This is a repository copy of *Different patterns of expression of cell cycle control and local invasion-related proteins in oral squamous cell carcinoma affecting young patients.*

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/119107/>

Version: Accepted Version

Article:

Miranda Galvis, M., Santos-Silva, A.R., Jardim, J.F. et al. (8 more authors) (2017) Different patterns of expression of cell cycle control and local invasion-related proteins in oral squamous cell carcinoma affecting young patients. *Journal of Oral Pathology and Medicine* . ISSN 0904-2512

<https://doi.org/10.1111/jop.12601>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

MISS MARISOL MIRANDA GALVIS (Orcid ID : 0000-0003-4798-584X)

DR. FELIPE PAIVA FONSECA (Orcid ID : 0000-0002-6657-4547)

Article type : Original Article

DIFFERENT PATTERNS OF EXPRESSION OF CELL CYCLE CONTROL AND LOCAL INVASION-RELATED PROTEINS IN ORAL SQUAMOUS CELL CARCINOMA AFFECTING YOUNG PATIENTS

RUNNING TITLE: PROTEINS EXPRESSION IN YOUNG WITH OSCC

Marisol Miranda Galvis, *DDS, MSc*¹

Alan Roger Santos-Silva, *DDS, PhD*^{1,6}

Juscelino Freitas Jardim, *DDS, MSc*¹

Felipe Paiva Fonseca, *DDS, PhD*²

Marcio A. Lopes, *DDS, PhD*¹

Oslei P. Almeida, *DDS, PhD*¹

Clóvis A. Lopes Pinto, *MD, PhD*³

Estela Kaminagakura, *DDS, PhD*⁴

Iris Sawazaki-Calone, *DDS, PhD*⁵

Paul M. Speight, *DDS, PhD*⁶

Luiz Paulo Kowalski, *MD, PhD*⁷

¹Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas, Piracicaba, Brazil.

²Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

³Department of Anatomic Pathology, A.C. Camargo Cancer Center, São Paulo, Brazil.

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.12601

This article is protected by copyright. All rights reserved.

⁴Departament of Bioscience and Oral Diagnosis, Science and Technology Institute, Univ Estadual Paulista, São José dos Campos, Brazil.

⁵Oral Pathology and Oral Medicine, Dentistry School, Western Paraná State University, Cascavel, Brazil.

⁶Academic Unit of Oral and Maxillofacial Pathology, School of Clinical Dentistry, The University of Sheffield, Sheffield, UK.

⁷Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Cancer Center, São Paulo, Brazil.

Correspondence

Dr. Luiz Paulo Kowalski, Department of Head and Neck Surgery and Otorhinolaryngology – A.C. Camargo Cancer Center. Rua Professor Antônio Prudente, 211, São Paulo 01509-900, Brazil. e-mail: lp_kowalski@uol.com.br. Fax: +55 11 21895172.

Abstract

Oral squamous cell carcinoma (OSCC) predominantly affects males in the fifth decade of life; nevertheless, an increased incidence in young patients has been reported worldwide and the clinical and behavioral characteristics of tumors in this group are controversial and the literature shows divergent results. **Purpose:** To investigate the clinicopathological features and prognostic significance of the immunoexpression of cell cycle and local invasion proteins in OSCC affecting young patients (≤ 40 years old). **Methods:** A tissue microarray was performed with 132 OSCC samples (61 cases of young patients vs. 71 cases of elderly patients) and submitted to immunohistochemical reactions with Ki67, p53, p16, Bcl-2, Cyclin D1, C-ErbB2, p21, Myc, EGFR, MMP-9, SMA, Cathepsin K and FGF-2 antibodies. **Results:** Clinicopathological features and survival rates were similar in both groups. Although overexpression of EGFR ($p = 0.042$) and MMP-9 ($p = 0.001$) was more frequent in young patients, only C-ErbB-2 ($p = 0.048$) and SMA ($p = 0.048$) expression correlated

with lower DFS in this group of patients. **Conclusion:** Clinicopathological features and survival rates are similar between younger and older patients with OSCC. The different patterns of C-ErbB2, EGFR, MMP-9 and SMA expression between the groups merits further investigation to understand their role in the early tumor onset in young patients.

Keywords: Oral squamous cell carcinoma; clinicopathologic characteristics; cell cycle proteins; local invasion proteins.

Introduction

Oral cancer is the sixth most prevalent human cancer¹, but it is the most common malignancy in some Asian countries² due to local cultural and social habits³. Oral squamous cell carcinoma (OSCC) is the most prevalent histological subtype (over 90% of cases) and typically affects males in the fifth and sixth decades of life, with a strong association with tobacco and alcohol use⁴. In the past, OSCC affecting patients younger than 40 years was uncommon, representing 4% of all patients⁵; however, recent epidemiological studies have demonstrated a higher incidence in this age group of up to 18.7%^{6,7,8}.

The clinical and behavioral characteristics of tumors in this group are controversial and the literature shows divergent results; in spite of a number of reviews suggesting the characteristics of the tumors in young people are the same as those found in the elderly^{9,10}, other studies describe important differences between the groups^{11, 12}.

A better understanding of the molecular basis of OSCC would contribute to our understanding of its biological profile and clinical behavior. Hence, analysis of known proteins of the cell cycle and local invasion that reflect the biological properties

Accepted Article

acquired during the complex development of tumors¹³, would determine if any significant difference exists between neoplasms affecting young and old subjects. Therefore, the aim of this study was to evaluate and compare the clinicopathological features and prognostic significance of the immunoexpression of a large panel of regulatory proteins involved in cell cycle control and local invasion in OSCC affecting young and elderly patients. We tested the hypothesis that OSCC from young patients presents different patterns of expression for cell cycle control and local invasion-related proteins when compared to OSCC of older patients.

Patients and methods

Tissue samples: Patients younger than 40 years and a control group older than 45 years, diagnosed with OSCC, were retrospectively retrieved from the archives of the A.C. Camargo Cancer Center (São Paulo - Brazil) over a 43-year period from 1968 to 2011. In addition, the young patients described by Santos-Silva, et al.¹⁴ in 2011 were included. The cutoff age of 40 years was used following previously published recommendations^{7, 9-11, 14}.

The original diagnoses were confirmed by reviewing 5- μ m-thick, H&E-stained slides and clinical data were retrieved from patient medical charts. The clinical stage was obtained according to Greene et al.¹⁵ and grouped as early (stages I and II) or advanced (stages III and IV). Histological differentiation was determined according to Barnes et al.⁴ as well differentiated (grade I), moderately differentiated (grade II) and poorly differentiated (grade III) tumors.

Tissue Microarray (TMA) construction: TMAs were created using the tissue microarrayer Beecher Instruments®, model MTA-I. (Silver Springs, MD, USA). Tumor at the invasive front region were selected and representative cylindrical cores of 1.0 mm

diameter were taken from each tissue block and arranged sequentially into a recipient paraffin block in duplicate¹⁶.

Immunohistochemistry: Sections were de-waxed with xylene and then re-hydrated through an ethanol series. After antigen retrieval endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Slides were incubated overnight with primary antibodies at 4°C. Antibody clones, dilutions, antigen retrievals, sources and positive controls are shown in **Table 1**. Slides were subsequently exposed to either Post Primary Block (NovoLink Max Polymer Leica Biosystems, UK) for 30 minutes at 37°C, or to avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit, DakoCytomation, USA). DAB chromogen (Diaminobenzidine Tetrahydrochloride, Sigma, St. Louis, USA) was used to visualize the reaction, counterstained with Carazzi's hematoxylin. Negative controls were obtained by omitting the primary specific antibody.

Immunohistochemical analysis: Slides were scanned, obtaining high-resolution images, using the Aperio Scanscope CS® Slide Scanner (Aperio Technologies Inc., Vista, CA, USA). All digital images obtained in .svs format were visualized with ImageScope software (Aperio Technologies Inc., Vista, CA, USA). Nuclear markers (Ki67, p53, Cyclin D1, p21 and Myc) were analyzed using the Nuclear Staining function, and the nuclear staining was classified as 0 (no staining), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong staining). Based on the percentages of nuclei classified as 1+, 2+ and 3+ the percentage of positive stained nuclei was expressed in the range 0–100%. The expression of nuclear markers was defined following the literature¹⁷⁻¹⁹ reporting positive expression when more than 10% of cells displayed nuclear staining and negative expression when immunoexpression was present in less than 10% of tumor cells.

Membrane (C-ErbB2 and Epidermal Growth Factor Receptor — EGFR) and cytoplasmic (Bcl-2, Matrix Metalloproteinase 9 — MMP-9, Anti-alpha Smooth Muscle Actin — SMA, Cathepsin K and Fibroblast Growth Factor 2 — FGF-2) markers were analyzed using the PixelCount V9 algorithm and staining was automatically quantified according to previously established input parameters²⁰. For statistical purposes, the median value of the final immunostaining results was used to split the cases into two groups, below and above the median, representing low and high expression levels respectively for each membrane and cytoplasmic marker analyzed²¹.

Semiquantitative analysis of SMA was jointly carried out by two observers and each case was classified as negative (0) (0 to 5% of stromal positivity) or positive (1) (>56% of stromal positivity)²².

Statistical analysis: Absolute and relative frequencies were established for clinicopathological features. Chi-square and Fisher tests were used to compare clinicopathological features between groups and the nonparametric Mann-Whitney test was used to compare the variables. Survival curves were acquired using the Kaplan-Meier method and a Log-rank test was carried out to evaluate the prognostic significance of the clinicopathological features and protein expression. The software SPSS Statistics, version 23.0 was employed for data analyses and a *p* value < 0.05 was considered statistically significant.

Ethical statement: The current study was performed in accordance with the ethical standards of the Human Research Ethics Committee of A.C Camargo Cancer Center (1957/14).

Results.

Sociodemographic and clinicopathological features: Sixty-one young patients (≤ 40 years old) and 71 elderly patients (> 45 years old) ($p = 0.001$) diagnosed with OSCC had complete clinicopathological information and representative tumor tissue samples for immunohistochemical analysis. Their sociodemographic and clinicopathological features are shown in **Table 2** and their correlation with expression of proteins in **Supplement 1**.

Survival analysis: Recurrences were detected in 24 young (57.1%) and 31 elderly patients (43.7%) ($p = 0.166$) and the disease-free survival (DFS) rate did not demonstrate a significant difference between the groups (27.3% in the young patients vs. 43.4% 5-year DFS in the control group) ($p = 0.104$). In the young patients, recurrence ranged from 0 to 41 months, with a mean time of 8.41 months, while in the control patients the recurrence time ranged from 0 to 163 months, with a mean time of 17.64 months.

Young patients with T3/T4 lesions demonstrated a lower DFS rate (19.7% vs. 53.5% 5-year DFS, $p = 0.040$). In the old groups a lower DFS was seen in tumors located at sites other than the tongue and floor of the mouth (8.5% vs. 53.5% vs. 58.8% 5-year DFS, $p = 0.038$), with T3/T4 lesions (33% vs. 58% 5-year DFS, $p = 0.028$), with poorly differentiated tumors (0% vs. 37.9% vs. 55.6% 5-year DFS, $p = 0.001$) and in tumors with positive surgical margins (37.5% vs. 53.5% 5-year DFS, $p = 0.002$). Considering all patients, those with T3/T4 lesions (26.8% vs. 57% 5-year DFS, $p = 0.001$) and positive margins demonstrated a lower DFS rate (23.1% vs. 44.5% 5-year DFS, $p = 0.001$).

The 5-year overall survival (OS) rate was 46.6% in the young patients group and 44.5% in the control group ($p = 0.681$). In the young group, mean survival was 27.45 months (Range 1–128 months), and in the control group it was 27.84 months (Range 0–172 months).

Patients with tumors located at sites other than the tongue and floor of the mouth (25.5% vs. 57.2% vs. 49.4% 5-year OS, $p = 0.043$), with T3/T4 lesions (23.9% vs. 74.9% 5-year OS, $p = 0.001$), with positive lymph nodes (40.1% vs. 77.8% 5-year OS, $p = 0.003$), advanced stage tumors (35.6% vs. 75.9% 5-year OS, $p = 0.001$) and with positive margins (0% vs. 52.1% 5-year OS, $p = 0.001$) demonstrated a lower OS rate. Regarding young patients only, those using tobacco (39.8% vs. 66.7% 5-year OS, $p = 0.034$), with T3/T4 lesions (24.7% vs. 74.3% 5-year OS, $p = 0.001$), advanced stage tumors (31.9% vs. 77.8% 5-year OS, $p = 0.001$) and with positive margins demonstrated (0% vs. 45.4% 5-year OS, $p = 0.001$) a lower OS rate. Considering old patients only, those affected by tumors located in sites other than the tongue and floor of the mouth (30.3% vs. 55.2% vs. 61.7% 5-year OS, $p = 0.010$), with T3/T4 lesions (22.8% vs. 75.2% 5-year OS, $p = 0.001$) and with positive surgical margins (0% vs. 59.1% 5-year OS, $p = 0.001$) demonstrated a lower OS rate.

Immunohistochemical expression of proteins: The immunohistochemical expression data of all evaluated proteins and comparisons between the groups of younger and older patients are presented in **Figure 1**. There were significant differences between young and old patients in the expression of EGFR ($p = 0.042$) and MMP-9 ($p = 0.001$).

EGFR was expressed in the membrane and cytoplasm of neoplastic cells with a median positivity of 174.95, with young patients demonstrating a higher expression than older patients (61.3% vs. 40.7% respectively) ($p = 0.042$) (**Figure 2**). EGFR was correlated with patients who reported alcohol consumption (56.3% vs. 17.6%, $p = 0.009$), but it did not influence survival in the studied sample (**Table 3**).

MMP-9 was expressed in the cytoplasm of neoplastic cells and showed a median of 107.97, with young patients showing significantly higher expression than older patients (68% vs. 34.5% respectively) ($p = 0.001$) (**Figure 2**). Considering all patients, higher expression of MMP-9 was associated with anatomical site, tumor size, metastatic lymph nodes and clinical stage. In young patients, it was associated with tumors located on the tongue, tumor size and advanced clinical stage, whereas in the old subjects it was associated only with tumor size. MMP-9 did not correlate with survival rates (**Table 3**).

Although there were no significant differences between young and control groups in the expression of C-ErbB2, Myc, SMA and FGF-2, their expression was correlated with survival. C-ErbB2 and SMA were associated with a lower DFS in the young group (8.8% vs. 43.4% 5-years DFS, $p = 0.048$ and 26% vs. 37.5% 5-year DFS, $p = 0.018$ respectively); Myc influenced the OS (0% vs. 47.6% 5-year OS, $p = 0.010$ in young and 0% vs. 44.9% 5-years OS $p = 0.001$ in old patients) in both groups of patients; and FGF-2 was associated with a lower OS in the young patients (33.3% vs. 55.9% 5-year OS, $p = 0.023$) and a decreased in DFS in old patients (22.6% vs. 52% 5-year DFS, $p = 0.032$) (**Table 3**). The other evaluated proteins did not show differences between the age groups and did not influence survival. The results are shown in **Figure 1** and **Table 3**.

Discussion

A higher incidence of OSCC among young people has been recently reported in different countries worldwide⁶⁻⁸, nevertheless the clinical and biological behavior of these tumors is still a matter of discussion. Therefore, studies that analyze the clinicopathological features and the molecular basis of OSCC affecting this specific population are of the utmost importance.

A major limitation for better understating of OSCC in young patients is a lack of consensus regarding the most appropriate cut-off age. The literature uses values ranging from 30–50 years^{5-9, 12}, making it difficult to compare results. Nevertheless, previously published studies from our research group^{10,14,22} have demonstrated interesting findings using 40 years as a cut-off age. As demonstrated in the present sample, OSCC affects mainly male patients, and this has also been true for young patients¹¹, possibly due to the higher incidence of tobacco and alcohol use by males⁴. However, in samples where these social habits are not present, young females were shown to be more affected¹⁴. This was also seen in our sample (data not shown).

Tobacco and alcohol consumption are considered the main etiological agents associated with the development of OSCC². However, the role of these habits in young patients is controversial due to the supposed absence or short time of exposure to these factors in younger populations. This assumption should be taken with care, since our sample of young patients showed a high prevalence of smoking and drinking; suggesting a significant increase in these habits in the young population, in agreement with Ribeiro et al.⁹. On the other hand, oral cancer is a recognizable multifactorial disease and other etiological agents might be playing important roles in cases not associated with well-known environmental factors. Moreover, our group has previously

Accepted Article

demonstrated a higher frequency of DNA ploidy abnormalities in young patients¹⁴, suggesting that increased genomic instability is present in these individuals compared with older patients²³.

To determine the distribution of OSCC among young and old patients, we aimed to characterize the clinicopathological factors that could influence the survival rates of this group. We identified that advanced tumor size (T3/T4) was significantly correlated with decreased DFS and OS, while positive surgical margins determined a lower OS rate in both groups. These results are in agreement with a previous report²⁴ and highlight the importance of early diagnosis and appropriate surgical management of tumors in any population. Nevertheless, the presence of lymph node metastases and advanced clinical stage tumors (III/IV) determined a worse OS in the young patients only, probably because of the presence of comorbidities in old patients that may influence OS rates.

Alterations in several cellular processes allow neoplastic onset and development; these processes were described by Hanahan and Weinberg in 2000 and denominated hallmarks of cancer²⁵. The first alteration is 'Self-Sufficiency in Growth Signals' including control of the cell cycle by the alteration of the proteins involved in its regulation, such as the receptors of growth factors (EGFR and C-ErbB2). In the present study, the expression of EGFR was higher in the young patients than in the elderly ones and the over-expression of C-ErbB2 was associated with a lower DFS in young individuals, suggesting that in this age group these members of ErbB family may be involved in the early development and progression of OSCC tumors. These findings have potential implications for the treatment, as Cetuximab (a selective EGFR-inhibitor) is a targeted therapy drug approved for the use in OSCC patients. In this scenario, the amount of EGF is a predictive biomarker for the response to chemotherapy²⁶ and

preclinical trials suggest that the combination EGFR/C-ErbB2 may potentiate the effect of Cetuximab and decrease resistance²⁷. To date, there are no published results concerning the use of Cetuximab in the treatment of OSCC affecting young patients.

Another important hallmark of cancer is the ability of the neoplastic cells to invade tissue and cause metastases²⁵, where the tumor microenvironment (TME) plays an important role in molecular interactions between tumor cells and different stromal constituents (immune cells, fibroblasts, myofibroblasts, blood vessels and the extracellular matrix)²⁸. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade and remodel the extracellular matrix and basement membrane. They can facilitate the invasion of tumor cells and consequently metastasis to distant organs. In particular, MMP-9 is overexpressed in 92% of cases of OSCC²⁹. The present investigation found a significantly higher expression of MMP-9 in young patients compared with older patients, which might account for the higher rate of recurrences found in young patients. Also, MMP-9 was correlated with anatomical site, tumor size and clinical stage in young patients, while in older subjects it was only associated with tumor size, suggesting that MMPs may be highly relevant to the development and dissemination of cancer cells in tumors affecting young patients.

Myofibroblasts are components of the TME that have been identified in carcinomas adjacent to nests of tumor cells, and are thought to facilitate invasion of malignant cells by the secretion of numerous factors that promote the growth of neoplastic cells, tumor invasion and metastasis. We found no significant differences in the expression of SMA between young and old patients affected by OSCC, similar to the results of Fonseca et al.²² However, expression was correlated with DFS, similarly to FGF-2, which is another component of TME that influenced the survival in the current sample, demonstrating possible prognostic potentials.

Furthermore, C-ErbB2 and Myc proteins influenced the survival of young patients affected by OSCC as published in previous studies, highlighting the multifactorial molecular background of tumor recurrence and the importance of future research to validate these results and analyze in detail the role of these proteins. The above-mentioned differences in tumor cells and components of the TME between young and old patients may have important roles in the biological basis of tumor susceptibility and might represents potential targeting proteins in young patients with OSCC.

Conversely, the present investigation did not show significant differences in the immunohistochemical expression of Ki67, p53, p16, Bcl-2, Cyclin D1, p21 and Cathepsin K between young and old patients with OSCC, illustrating the high heterogeneity of this disease and the different ways that tumorigenesis may occur in this context.

Interesting, Kaminagakura et al.³⁰ described Cyclin D1 overexpression in young patients treated in the same institution and over the same time frame. This contrasting result can be explained by differences in the methodology used to analyze the immunohistochemistry reactions (their cutoff point for overexpression was 50%) and the inclusion of patients from other institutions in our study.

We demonstrated that young patients with OSCC might have similar survival rates to older patients, confirming that OSCC behavior may not be influenced by the age of the affected patients. However, we have described differences in C-ErbB2, EGFR, MMP-9 and SMA expression between these groups of patients, which might be associated with the unusual early development of oral cancer in young patients. These results demand additional investigation in order to better characterize these molecular

Accepted Article

differences and future applicability in the treatment of OSCC affecting young individuals.

Acknowledgements

This study was supported by the Brazilian Coordination of Higher Education (CAPES-Brazil)

Conflict of Interest Statement

The authors declare no conflicts of interest

References

1. DUVVURI U, MYERS JN. Cancer of the head and neck is the sixth most common cancer worldwide. *Curr Probl Surg.* 2009; 46(2):114-7.
2. WARNAKULASURIYA S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol.* 2009; 45(4-5): 309-16.
3. NAIR U, BARTSCH H, NAIR J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. *Mutagenesis.* 2004; 19(4): 251-62.
4. BARNES L, EVESON JW, REICHART P, SIDRANSKY D. *Pathology and Genetics of Head and Neck Tumours.* IARC Press, Lyon. 2005.
5. CUSUMANO RJ, PERSKY MS. Squamous cell carcinoma of the oral cavity and oropharynx in young adults. *Head Neck Surg.* 1988; 10(4) :229-34.
6. MARTIN-GRANIZO R, RODRIGUEZ-CAMPO F, NAVAL L, DIAZ GONZALEZ FJ. Squamous cell carcinoma of the oral cavity in patients younger than 40 years. *Otolaryngol Head Neck Surg.* 1997; 117(3 Pt 1): 268-75.

7. MYERS JN, ELKINS T, ROBERTS D, BYERS RM. Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. *Otolaryngol Head Neck Surg.* 2000; 122(1): 44-5.1.
8. PONTES FSC, CARNEIRO JT, FONSECA FP, SILVA TSP, PONTES HAR, PINTO DS. Squamous cell carcinoma of the tongue and floor of the mouth: Analysis of survival rate and independent prognostic factors in the Amazon region. *J Craniofac Surg.* 2011; 22 (3): 925-930.
9. RIBEIRO AC, SILVA AR, SIMONATO LE, SALZEDAS LM, SUNDEFELD ML, SOUBHIA AM. Clinical and histopathological analysis of oral squamous cell carcinoma in young people: a descriptive study in Brazilians. *Br J Oral Maxillofac Surg.* 2009; 47(2): 95-8.
10. KAMINAGAKURA E, VARTANIAN JG, DA SILVA SD, DOS SANTOS CR, KOWALSKI LP. Case-control study on prognostic factors in oral squamous cell carcinoma in young patients. *Head Neck.* 2010; 32(11): 1460-6.
11. SARKARIA JN, HARARI PM. Oral tongue cancer in young adults less than 40 years of age: rationale for aggressive therapy. *Head Neck.* 1994; 16(2): 107-11.
12. HILLY O, SHKEDY Y, HOD R, SOUDRY E, MIZRACHI A, HAMZANY Y, BACHAR G, SHPITZER T. Carcinoma of the oral tongue in patients younger than 30 years: comparison with patients older than 60 years. *Oral Oncol.* 2013; 49(10): 987-90.
13. DANIEL F, FAVA M, HOFFMANN R, CAMPOS M, YURGEL L. Main Molecular Markers of Oral Squamous Cell Carcinoma. *Applied Cancer Research* 2010;30(3)279-288.
14. SANTOS-SILVA AR, RIBEIRO AC, SOUBHIA AM, MIYAHARA GI, CARLOS R, SPEIGHT PM, HUNTER KD, TORRES-RENDON A, VARGAS PA, LOPES MA. High incidences of DNA ploidy abnormalities in tongue squamous cell carcinoma of young patients: an international collaborative study. *Histopathology.* 2010; 58(7): 1127-35.
15. GREENE FL, PAGE DL, FLEMING ID, FRITZ A, BALCH CM. *AJCC cancer staging handbook.* Springer Verlag. 2002.

16. MONTEIRO LS, DINIZ-FREITAS M, GARCIA-CABALLERO T, FORTEZA J, FRAGA M. EGFR and Ki-67 expression in oral squamous cell carcinoma using tissue microarray technology. *J Oral Pathol Med*. 2010 Aug 1;39(7):571-8.
17. IAMAROON A, KHEMALEELAKUL U, PONGSIRIWET S, PINTONG J. Co-expression of p53 and Ki67 and lack of EBV expression in oral squamous cell carcinoma. *J Oral Pathol Med*. 2004; 33(1): 30-6.
18. HUANG SF, CHENG SD, CHUANG WY, CHEN IH, LIAO CT, WANG HM, HSIEH LL. Cyclin D1 overexpression and poor clinical outcomes in Taiwanese oral cavity squamous cell carcinoma. *World J Surg Oncol*. 2012 Feb 16;10:40.
19. YUEN PW, CHOW V, CHOY J, LAM KY, HO WK, WEI WI. The clinicopathologic significance of p53 and p21 expression in the surgical management of lingual squamous cell carcinoma. *Am J Clin Pathol*. 2001 Aug;116(2):240-5.
20. FONSECA FP, DE ANDRADE BA, RANGEL AL, DELLA COLETTA R, LOPES MA, DE ALMEIDA OP, VARGAS PA. Tissue microarray is a reliable method for immunohistochemical analysis of pleomorphic adenoma. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2014; 117(1): 81-8.
21. KELNER N, RODRIGUES PC, BUFALINO A, FONSECA FP, SANTOS-SILVA AR, MIGUEL MC, et al. Activin A immunoexpression as predictor of occult lymph node metastasis and overall survival in oral tongue squamous cell carcinoma. *Head Neck*. 2015; 37: 479–486.
22. FONSECA FP, COLETTA RD, AZEVEDO MB, PRADO RIBEIRO AC, PIRES SOUBHIA AM, MIYAHARA GI, CARLOS R, et al. Stromal myofibroblasts in squamous cell carcinoma of the tongue in young patients - a multicenter collaborative study. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2014; 118(4): 483-9.
23. TONER M, O'REGAN EM. Head and neck squamous cell carcinoma in the young: a spectrum or a distinct group? Part 2. *Head Neck Pathol*. 2009; 3(3): 249-51.
24. JADHAV KB, GUPTA N. Clinicopathological prognostic implicators of oral squamous cell carcinoma: need to understand and revise. *N Am J Med Sci*. 2013; 5(12): 671-9.

25. HANAHAN D, WEINBERG RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70.
26. ANSELL A, JEDLINSKI A, JOHANSSON AC, ROBERG K. Epidermal growth factor is a potential biomarker for poor cetuximab response in tongue cancer cells. J Oral Pathol Med. 2016 Jan;45(1):9-16.
27. POLLOCK NI, GRANDIS JR. HER2 as a therapeutic target in head and neck squamous cell carcinoma. Clin Cancer Res. 2015 Feb 1;21(3):526-33.
28. SALO T, VERED M, BELLO IO, NYBERG P, BITU CC, ZLOTOGORSKI HURVITZ A, DAYAN D. Insights into the role of components of the tumor microenvironment in oral carcinoma call for new therapeutic approaches. Exp Cell Res. 2014; 325(2):58-64.
29. ROSENTHAL EL, MATRISIAN LM. Matrix metalloproteases in head and neck cancer. Head Neck. 2006; 28(7): 639-48.
30. KAMINAGAKURA E, WERNECK DA CUNHA I, SOARES FA, NISHIMOTO IN, KOWALSKI LP. CCND1 amplification and protein overexpression in oral squamous cell carcinoma of young patients. Head Neck. 2011;33(10):1413-9.

Figure 1. Differences in the immunohistochemical expression of proteins between age groups. (A) Nuclear markers. (B) Membrane and cytoplasmic markers.

Figure 2. Comparison of the immunohistochemical expression of EGFR in the membrane and cytoplasm of neoplastic epithelial cells, showing strong positivity in young patients (A) and moderate positivity in old subjects (B). MMP-9 immunopositivity was observed in the cytoplasm of tumor cells with a moderate reactivity in young patients (C) and weak reactivity in control patients (D).

Table 1. Antibodies used for the immunohistochemical analysis

<i>Antibody</i>	<i>Clone</i>	<i>Dilution</i>	<i>Antigen retrieval</i>	<i>Source</i>	<i>Positive control</i>
Ki67	MIB-1	1:300	Citric acid	Dako	Tonsil
p53	DO-7	1:300	Citric acid	Dako	Carcinoma
p16	E6H4	Ready	CC1	Ventana*	Cervical carcinoma
Bcl-2	124	1:50	Citric acid	Dako	Tonsil
Cyclin D1	RBT14	Ready	EDTA/Tris	Biosb	Tonsil
C-ErbB2		1:1500	Citric acid	Dako	Breast Carcinoma
p21	SX118	1:50	EDTA/Tris	Dako	Breast Carcinoma
Myc	9E.10.3	1:50	EDTA/Tris	Thermo	Burkitt lymphoma
EGFR	EGFR.25	1:50	Citric acid	Leica	Placenta
MMP-9		1:200	Citric acid	Thermo	Placenta
SMA	1A4	1:400	Citric acid	Dako	Endometrium
Cathepsin K	3F9	1:500	Citric acid	Biovendor	OSCC
FGF-2		1:500	Citric acid	Chemicon	Colon tumor

*Automated immunohistochemistry

Table 2. Sociodemographic and clinicopathological features

<i>Feature</i>	<i>Young</i>	<i>Older</i>	<i>p value</i>
<i>n (%)</i>			
Age			
Mean	34.1	61.2	0.001*
Median	36	59	
Range	16-40	47-80	
Sex			
Male	45 (73.8)	56 (78.9)	0.490
Female	16 (26.2)	15 (21.1)	
Tobacco consumption			
Yes	44 (72.1)	56 (78.9)	0.139
No	14 (23.0)	8 (11.3)	
NA	3 (4.9)	7 (9.9)	
Alcohol consumption			
Yes	37 (60.7)	42 (59.2)	0.552
No	21 (34.4)	22 (31.0)	
NA	3 (4.9)	7 (9.9)	
Anatomical site			
Tongue	33 (54.1)	35 (49.3)	0.520
Floor of the mouth	16 (26.2)	16 (22.5)	
Other	12 (19.7)	20 (28.2)	
T classification			
T1/T2	26 (42.6)	30 (42.3)	0.966
T3/T4	35 (57.4)	41 (57.7)	
N classification			
N0	28 (45.9)	25 (35.2)	0.284
N1-N3	33 (54.1)	46 (64.8)	
Clinical stage			
I/II	16 (26.2)	14 (19.7)	0.495
III/IV	45 (73.8)	57 (80.3)	

Histological differentiation			
I	35 (57.5)	36 (50.8)	0.477
II	18 (29.5)	26 (36.6)	
III	6 (9.8)	4 (5.6)	
NA	2 (3.2)	5 (7)	
Surgical margins			
Negative	39 (63.9)	42 (59.2)	0.261
Positive	7 (11.5)	4 (5.6)	
NA	15 (24.6)	25 (35.2)	
Treatment			
Surgery	18 (29.5)	23 (32.4)	ND
Radiotherapy	1 (1.6)	10 (14.1)	
Surgery + radiotherapy	29 (47.5)	30 (42.3)	
Surgery + radiotherapy	2 (3.3)	8 (11.2)	
+ chemotherapy			
NA	11 (18.1)	0 (0)	
Recurrence			
Yes	24 (39.4)	31 (43.7)	0.166
No	18 (29.5)	40 (56.3)	
NA	19 (31.1)	0 (0)	

Abbreviations: NA, not available; ND, not determined. *Statistically significant difference.

Table 3. Correlation between expression of proteins and 5-year survival.

Protein	Category	Young				Control				All			
		OS (%)	p value	DF S (%)	p value	OS (%)	p value	DF S (%)	p value	OS (%)	p value	DF S (%)	p value
Ki67	Low	25	0.33	33.	0.90	41.	0.96	41.	0.90	36.	0.71	35.	0.8
	High	45.6	2	3	0	7	9	5	2	9	4	3	45
p53	Low	38.	0.39	24.	0.23	42	0.54	44	0.87	40.	0.93	35.	0.6
	High	8	5	8	5	42.	3	39.	5	1	3	8	72
p16	Low	52.4		30.4		4		3		46.3		36.1	
	High	37.5	0.28	24.7	0.92	39.7	0.78	33.2	0.86	38.5	0.63	30.44.	0.9
Bcl2	Low	62.5		0		44.4		52.7		49.7		7	
	High	34.2	0.59	33.8	0.68	39.3	0.75	38.5	0.76	37.1	0.84	36.6	0.8
Cyclin D1	Low	47.8		30.3		40.7		35.5		44.3		33.7	
	High	42.9	0.87	33.8	0.99	49.7	0.30	55.29.	0.33	45.7	0.50	43.2	0.4
C-	Low	48.5		20.7		35.9		1		41		26.6	
	High	26.	0.19	8.8	0.04	36.	0.70	30.	0.34	32.	0.28	23.	0.0

ErbB2	High	9 60. 2	2	43. 4	8*	5 53. 2	7	6 51. 5	5	7 56. 7	<i>1</i>	3 48. 7	58
p21	Low	32	<i>0.34</i>	20.	<i>0.50</i>	47.	<i>0.16</i>	54.	<i>0.25</i>	39.	<i>0.77</i>	34.	<i>0.7</i>
	High	51. 2	3	8 32. 3	8	1 37. 9	6	5 33. 5	3	2 42. 7	6	3 33. 1	44
Myc	Low	0	<i>0.01</i>	-	<i>0.37</i>	0	<i>0.00</i>	-	<i>0.81</i>	0	<i>0.00</i>	0	<i>0.4</i>
	High	47. 6	<i>0*</i>	25	2	44. 9	<i>1*</i>	43. 4	2	46	<i>1*</i>	36. 6	25
EGFR	Low	44.	<i>0.87</i>	41.	<i>0.22</i>	39.	<i>0.64</i>	32.	<i>0.23</i>	42.	<i>0.87</i>	35.	<i>0.8</i>
	High	9 46. 8	3	7 0	5	7 50. 9	0	3 59. 6	8	1 48. 6	7	3 37. 5	60
MMP-9	Low	40	<i>0.76</i>	32.	<i>0.93</i>	33.	<i>0.65</i>	39.	<i>0.88</i>	34.	<i>0.59</i>	37.	<i>0.6</i>
	High	48. 3	4	9 26. 4	9	7 61. 8	5	2 55. 3	6	8 53. 3	8	1 37. 2	96
SMA	Low	63.	<i>0.25</i>	37.	<i>0.01</i>	66.	<i>0.14</i>	75	<i>0.29</i>	64.	<i>0.13</i>	55.	<i>0.4</i>
	High	6 42. 5	<i>1</i>	5 26	<i>8*</i>	7 44	7	38. 3	<i>1</i>	7 43. 3	8	6 33. 7	41
Cathepsin K	Low	48.	<i>0.85</i>	32.	<i>0.73</i>	33.	<i>0.43</i>	42.	<i>0.32</i>	38.	<i>0.45</i>	38.	<i>0.3</i>
	High	1 47. 6	4	4 31. 3	3	4 53. 2	<i>1</i>	9 33. 7	4	7 50	9	5 33. 9	76
FGF-2	Low	55.	<i>0.02</i>	46.	<i>0.16</i>	36.	<i>0.36</i>	22.	<i>0.03</i>	41	<i>0.38</i>	31.	<i>0.3</i>
	High	9 33. 3	<i>3*</i>	2 14. 3	<i>1</i>	1 49. 1	7	6 52	<i>2*</i>	42. 6	6	7 38. 6	76

Abbreviations: DFS, disease-free survival; OS overall survival.

*Statistically significant difference.

*Statistically significant difference
Numbers in brackets are median

Young
Old