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ORIGINAL ARTICLE

Pharmacodiagnostic evaluation of EGFR expression in oral squamous cell carcinoma

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OBJECTIVE: To determine epidermal growth factor receptor (EGFR) expression in oral squamous cell carcinomas (OSCC), and its possible relationships with clinical findings, histological findings, disease course and prognosis.

MATERIALS AND METHODS: Surgical specimens of 47 OSCCs were studied immunohistochemically for detection of EGFR using a standardized immunohistochemical detection system (EGFR PharmaDx™ kit). Statistical analysis was used to investigate possible relationships between EGFR expression and clinical findings, histological findings, cell proliferation (MIB1 labelling index), disease course and patient survival.

RESULTS: Epidermal growth factor receptor expression was absent or weak in 12 cases (25.5%) and moderate or intense in 35 cases (74.5%). However, EGFR expression did not show statistically significant associations with any of the clinical, histological, biological or prognostic variables considered.

CONCLUSION: First, despite previous suggestions that EGFR is a useful indicator of biological tumour behaviour, the present results suggest that EGFR is not a useful indicator of prognosis in OSCC. Secondly, the high prevalence of EGFR overexpression suggests that the possibility of anti-EGFR therapy in OSCC merits further investigation.

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Keywords: squamous cell carcinoma; epidermal growth factor receptor; cell proliferation (MIB1 labelling index); immunohistochemistry; anti-EGFR therapy

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Introduction

Despite recent advances in cancer treatment, the 5-year survival rate for oral cancer has not changed significantly during the last four to five decades (Moore *et al*, 2000). The assessment of a patient's prognosis is of great importance, as it will be a major factor in individual therapy selection (Bettendorf *et al*, 2004). Conventional clinicopathologic factors have only limited prognostic value for this type of cancer (Hibbert *et al*, 1983). Hence, it is clinically important to identify new prognostic factors that accurately predict the biological behaviour of the disease and allow for a more precise prognostic and therapeutic characterization of individual tumours.

In this connection, special attention has been paid to the role of the HER receptor family in head, neck and oral cancer. The HER growth receptor family consists of four cell surface receptors, including epidermal growth factor receptor (EGFR, c-erb-1/HER1), c-erbB-2 (HER2/neu), c-erbB-3 (HER3) and c-erbB-4 (HER-4). EGFR is an important receptor involved in signalling pathways implicated in the proliferation and survival of cancer cells (Shintani *et al*, 2004).

Epidermal growth factor receptor overexpression has been observed in a variety of human cancers, including oral squamous cell carcinoma (OSCC). However, the prognostic significance of EGFR in OSCC is still a matter of debate. Previous experimental and clinical studies (Shintani *et al*, 2003, 2004) support the view that EGFR may be a relevant target for cancer therapy. For this purpose, it is necessary to determine the expression status of the tumour-associated target in each patient (Schartinger *et al*, 2004). The aim of this study was to investigate EGFR expression in OSCC and its possible relationship with clinicopathologic features and outcome in a group of 47 patients.

Material and methods

Patients

A total of 47 patients, diagnosed with and treated for primary OSCC in the Oral and Maxillofacial Service of

the University Hospital Complex of Santiago de Compostela –Spain, between 1995 and 2000, were included in this study. None had received any treatment for OSCC before surgical intervention. Clinicopathologic information on each case (including age, gender, smoking and alcohol intake history, tumour node metastasis (TNM) classification, location, histologic grade, treatment, presence or absence of tumoral recurrence, and survival) was obtained from patients' files. Tumour stage was classified according to the 5th edition of the TNM classification of the International Union Against Cancer (Sobin and Wittekind, 1997). The site of recurrence was recorded and specified as local (primary site), regional (neck lymph node area), or distant metastasis.

All the patients were followed up until their death or for a minimum of 3 years after treatment. The median follow-up period was 37.80 months, with a range of 6–108 months.

Tissue samples

Tissue samples were obtained from the Pathology Service of the University Hospital Complex of Santiago de Compostela (courtesy Dr J. Forteza). Tissues were fixed in 10% buffered formalin and processed with standard procedures for paraffin wax embedding. Section of 5 μm were cut and stained with haematoxilin–eosin (HE) to confirm the initial diagnosis of OSCC. Histologic differentiation of tumours was defined according to the classification of the World Health Organization (Pindborg *et al*, 1997).

Tumour thickness measurement

For tumour thickness measurement, multiple sections were studied, selecting the thickest tissue section in which mucosa adjacent to the tumour could be observed and which was considered not to have been cut tangentially. A horizontal optical micrometer (Graticules Ltd, Tonbridge, UK) was used to measure the distance from an imaginary line, reconstructing the basal membrane of healthy oral mucosa to the deepest point of invasion (Gonzalez-Moles *et al*, 2002).

Immunohistochemistry

For immunohistochemical study, 5-μm sections were cut from formalin-fixed paraffin-embedded tumour samples, mounted on ChemMate capillary gap microscope slides (DakoCytomation, Glostrup, Denmark) and heated in an oven at 60°C for 24 h. The sections were deparaffinized in xylene, dehydrated in an ethanol series and rinsed in distilled water. All immunohistochemistry procedures were performed automatically using a Techmate 500 (DakoCytomation).

Immunohistochemical demonstration of EGFR

For EGFR immunostaining, the EGFR PharmaDx^{TM} kit (DakoCytomation) was used, following manufacturer's instructions. All reagents used were those supplied with the kit. Briefly, the procedure comprised: (1) epitope retrieval with proteinase-K for 5 min; (2) incubation with peroxidase-blocking agent for 5 min; (3) incubation with primary antibody (mouse anti-EGFR mAB, clone 2–18C9) at the supplied dilution for 30 min; (4) incubation with labelled polymer-horseradish peroxidase (HRP) (dextran polymer conjugated with HRP and affinity-isolated goat anti-mouse immunoglobulins) for 30 min; (5) incubation with diaminobenzidine (DAB) chromogen substrate solution for 10 min; 6) counterstaining with Harris haematoxylin for 2 min. Negative and positive controls provided in the kit were used in each staining run.

Immunohistochemical demonstration of MIB1

The procedure for MIB1 staining was as follows: (1) epitope retrieval with Tris-ethylenediaminetetraacetic acid (EDTA) (pH 9; Target Retrieval Solution, S3308; DakoCytomation) in a hot water bath at 99°C for 40 min; (2) incubation with the primary monoclonal antibody (mouse anti-Ki-67, clone MIB1, dilution 1/10; DakoCytomation) at room temperature for 30 min; (3) blocking of endogenous peroxidase with 3% H_2O_2 for 5 min; (3) incubation with goat secondary antibody (anti-rabbit-Ig and anti-mouse-Ig) conjugated to peroxidise-labelled dextran polymer (EnVisionTM Detection Kit, DakoCytomation) for 30 min; (4) visualization of bound antibody with diaminobenzidine tetrahydrochloride (DakoCytomation) for 5 min; and (5) counterstaining with Harris haematoxylin for 2 min.

Evaluation of EGFR expression

Epidermal growth factor receptor expression was evaluated on the basis of extent and intensity of EGFR immunolabelling in tumour cell membranes, classified on a four-point scale (Gamboa-Rodríguez *et al*, 2004): 0 (no labelling, or labelling in $<10\%$ of tumour cells); 1 (weak labelling, homogeneous or patchy, in $>10\%$ of tumour cells); 2 (moderate labelling, homogeneous or patchy, in $>10\%$ of tumour cells); 3 (intense labelling, homogeneous or patchy, in $>10\%$ of tumour cells). For data analysis, these categories were subsequently grouped into two wider categories: absent/weak labelling (0 or 1) and moderate/intense labelling (2 or 3). All samples were evaluated by a single person blinded to sample characteristics. Cases of difficult interpretation were discussed by two observers and a final scoring was made on a consensus basis.

Cell proliferation (MIB1 labelling index)

The evaluation of each section was performed in 10–15 fields at a magnification of ×400 (× 40 objective lens, ×10 eyepiece). A test grid sample (Mertz Graticule, Klarman Rulings Inc., Litchfield, NH, USA) which contained 36 points was used to register MIB1-positive and -negative cancer cell nuclei. Only nuclei coinciding with the points in the graticule were counted. At least 150 nuclei were counted in each case. The MIB1 labelling index (LI) is defined as [(MIB1-positive)/(MIB1-positive + MIB1-negative)] × 100.

Statistical analysis

Continuous variables are given as mean and standard deviations. Student's *t*-test was used to analyse the

significance of differences in mean values of continuous variables (age, tumour depth, MIB1 labelling index, etc.). Chi-squared or Fisher's exact test was used to analyse differences in the distribution of categorical variables (sex, TNM stage, clinical stage, EGFR expression, etc.).

Mean length of disease-free period and mean survivals were estimated by the Kaplan–Meier method (Kaplan and Meier, 1958). For patient groups defined by categorical variables, the significance of differences between survival curves was determined by the log-rank test with 95% confidence intervals. For analysis of the effects of continuous variables, univariate Cox proportional-hazards regression was used. Categorical and continuous variables with significant effects in the univariate analyses ($P < 0.05$) were entered into Cox proportional-hazards multivariate regression analyses for identification of the best predictors.

Results

Clinicopathological features and disease course

The clinicopathological characteristics of the 47 patients have been previously reported (Diniz-Freitas *et al*, 2006). Age ranged from 31 to 85 years with an average of 57.68 years; 39 patients (83%) were male and eight (17%) female. The most affected region was the tongue ($n = 18$, 38.3%), followed by the floor of the mouth ($n = 13$, 27.7%), gingiva ($n = 9$, 19.1%), retromolar region ($n = 3$, 6.4%), hard palate ($n = 3$, 6.4%) and buccal mucosa ($n = 1$, 2.1%).

In all, 22 (46.8%) tumours were graded as well differentiated and 25 (53.2%) as moderately differentiated. The thickness of 10 tumours could not be evaluated because of the absence of normal mucosa in these samples. The mean overall thickness was 6.33 mm (range 1.25–16.37 mm).

Twenty-one patients (42.5%) presented with advanced tumours (stage III or IV) and 26 (57.5%) with early tumour (stage I or II). All the patients underwent surgical excision of the primary tumour, and 15 patients (31.9%) underwent ipsilateral or bilateral therapeutic neck dissection. Elective neck dissection was carried out in 17 patients (36.2%). Of the remaining 15 patients, seven (46.7%) developed disease in the neck subsequently (i.e. after excision of the primary tumour), and all seven patients underwent therapeutic neck dissection.

A total of 12 patients (25.5%) underwent postoperative radiotherapy directed to the primary site, neck or both. Sixteen patients (34%) suffered local recurrence during the study period, with the mean time between excision and recurrence being 13 months (range 2–29 months).

Metastatic lymph nodes were confirmed histologically in 13 of 13 (100%) and one of 19 (5.3%) of subjects who underwent therapeutic and elective neck dissections, respectively; and as noted, seven of the 15 patients who did not initially undergo neck dissection went on to develop neck disease. Overall, therefore, a total of 21 patients (44.7%) had pathologic evidence of cervical node involvement at some time in the course of their disease.

At the end of the study period, 21 patients (44.7%) were alive, one (2.1%) was alive but with disease recurrence, and 25 patients (53.2%) had died as a result of the cancer. No deaths as a result of other causes were recorded during the follow-up.

Correlations between EGFR expression and clinicopathological features

Epidermal growth factor receptor expression was negative in one case (2.1%), weak in 11 cases (23.4%), moderate in 19 cases (40.5%), and intense in 16 cases (34.0%) (Figure 1). Grouping these categories together,

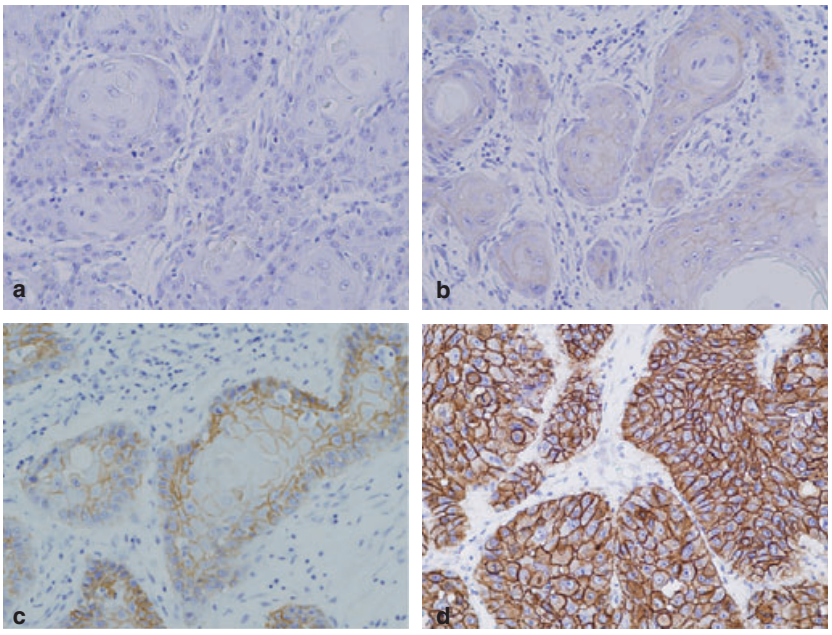

Figure 1 Immunohistochemical staining of EGFR in oral squamous cell carcinomas evaluated on the basis of intensity of EGFR immunolabelling in tumour cell membranes. (**a**) 0 (no labelling, or labelling in $<10\%$ of tumour cells); (**b**) 1 (weak labelling, homogeneous or patchy, in $>10\%$ of tumour cells); 2 (moderate labelling, homogeneous or patchy, in $>10\%$ of tumour cells); 3 (intense labelling, homogeneous or patchy, in $>10\%$ of tumour cells) (objective magnification ×20)

EGFR expression was absent or weak in 12 cases (25.5%) and moderate or intense in 35 cases (74.5%).

Epidermal growth factor receptor expression was analysed in relation to clinicopathologic factors including age, gender, tumour size and thickness, intra-oral location, lymph node metastasis, clinical stage and histological differentiation. None of these factors was correlated with EFGR expression (Table 1). No significant relationships were found between EGFR expression intensity and the appearance of local recurrences ($\chi^2 = 0.004$, $P = 0.952$) or regional recurrences ($\chi^2 = 0.066$, $P = 0.797$).

Correlations between cell proliferation and clinicopathological features

All except one specimen showed MIB1-positive cells, with the MIB1 labelling index ranging from 23.60 to 85.92% (mean 57.22%). MIB1 nuclear staining was seen either in a random pattern, or with greater intensity at the periphery of the tumour islands. MIB1 labelling index was analysed in relation to clinicopathologic factors including age, gender, tumour size and thickness, location, lymph node metastasis, clinical stage, and histological differentiation. A significant association was observed only with gender: in males, the MIB1 labelling index (mean 60.07%, s.d. 15.29) was significantly higher ($P = 0.04$, Student's *t*-test) than in females (mean 41.24%, s.d. 11.60).

Table 1 Correlations between EGFR expression and clinicopathological features

		EGFR expression			
Variable	*N*	*Absent/ weak*	*Moderate/ intense*	χ^2	*P*
Age					
≤45 years	9	–	9 (100)	3.817	0.052
>45 years	38	12 (31.6)	26 (68.4)		
Sex					
Men	39	10 (25.6)	29 (74.4)	0.001	0.970
Women	8	2 (25)	6 (75)		
Location					
Tongue	18	5 (27.8)	13 (72.2)	3.643	0.602
Floor of the mouth	13	5 (38.5)	8 (61.5)		
Alveolar ridge	9	1 (11.1)	8 (88.9)		
Retromolar trigone	3	1 (33.3)	2 (66.7)		
Hard palate	1	–	1 (100)		
Buccal mucosa	1	–	1 (100)		
Primary tumour size (T stage)					
T1	18	5 (27.8)	13 (72.2)	7.721	0.052
T2	12	6 (50)	6 (50)		
T3	7	1 (14.3)	6 (85.7)		
T4	10	–	10 (100)		
Cervical lymph node metastasis (N stage)					
N0	34	9 (26.5)	25 (73.5)	0.097	0.952
N1	8	2 (25)	6 (75)		
N2	5	1 (20)	4 (80)		
Clinical stage					
Stage I	17	5 (29.4)	12 (70.6)	2.851	0.415
Stage II	10	4 (40)	6 (60)		
Stage III	9	2 (22.2)	7 (77.8)		
Stage IV	11	1 (9.1)	10 (90.9)		
Degree of tumour differentation					
Well differentiated	22	7 (31.8)	15 (68.2)	0.860	0.354
Moderately differentiated	25	5 (20)	20 (80)		
Tumour thickness (37 samples)					
<8 mm	28	8 (28.6)	20 (71.4)	0.074	0.786
≥8 mm	9	3 (33.3)	6 (66.7)		

Values in Parentheses are Percentages.

Table 2 Results of Cox multivariate regression to identify the best independent predictors of survival

Variable	*Wald* χ^2	*P*	*Exp(β)*	*95% CI de Exp (β)*
Stage I *vs* Stage II	8.705	0.003	0.148	0.041–0.526
Stage II *vs* Stage III	6.131	0.013	0.063	0.007–0.561
Stage III *vs* Stage IV	1.154	0.283	0.503	0.143–1.763

Analysis of overall survival

The cumulative 3-year survival rate for the 47 patients was 46.8%. T stage ($P = 0.0092$) and N stage ($P = 0.0004$), clinical stage ($P = 0.0005$) and histological differentiation grade ($P = 0.0281$) were all significant predictors of survival. Tumours ≥ 8 mm had a significantly poorer prognosis than tumours <8 mm ($P = 0.029$). There was no significant correlation between MIB1 labelling index and overall survival [Cox regression, Exp(β) = 1.021, $P = 0.114$).

The mean survival time was 63.31 months (42.86–83.76 months) for patients with absent/weak EGFR expression, and 49.72 months (36.31–63.13 months) for patients with moderate/intense EGFR expression; this difference was not statistically significant ($P = 0.33$).

Multivariate analysis

Cox proportional-hazards multiple regression indicated that the clinical stage was the best independent predictor of overall survival (Table 2).

Discussion

One of the aims of the present study was to evaluate EGFR expression in a series of 47 patients diagnosed as suffering from OSCC. We found that 97% of the tumours showed EGFR expression. Previous studies of EGFR expression in OSCC have obtained rather contradictory results. Sakai *et al* (1990), reported EGFR in 15% of tumours and Kusukawa *et al* (1996), in 31% of tumours; by contrast, other studies have reported EGFR expression in all head and neck SCCs, including OSCCs (Field and Spandidos, 1987; Störkel *et al*, 1993).

Some authors have related EGFR expression to advanced tumour stage and the presence of cervical lymph node metastases (Kusukawa *et al*, 1996), while others have not confirmed these relationships and conclude that the study of EGFR expression does not give additional information on the clinicopathological status of patients with oral cancer (Christensen *et al*, 1995). In the present study, EGFR expression was not significantly associated with any of the clinicopathological variables considered.

Growth factors and their receptors may of course be involved in cell proliferation and cancer development. Thus, we would expect to find a higher rate of cell proliferation (as measured by MIB1 immunoreactivity)

in tumours with strong EGFR expression; in fact, however, we did not detect any difference in MIB-1 immunoreactivity between tumours with absent/weak and moderate/intense EGFR expression. This finding is in line with Keçicki *et al* (1999), who found no differences in cell proliferation between EGFR-positive and -negative laryngeal SCCs.

The prognostic utility of EGFR in OSCC is likewise controversial. While some authors have found that high EGFR expression is associated with poor prognosis, others have not (Partridge *et al*, 1988; Bergler *et al*, 1998) or indeed have found that high EGFR expression is associated with good prognosis (Maiorano *et al*, 1998). Specifically, Partridge *et al* (1988) studied 20 cases and did not find any correlation between EGFR expression and degree of differentiation, disease-free period or survival. Bergler *et al* (1998) did not detect any correlation between EGFR expression and tumour stage. Maiorano *et al* (1998) evaluated EGFR expression in the cytoplasm and membrane of neoplastic cells, and found positivity in 36% of tumours. In addition, patients with EGFR expression in the membrane and/or cytoplasm showed better prognosis; the strength of the correlation was improved by considering only expression in the membrane. Ulanovski *et al* (2004) found EGFR expression in 34% of 23 tongue SCCs. They observed a significant association between degree of differentiation and EGFR expression, i.e. less differentiated tumours showed weaker EGFR expression. However, they did not find any correlation between EGFR expression and tumour thickness or N stage, two important indicators of tumour progression. They likewise did not find any association between EGFR expression and tumour recurrence, cervical metastases or survival. Bankfalvi *et al* (2003), in a study of 75 patients with OSCCs, observed a correlation between EGFR expression and mode of tumour invasion. In addition, a correlation was observed between EGFR expression and T stage and overexpression was correlated with unfavourable prognosis. Störkel *et al* (1993) investigated the prognostic value of EGFR expression in 100 patients with OSCCs. All cases showed EGFR expression; furthermore, EGFR expression was correlated with histological grade and with a 5-year survival.

Yamada *et al* (1992) found EGFR expression in 51% of the OSCCs studied. Expression did not show any correlation with degree of differentiation.

Xia *et al* (1999) studied the predictive value of EGFR (HER-1), HER-2, HER-3 and HER-4 in 47 cases of OSCC, and in all four cases found significant relationships between expression and survival. However, these authors found that the joint presence of EGFR, HER-2 and HER-3 was a more effective predictor of survival than any of the markers individually.

Thus, although some previous studies have suggested that overexpression of EGFR is an indicator of poor prognosis in squamous cell carcinoma of the head and neck, not all studies have supported this view. These discrepancies may be as a result of methodological factors (e.g. technique sensitivity and specificity, antibodies used, tissue fixation times and pretreatments), sample size, follow-up time, statistical analyses, and/or differences in tumour location, stage and treatment. The use of a standard Food and Drug Administration (FDA)-approved kit is the first step towards standardization of the methodology.

Independently of its value as a prognostic marker, the key role of EGFR in carcinogenesis has led to research aimed at identifying selective inhibitors of EGFR-mediated pathways. The most promising strategies include immunotherapy with monoclonal antibodies, and targeted chemotherapy based on tyrosine kinase inhibitors. Optimal application of these potential therapies will require quantitative determination of EGFR in each tumour, as we can only expect these therapies to be effective in cases in which EGFR is overexpressed. To this end, various studies have performed semi-quantitative determination of EGFR by immunohistochemical methods in patients with OSCC or SCC of the head or neck. As there are no standardized procedures for evaluating EGFR expression, in the present study we used a semiquantitative four-point scale, grouped for data analysis into two categories, absent/weak and moderate/intense. About 98% of our tumours showed EGFR expression, while overexpression (i.e. moderate/intense expression) was observed in about 74% of tumours. Schartinger *et al* (2004) using the same PharmaDx EGFR detection kit as was used in the present study, detected EGFR in 71% of oral and oropharyngeal SCCs. As in the present study, these authors did not detect any significant relationship between EGFR expression and survival; however, they concluded that the high prevalence of EGFR overexpression in tumours of this type raises the possibility that EGFR-targeted treatments may be effective in these patients.

In view of the conflicting results on the prevalence of EGFR overexpression in OSCC, we consider that there is a clear need for a standardized procedure for immunohistochemical evaluation of EGFR expression level. The high percentage of oral squamous cell carcinomas showing EGFR expression suggests that the proposed new EGFR-targeted treatments may possibly be effective in these tumours. Further studies are needed to investigate whether EGFR gene amplification, determined by fluorescent *in situ* hybridization (FISH), and/or EGFR gene mutations identified by sequencing, may be of prognostic value in OSCC.

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