Int. J. Oral Maxillofac. Surg. 2018; xxx: xxx–xxx
https://doi.org/10.1016/j.ijom.2018.01.002, available online at https://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Clinical Paper
Head and Neck Oncology

EGFR amplification and expression in oral squamous cell carcinoma in young adults☆

V. Costa[1], L. P. Kowalski[2], C. M. Coutinho-Camillo[3,4], M. D. Begnami[4], V. F. Calsavara[5], J. I. Neves[4], E. Kaminagakura[1]

[1]Department of Biosciences and Oral Diagnosis, Institute of Science and Technology, São Paulo State University (Unesp), São José dos Campos, Brazil; [2]Department of Head and Neck Surgery and Otorhinolaryngology, A.C. Camargo Cancer Center, São Paulo, Brazil; [3]International Research Center, A.C. Camargo Cancer Center, São Paulo, Brazil; [4]Department of Anatomic Pathology, A.C. Camargo Cancer Center, São Paulo, Brazil; [5]Department of Epidemiology and Statistics, A.C. Camargo Cancer Center, São Paulo, Brazil

V. Costa, L. P. Kowalski, C. M. Coutinho-Camillo, M. D. Begnami, V. F. Calsavara, J. I. Neves, E. Kaminagakura: EGFR amplification and expression in oral squamous cell carcinoma in young adults. Int. J. Oral Maxillofac. Surg. 2018; xxx: xxx–xxx.

Abstract. The aim of this study was to investigate epidermal growth factor receptor (EGFR) gene alterations in two groups of patients with oral squamous cell carcinoma (OSCC) (a test group of subjects aged ≤40 years and a control group of subjects aged ≥50 years) and to associate the results with EGFR immunostaining, clinicopathological features, and the prognosis. Sixty cases of OSCC were selected (test group, $n = 21$; control group, $n = 39$). The tissue microarray technique was applied to ensure the uniformity of results. Gene amplification was analyzed by fluorescence in situ hybridization (FISH), and immunohistochemical staining for EGFR was analyzed using an automated imaging system. *EGFR* amplification was higher in the test group than in the control group ($P = 0.018$) and was associated with advanced clinical stage ($P = 0.013$), regardless of age. Patients with EGFR overexpression had worse survival rates, as did patients who had T3–T4 tumours and positive margins. EGFR overexpression has a negative impact on disease progression. Despite the higher amplification of EGFR in young adults, it does not significantly impact the survival rates of affected patients.

Key words: oral cancer; squamous cell carcinoma; EGFR gene; fluorescence in situ hybridization; immunohistochemistry; prognosis.

Accepted for publication 3 January 2018

Oral squamous cell carcinoma (OSCC) is a malignant epithelial neoplasm that mainly affects alcohol- and tobacco-using patients in the fifth and sixth decades of life[1]. Rising trends of oral cancer have been reported among young and middle-aged individuals under the age of 45 years[2], women, and patients who have never been exposed to aetiological factors[3–5]. In this respect, cancer pathogenesis and genetic alterations have been studied to determine the probable causes of cancer in young adults (≤40 years of age)[6,7].

The epidermal growth factor receptor (EGFR) plays an important role in the growth and progression of solid tumours[8]. EGFR is a tyrosine kinase receptor and a member of the ErbB receptor family, which includes EGFR/erbB-1, HER2/erbB-2, HER3/erbB-3, and HER4/erbB-4. This receptor contains an external ligand-binding domain, a transmembrane domain, and a cytoplasmic domain, and is considered a potential therapeutic target in carcinomas[8–12]. Amplification of the EGFR gene (*EGFR*) is observed in ap-

☆ All authors reviewed and approved the final paper prior to its submission.

0901-5027/000001+07

Please cite this article in press as: Costa V, et al. *EGFR* amplification and expression in oral squamous cell carcinoma in young adults, *Int J Oral Maxillofac Surg* (2018), https://doi.org/10.1016/j.ijom.2018.01.002

proximately 15.4% to 31.2% of OSCC cases[13,14].

Radiation combined with EGFR inhibitors has been used successfully for the local treatment of advanced head and neck tumours[15]. However, in view of the high cost of anti-EGFR therapy[16], it is necessary to identify the group that will best respond to this therapy, as suggested by Galvis et al.[17]. The aim of this study was to investigate *EGFR* alterations in two groups of patients with OSCC (a test group of subjects aged ≤40 years and a control group of subjects aged ≥50 years) and to associate the results with EGFR immunostaining, clinicopathological features, and the prognosis.

Materials and methods

A retrospective study was performed involving 60 patients (test group, $n = 21$; control group, $n = 39$) with OSCC treated from 1970 to 2007 at the Department of Head and Neck Surgery and Otorhinolaryngology of the A.C. Camargo Cancer Center, São Paulo, Brazil. Demographic characteristics (age, sex, and race), lifestyle habits (smoking habit and alcohol consumption), clinical variables (tumour site and clinical stage), treatment, pathological factors (histological grade), and follow-up (minimum of 60 months) were analyzed. The Ethics Committee of the Institute of Science and Technology, Unesp approved the study.

The clinical characteristics of the patients were obtained from their medical records and the tumours were staged according to the American Joint Committee on Cancer (AJCC) *Cancer Staging Manual*[18]. The histopathological diagnoses were reviewed and the histological grade was determined based on the classification proposed by the World Health Organization for head and neck tumours[1]. Two previously calibrated examiners independently performed this analysis. The kappa test was used to determine agreement between the examiners (VC, EK). In the case of any discrepancy, a third observer was consulted (MDB).

The tissue microarray (TMA) blocks were constructed as described by Kaminagakura et al. (2010)[19].

Dual-colour fluorescence in situ hybridization (FISH)

One slide from each TMA block was subjected to hybridization. A ZytoLight SPEC EGFR/CEN7 Dual Color Probe (ZytoVision GmbH, Bremerhaven, Germany), which contains both the fluorescently labelled *EGFR* gene and chromosome 7 centromere probes, was used for FISH. Briefly, the sections were incubated at 56 °C overnight and deparaffinized by washing in xylene, ethanol, and distilled water. After incubation in 0.2 M HCl for 20 min at room temperature, the sections were heat pre-treated in saline–sodium citrate buffer (2× SSC, pH 6.0) for 1 h at 80 °C. Next, the sections were digested with pepsin for 8 min at room temperature, rinsed in 2× SSC for 2 min at room temperature, and dehydrated in an increasing ethanol series (75%, 80%, and 100%) for 2 min each. The *EGFR*/*CEN7* probe mix was applied to dry slides and the tissue area was coverslipped and sealed with rubber cement. The slides were then incubated in a hybridizer (S2450; Dako, Glostrup, Denmark) for denaturation at 75 °C for 10 min and hybridization at 37 °C for approximately 18 h. Post-hybridization washes were performed in urea/0.1× SSC for 30 min at 45 °C and in 2× SSC for 2 min at room temperature. The slides were dehydrated in serial ethanol solutions, following which 15 μl of mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) was applied, and the tissue area was coverslipped.

The criteria proposed by Jiang et al. were used to establish whether the FISH results were evaluable[20]. In each case, 30 non-overlapping, intact interphase tumour nuclei identified by DAPI staining were evaluated and the copy numbers of *EGFR* (green signal) and *CEN7* (red signal) in each nucleus were determined. Amplification was defined when the average copy number ratio, *EGFR/CEN7*, was ≥2.0 in all nuclei evaluated, or when the *EGFR* signals formed a tight gene cluster.

Immunohistochemistry

The TMA sections were preheated for 24 h at 60 °C, deparaffinized, and rehydrated in serial ethanol solutions. Antigen retrieval was performed in a pressure cooker in 10 mM citric acid solution (pH 6.0). Endogenous peroxidase activity was blocked with 6% hydrogen peroxide, followed by a washing step in 10 mM Tris-buffered saline (pH 7.4) for 5 min. Next, the sections were incubated with ready-for-use primary antibodies against EGFR protein (1:500, clone NB100-595; Novus Biologicals, Littleton, CO, USA) at 4 °C overnight. After this period, the sections were washed in Tris-buffered saline and incubated with biotin-free horseradish peroxidase (HRP-EnVision; DakoCytomation, Carpinteria, CA, USA) for 30 min. The reaction was developed using diaminobenzidine (DakoCytomation) as chromogen. The sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted. Positive and negative controls were included in all reactions.

An automated imaging system (ACIS III; Dako, Carpinteria, CA, USA) was used for quantitative analysis. This system detects levels of hue, saturation, and luminosity, converting the signals into a numerical measure of density (staining intensity) ranging from 60 to 256. The analysis was performed according to the criteria proposed by Fukazawa et al.[21]. To analyze immunohistochemical EGFR expression, the *membrane histo* program was used to measure optical membrane density. The operator quantified at least five areas showing the highest staining intensity, as recommended by the manufacturer of the equipment (ACIS III; Dako). The selected areas were restricted to OSCC cells.

Statistical analysis

The baseline patient characteristics are expressed as absolute and relative frequencies for qualitative variables and as the median, minimum and maximum for quantitative variables. The association between qualitative variables was evaluated by χ^2 test or Fisher's exact test, as appropriate. The non-parametric Mann–Whitney *U*-test was applied to compare the variable age between the groups (test and control).

Regarding the expression of EGFR, the determination of two groups of observations with respect to a simple cut-off point was estimated using the maximum of the standardized log-rank statistic proposed by Lausen and Schumacher[22]. In each analysis, the maximally selected log-rank statistic for cut-off points between 5% and 95% of continuous measure was considered. The Kaplan–Meier estimator of the survival function was considered for survival analysis and the log-rank test was used to compare the survival distribution between groups. The Cox semiparametric proportional hazards model was used to describe the relationship between survival and relapse times and the covariate defined with respect to a cut-off point[23]. The assumption of proportional hazards was assessed based on the so-called Schoenfeld residuals[24,25]. There was evidence that covariates had a constant effect over time in all cases.

Overall survival was calculated from the date of primary treatment to death.

Please cite this article in press as: Costa V, et al. *EGFR* amplification and expression in oral squamous cell carcinoma in young adults, *Int J Oral Maxillofac Surg* (2018), https://doi.org/10.1016/j.ijom.2018.01.002