histopathological characteristics of the sample (Table 1).



**Figure 1:** TGF- $\beta$ 1 is increased in oral squamous cell carcinoma (OSCC) compared to normal oral mucosa. Percentage of cases presenting high ( $\geq 50\%$  of positive cells) or low ( $< 50\%$ ) TGF- $\beta$ 1 expression. Different letters denote significant difference (chi-square test,  $p < 0.05$ ). (A). Representative examples of TGF- $\beta$ 1 expression in normal oral mucosa and OSCC according to prognosis (original magnification x200) (B).

**Histopathological Analysis**

Table 2 shows the histopathological grade according to TGF-β1 expression. Most cases classified as TGF-β1 low expression had a moderate malignancy grade (Grade II). Analyzing the distribution within cases classified as TGF-β1 high expression, we observed that most cases had a low malignancy grade (Grade I). Despite this tendency, no significant difference was observed between groups (p=0.06).

**Proliferation Analysis**

Ki-67 labelling was detected in all cases of normal oral mucosa and OSCC. The average of Ki-67 was 8.6 (± 2.76) for normal oral mucosa, 50.93 (±16.83)

for OSCC with favourable prognosis and 50.85 (±19.69) for unfavourable prognosis. Spearman's correlation coefficients were calculated to determine whether TGF-β1 could be implicated in changes in cell proliferation. Epithelial TGF-β1 was inversely correlated with Ki-67 within OSCC cases (r=-0.094; p=0.009) (Figures 2A and B).

**Survival Analysis**

During the follow-up period, 31 (48.5%) patients presented recurrence with an average time of 22.13 months after diagnosis. Eighteen patients (26.47%) deceased because of the tumor with a mean age of 56.61 years and a mean time to death of 19.07 months

**Table 1:** Demographic and clinical characteristics of patients with oral squamous cell carcinoma (OSCC) according to TGF-β1 expression.

Demographic and clinical characteristics	TGF-β1 low	TGF-β1 high	p value*
Gender			
Male	28 (80.0%)	28 (90.3%)	0.07
Female	7 (20.0%)	3 (9.7%)	
Smoking			
Yes	27 (77.1%)	27 (87.1%)	0.50 <sup>#</sup>
No	0 (0%)	1 (3.2%)	
Unknown	8 (22.9%)	3 (9.7%)	
Alcohol			
Yes	20 (57.1%)	21 (67.7%)	0.55 <sup>#</sup>
No	4 (11.4%)	5 (16.1%)	
Unknown	11 (31.4%)	5 (16.1%)	
Pain			
Yes	22 (62.9%)	22 (71.0%)	0.52 <sup>#</sup>
No	5 (14.3%)	4 (12.9%)	
Unknown	8 (22.9%)	5 (16.1%)	
Site			
Mouth floor	9 (25.8%)	12 (38.7%)	0.38 <sup>#</sup>
Tongue	8 (22.9%)	5 (16.1%)	
Palate	12 (34.3%)	11 (35.5%)	
Retromolar	5 (14.3%)	2 (6.5%)	
Buccal mucosa	1 (2.9%)	0 (0.0%)	
Alveolar ridge	0 (0.0%)	1 (3.2%)	
TNM			
I/II	5 (14.3%)	4 (12.9%)	0.56 <sup>#</sup>
III/IV	28 (80.0%)	26 (83.9%)	
Unknown	2 (5.7%)	1 (3.2%)	
Prognosis			
Favorable	18 (51.4%)	11 (35.5%)	0.14
Unfavorable	17 (48.6%)	20 (64.5%)	

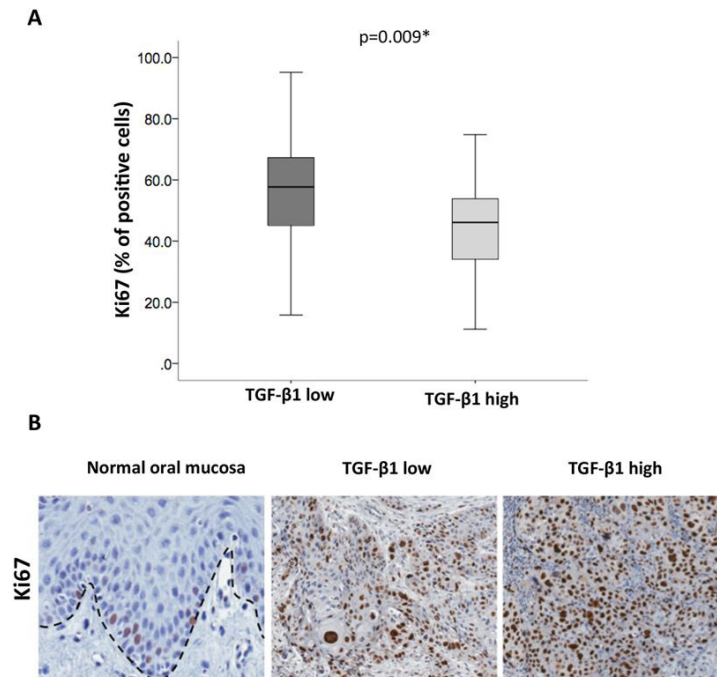
\*Chi-square test; <sup>#</sup>missing cases were excluded for statistical analysis; TNM = Tumour, Node, Metastasis clinical staging system.

**Table 2:** Histopathological classification of cases of oral squamous cell carcinoma (OSCC) according to Bryne histopathological grading system and TGF-β1 expression.

	TGF-β1 low	TGF-β1 high	p value*
Grade I	6 (17.1%)	13 (41.9%)	0.062
Grade II	18 (51.4%)	9 (29.0%)	
Grade III	11 (31.4%)	9 (29.0%)	

\*Chi-square test.





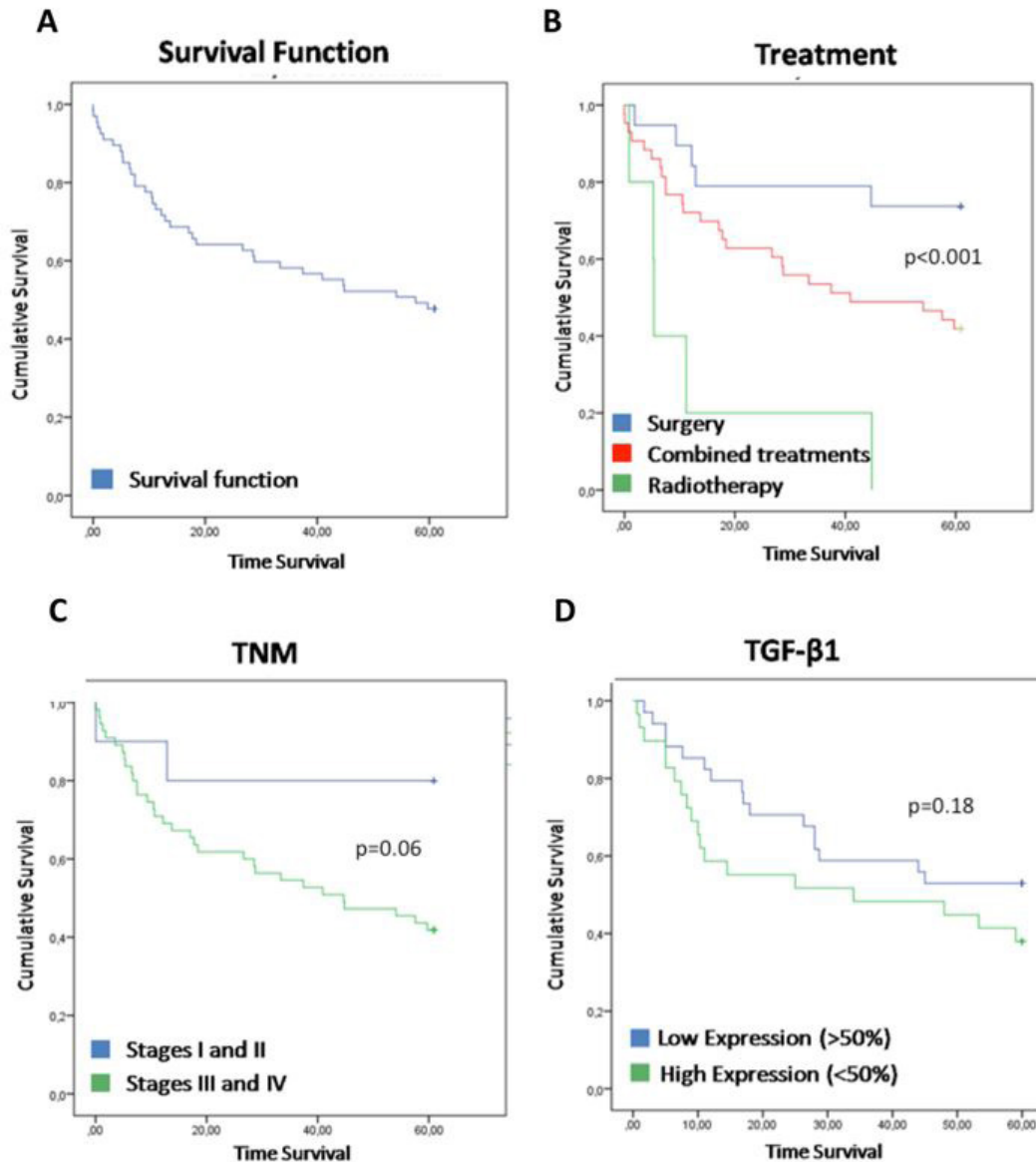
**Figure 2:** Cell proliferative activity is decreased in oral squamous cell carcinoma (OSCC) with TGF-β1 high expression. Box plot of Ki67 percentage of positive cells according to TGF-β1 expression. \*Spearman's correlation test (A). Representative examples of Ki-67 expression in normal oral mucosa and OSCC according to TGF-β1 expression (original magnification x200) (B).

after the diagnosis. Kaplan-Meier survival curves were constructed for all clinic-demographic, histopathological and immunohistochemical features. Only the type of treatment was significantly correlated with survival in the present sample ( $p < 0.001$ ). Nevertheless, differences in TNM and TGF-β1 led to a tendency of distinctive survival. Kaplan-Meier overall survival curve according to type of treatment, TNM and TGF-β1 expression are shown in Figure 3. We observed that patients who were treated with surgery alone presented better survival rates (Figure 3B). Patients diagnosed in stage I/II presented a tendency of better overall survival compared to patients diagnosed in advanced stages (III/IV) (Figure 3C). Likewise, a clear tendency of better overall survival was observed for patients who demonstrated low TGF-β1 expression (Figure 3D).

## DISCUSSION

The study of OSCC is extremely important due to its high morbidity and mortality<sup>1,23</sup>. Moreover, there is a lack of strong prognostic factors that can help out clinicians to recognize which patients might benefit for additional treatments, for example. This critical issue was confirmed in the present study, as we could observe that patients who evolved differently shared the same clinical and demographic features

at the time of diagnosis. The clinical stage (TNM), well recognized as an important prognostic factor in OSCC, was not significantly associated with patients' survival. This unexpected result can rely on the fact that the majority of our sample was composed by advanced cases (III/IV). This limitation is imposed by other alarming situation regarding OSCC: the majority of OSCC cases are diagnosed in late stages<sup>24</sup>, which is also the reality of the tertiary referral hospital from which the cases were selected. Moreover, the total number of patients included in the study can be pointed as a limitation. In a larger sample, probably the TNM stage would be significantly associated the survival, once the p value obtained herein was very close to the significance. Other limitation of the present study is due to its retrospective nature. Thus, the TNM system used herein is based on the 7th edition of the AJCC Staging Manual. Recently, an 8th edition was published and has been associated with an improved predictive value<sup>25</sup>. In the present sample, patients' survival was only significantly associated with treatment modality. This result supports that tumors which can be surgically removed, such as initial tumors, present a better prognosis and also highlight the problem of ineffective therapies for more advanced cases. Yet, there is a possible bias here associated with the therapeutic choice for initial and advanced cases. Larger and inoperable tumors



**Figure 3:** Kaplan-Meier analysis of oral squamous cell carcinoma (OSCC) overall survival (A) and according to type of treatment (B), Tumour, Node, Metastasis clinical staging system (TNM) (C) and TGF- $\beta$ 1 expression (D) (Log rank test).

or patients with disseminated disease have poor prognosis despite the treatment modality.

In the past decades, several biomarkers have been evaluated in OSCC in search of better understanding the tumor development and behavior<sup>26</sup>. Alterations in TGF- $\beta$ 1 signaling pathway can contribute to the development of many cancers<sup>27</sup> and appear to play a role during oral carcinogenesis<sup>16</sup>. TGF- $\beta$ 1 polymorphism has been associated with increased susceptibility to OSCC, and individuals who carry these alleles have a 2.73-fold increased risk of developing oral cancer<sup>28</sup>. In other types of malignancies, including renal, pancreatic and hepatic cancer, increased expression of TGF- $\beta$ 1 has already been associated

with poor prognosis<sup>9,12,13</sup>. In OSCC, the prognostic value of TGF- $\beta$ 1 remains unclear. A previous study conducted with a sample of OSCC patients from Iran demonstrated no association of TGF- $\beta$ 1 with patients' survival<sup>17</sup>. On the other hand, a study performed with patients from Taiwan found that this protein was capable to predict a shorter survival time of OSCC patients with advanced disease<sup>18</sup>. These conflicting results led us to conduct this study in a different sample and evaluate the prognostic value of TGF- $\beta$ 1. We observed that patients with unfavorable prognosis presented higher expression of this protein, however no significant difference was observed. In the same way, the Kaplan-Meier survival analysis revealed a

clear tendency of poor overall survival in patients presenting higher TGF- $\beta$ 1 expression. Our results demonstrate that statistically this growth factor is not a prognostic marker for OSCC. Nevertheless, from the observations of the present study allied to previously published ones, we believe that this protein plays an important role in OSCC development.

In normal conditions, TGF- $\beta$ 1 is a potent growth inhibitor, exerting a tumor suppressor role. A genetic loss of TGF- $\beta$ 1 signaling components or a downstream perturbation of the pathway confers to this growth factor a pro-tumorigenic function, associated with enhanced invasion and metastasis. Due to this dual role we decided to evaluate the effect of TGF- $\beta$ 1 expression on OSCC cell proliferation by correlating its levels with Ki-67 immunolabeling. Surprisingly, we found a significant inverse correlation between these proteins, suggesting that even in established cases of cancer, TGF- $\beta$ 1 might participate in cell proliferation suppression. We believe that TGF- $\beta$ 1 can maintain its function of cell proliferation suppression, but at the same time it presents a pro-tumorigenic role by promoting the epithelial-mesenchymal transition (EMT), a hallmark of cancer that is associated directly to cell invasion and tumor metastasis. As a matter of fact, TGF- $\beta$ 1 activity in oral cancer cells is associated with a decrease in E-cadherin expression and increase in vimentin expression<sup>18</sup> and TGF- $\beta$ 1 stimulus induces squamous cells to undergo a mesenchymal switch

and convert into a fibroblast phenotype<sup>29</sup>. Recently, a phase II clinical trial has demonstrated that in hepatocellular cancer TGF- $\beta$ 1 inhibitor reduces plasma levels of E-cadherin<sup>30</sup>.

In conclusion, we believe that TGF- $\beta$ 1 activity is associated with proliferative behaviour however this protein did not present a prognostic value in the present sample of OSCC. TGF- $\beta$ 1 appears to maintain its suppressive role concerning cell proliferation even in established cases of OSCC.

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### Conflict of interest

All authors state they have no potential conflicts of interest to declare.

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