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

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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.

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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-

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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.

Disease-free survival was calculated from the date of primary treatment to the date of first recurrence. Disease-specific survival was calculated from the date of primary treatment to death from OSCC.

The significance level was fixed at 5% for all tests. Statistical analyses were performed using IBM SPSS Statistics version 23.0 (IBM Corp., Armonk, NY, USA) and R software version 3.4 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The clinicopathological data of the participants are summarized in Table 1. Twenty-one samples from the test group (≤40 years old) and 39 samples from the control group (≥50 years old) were suitable for evaluation. Regardless of age, univariate and multivariate Cox proportional hazards regression models were performed, related to clinicopathological variables (Table 2). In the multivariate analysis, patients who presented T3/T4 tumours and those who had positive margins were at a significantly increased risk of death from disease (hazard ratio 6.52 and 5.94, respectively; both $P < 0.05$).

Gene amplification was found in six cases (28.6%) in the test group and in two cases (5.1%) in the control group (Fig. 1). Significantly higher gene amplification ($P = 0.018$) was observed in the test group (Table 3). Membrane overexpression of EGFR was observed in 66.7% of the test group and in 53.8% of the control group (Fig. 1), with no significant difference between the groups ($P = 0.493$) (Table 4). There was a tendency towards an association between gene amplification and EGFR overexpression; however this was not statistically significant ($P = 0.138$), probably due to the low number of cases of gene amplification.

Analysis of the association between the clinicopathological data and FISH results showed that *EGFR* amplification was significantly more prevalent in advanced tumours ($P = 0.013$), regardless of age (Table 5). The mean duration of follow-up was 40.7 months (range 1 to 136 months), and the mean time to recurrence was 14 months in the test group and 27 months in the control group, but the difference between groups was not statistically significant ($P = 0.248$).

The impact of EGFR overexpression on disease progression and survival in all of the patients was determined by Cox proportional hazards regression analysis (Table 6). Multivariate analysis revealed a 5.0-fold increased risk of death from disease in all patients who had membrane EGFR overexpression.

No statistically significant association was observed between *EGFR* amplification and survival rates ($P > 0.05$). Kaplan–Meier survival curves for patients who had membrane EGFR overexpression are shown in Fig. 2 ($P < 0.05$).

Table 1. Demographic and clinical characteristics.

Characteristics	Test group ($n = 21$)	Control group ($n = 39$)	*P*-value
Age, years			
Mean	35.0	60.0	<0.001
Range	20–40	51–80	
Sex, *n* (%)			0.582
Male	14 (67%)	30 (77%)	
Female	7 (33%)	9 (23%)	
Race, *n* (%)			0.493
White	16 (76%)	33 (85%)	
Other	5 (24%)	6 (15%)	
Location, *n* (%)			0.807[a]
Tongue	11 (52%)	22 (56%)	
Floor of the mouth	4 (19%)	5 (13%)	
Retromolar area	2 (10%)	7 (18%)	
Buccal mucosa	1 (5%)	1 (3%)	
Not reported	3 (14%)	4 (10%)	
Tobacco use, *n* (%)			1.000
Tobacco exposure	17 (81%)	31 (79%)	
No tobacco exposure	4 (19%)	8 (21%)	
Alcohol use, *n* (%)			0.911
Alcohol exposure	14 (67%)	24 (62%)	
No alcohol exposure	7 (33%)	15 (38%)	
T classification, *n* (%)			0.294
T1–T2	6 (29%)	18 (46%)	
T3–T4	15 (71%)	21 (54%)	
N classification, *n* (%)			0.743
N0	8 (38%)	18 (46%)	
N+	13 (62%)	21 (54%)	
M classification, *n* (%)			NA
M0	21 (100%)	39 (100%)	
M+	0 (0%)	0 (0%)	
Clinical stage, *n* (%)			0.340
I–II	3 (14%)	11 (28%)	
III–IV	18 (86%)	28 (72%)	
Histological grade, *n* (%)			0.042[a]
Well-differentiated	13 (62%)	23 (59%)	
Moderately differentiated	3 (14%)	14 (36%)	
Poorly differentiated	5 (24%)	2 (5%)	
Treatment, *n* (%)			0.731[a]
Surgery	8 (38%)	19 (49%)	
RT	1 (5%)	3 (8%)	
Surgery + RT	11 (52%)	16 (41%)	
Surgery + RT + ChT	1 (5%)	1 (2%)	

NA, not applicable; RT, radiotherapy; ChT, chemotherapy.
[a] Fisher's exact test.

Discussion

OSCC is uncommon in young adults (≤40 years old)[26]. Some authors have suggested that the disease has a more aggressive course in this age group[3,27,28], but no consensus exists[19,29–33]. Studies have shown an increase in the number of OSCC cases among young female patients[3–5], while the present study found a higher incidence in males, Caucasians, smokers, and alcohol drinkers. Despite the high incidence of OSCC among smokers and drinkers, there are no relationships between EGFR expression or amplification and alcohol and tobacco use[14,34,35], as observed in this study.

There are no studies in the English language literature investigating *EGFR* amplification specifically in young adults. In the present study, amplification was higher in the test group (≤40 years old) than in control patients. In a previous study of patients with colorectal cancer, Cappuzzo et al. suggested that patients with a high *EGFR* gene copy number have an increased likelihood of responding to monoclonal antibody therapy[36].

Table 2. Cox proportional hazards regression analysis for all patients, related to clinical characteristics.

Variable	Overall survival			Disease-free survival			Disease-specific survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
T classification									
T3–T4	6.14	2.52–14.99	<0.001	2.18	1.01–4.71	0.048	7.20	2.45–21.17	<0.001
Univariate model									
T3–T4	5.87	2.17–15.89	<0.001	1.88	0.86–4.10	0.112	6.52	1.84–23.15	0.004
Multivariate model									
Margins									
Positive	6.93	1.77–27.12	0.005	5.51	1.52–20.00	0.009	10.43	2.46–44.34	0.001
Univariate model									
Positive	4.03	1.03–15.89	0.046	5.89	1.57–22.18	0.009	5.94	1.39–25.49	0.016
Multivariate model									

HR, hazard ratio (estimated from the Cox proportional hazards regression model); CI, confidence interval of the estimated HR.

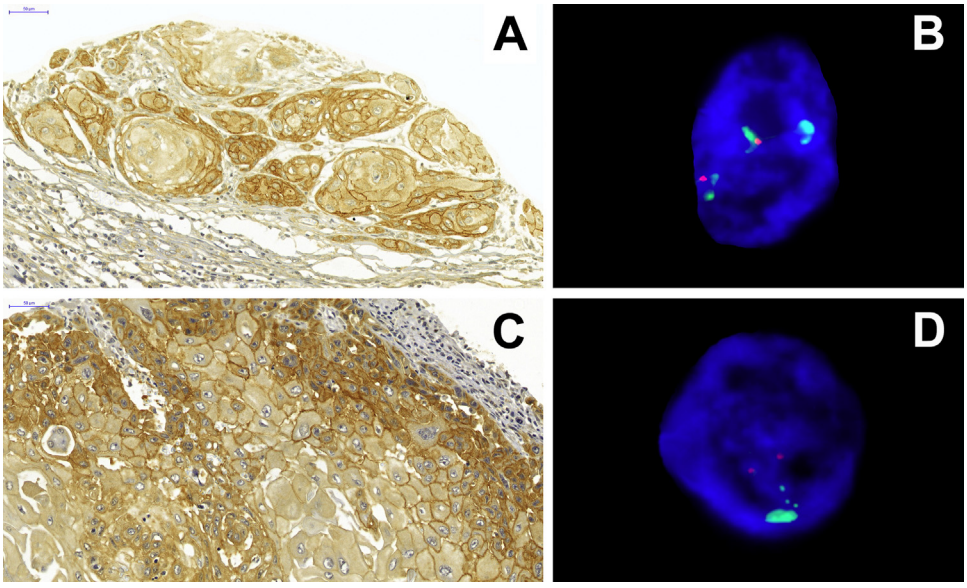

Fig. 1. EGFR protein expression and *EGFR* gene amplification in OSCC. (A) Membrane overexpression in OSCC cells of the test group; (B) *EGFR* amplification in the test group; (C) membrane overexpression in OSCC cells of the control group; (D) *EGFR* amplification in the control group. Immunohistochemistry: Mayer's haematoxylin counterstain. FISH: DAPI stain, *CEN7* (red) and *EGFR* (green) probes.

Significant association between gene amplification and clinicopathological data is not a common finding[14,37,38]. However, Huang et al. reported that *EGFR* gene amplification was significantly more prevalent in advanced tumours[13], as also demonstrated in this study. The present study findings showed this gene to be more amplified in the younger age group; however, the mechanism by which *EGFR* FISH status contributes to the oncogenic effect in expressing cells remains unclear[39].

Although no association between EGFR expression and clinicopathological data was observed in this study, Xia et al. found a significant association between EGFR expression levels and metastasis[11]. This could be explained by the role of EGFR in oncogenesis, in which mutations in this gene can activate multiple pathways, such as Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTOR[40]. The activation of these pathways can induce numerous cellular processes, including differentiation, transformation, proliferation, invasion, migration, angiogenesis, and inhibition of apoptosis[8,15], and this could also explain the poor outcomes of patients with EGFR overexpression, which occurs mainly in advanced tumours. Moreover, abnormal activation of *EGFR* signalling in head and neck squamous cell carcinoma may be accomplished by different mechanisms, including overexpression of EGFR and its ligands, the establishment of autocrine/paracrine loops, *EGFR* mutation or polymorphism, *EGFR* amplification, and

Table 3. EGFR FISH results for young adults and controls.

Group	No *EGFR* amplification	*EGFR* amplification	*P*-value
Test, *n* (%)	15 (71.4%)	6 (28.6%)	0.018
Control, *n* (%)	37 (94.9%)	2 (5.1%)	

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization.

Table 4. EGFR immunostaining results for young adults and controls.

Group	EGFR underexpression	EGFR overexpression	*P*-value
Test, *n* (%)	7 (33.3%)	14 (66.7%)	0.493
Control, *n* (%)	18 (46.2%)	21 (53.8%)	

EGFR, epidermal growth factor receptor.

Table 5. EGFR FISH results versus clinicopathological features in all patients, regardless of age.

		EGFR		*P*-value
		No amplification	Amplification	
T classification	T1–T2	23 (44.2%)	1 (12.5%)	0.128
	T3–T4	29 (55.8%)	7 (87.5%)	
N classification	N0	24 (46.2%)	2 (25%)	0.446
	N+	28 (53.8%)	6 (75%)	
M classification	M0	52 (100%)	8 (100%)	NA
	M+	0 (0%)	0 (0%)	
Clinical stage	I–II	13 (25%)	1 (12.5%)	0.013
	III–IV	39 (75%)	7 (87.5%)	

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; NA, not applicable.

Table 6. EGFR expression: multivariate Cox proportional hazards regression analysis for all patients.

Variable	Cut-off expression	HR	95% CI	*P*-value
Overall survival	EGFR >72	3.8	1.17–12.48	0.027
Disease-free survival	EGFR >76	2.6	1.16–5.91	0.021
Disease-specific survival	EGFR >73	5.0	1.18–21.2	0.028

EGFR, epidermal growth factor receptor; HR, hazard ratio (estimated from the Cox proportional hazards regression model); CI, confidence interval of the estimated HR.

transactivation by other tyrosine kinase receptors[39].

Interestingly, in addition to membrane staining, some of the present cases exhibited cytoplasmic EGFR staining. Cytoplasmic EGFR staining may indicate the translocation of EGFR from the membrane to the cytoplasm during cell–stromal interactions, leading to cytoplasmic EGFR overexpression and a more aggressive tumour behaviour[41]. Other authors have hypothesized that a rise in the 150-kDa form of EGFR is due to increased degradation of the mature 170-kDa form of the receptor through saturation of the degradation mechanism in the lysosomal compartment, resulting in cytoplasmic EGFR accumulation[42]. Chiang et al. reported nuclear or nuclear and cytoplasmic EGFR expression in 67% of OSCC samples[43]. The presence of nuclear staining is a common finding and is related to resistance to radiotherapy[44]. However, nuclear staining was not observed in any of the present cases.

In this study, there was a tendency towards an association between gene amplification and EGFR overexpression; however it was not statistically significant. A post-translational mechanism could be involved in this imbalance. For example, ring finger protein RNF11 overexpression is sufficient to increase the expression of EGFR and is accompanied by RNF11-dependent up-regulation of the inner coat protein complex II (COPII) paralogues SEC2B, SEC24B, and SEC24D, which are required for EGFR degradation and transport to the plasma membrane[45]. In addition, cylindromatosis tumour suppressor protein (CYLD) facilitates the interaction of EGFR with CBL-B through its Tyr15 phosphorylation in response to epidermal growth factor, which leads to EGFR ubiquitination and subsequent degradation[46].

Szabó et al. associated the relationship between EGFR overexpression and gene amplification with a poor outcome[34]. However, some authors have only found a poor outcome in association with *EGFR* amplification[37,47]. In the present study, OSCC survival was significantly influenced by the level of EGFR expression, similar to the findings of other studies[13,34]. No association was observed between *EGFR* amplification and survival rates, which is in accordance with earlier findings[13,35,38].

In summary, despite the higher amplification of *EGFR* in young adults, it was found not to significantly impact the survival rates of affected patients. Tumours with positive margins, a larger size, and higher EGFR expression were found to have a negative impact on disease progression and survival rates. Furthermore, patients with OSCC who show membranous EGFR overexpression could possibly benefit from anti-EGFR immunotherapy, regardless of their age.

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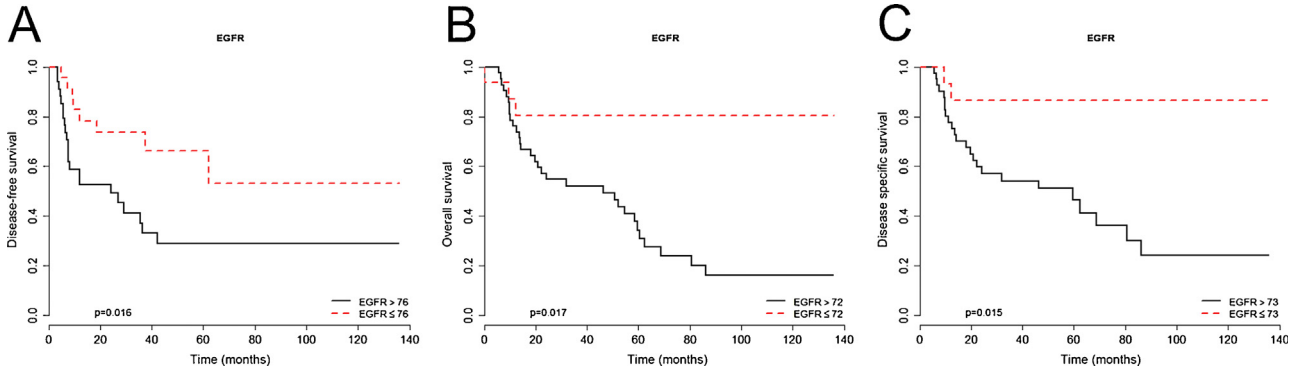

Fig. 2. Kaplan–Meier survival curves for patients with EGFR overexpression, regardless of age. (A) Disease-free survival ($P = 0.016$); (B) overall survival ($P = 0.017$); (C) disease-specific survival ($P = 0.015$). The effect of EGFR overexpression was significant in all the survival curves.

Competing interests

None.

Ethical approval

The Ethics Committee of the Institute of Science and Technology, Unesp approved the study under protocol number 1.227.336.

Patient consent

Not required.

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