Oral Oncology (2007) **43**, 193–198

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ORAL ONCOLOGY

High EGFR expression predicts poor prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx: A TMA-based immunohistochemical analysis

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Received 15 December 2005; received in revised form 14 February 2006; accepted 15 February 2006
Available online 18 July 2006

KEYWORDS
EGFR;
Tissue microarray;
Oral squamous cell carcinoma;
Prognosis

Summary This retrospective study was designed to investigate the prognostic significance of EGFR overexpression in human oral squamous cell carcinoma on a long-term follow-up. EGFR expression was examined immunohistochemically on a tissue microarray (TMA) of paraffin embedded tissue specimens from 109 patients who underwent surgical treatment for squamous cell carcinoma of the oral cavity and oropharynx in the period between 1980 and 1997. High EGFR expression was found in 80 (73.42%) of the tumour samples. Kaplan–Meier curves showed that EGFR overexpression was significantly related to decreased overall survival ($p = 0.05$). Multivariate analysis showed that EGFR overexpression is an independent prognostic marker in these patients ($p = 0.02$, RR 3.6). These results confirm that EGFR overexpression is an independent prognostic marker in patients with squamous cell carcinoma of the oral cavity and oropharynx. The EGFR antigen represents an attractive target for targeted therapies with monoclonal antibodies or specific tyrosine-kinase inhibitors in these patients.

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1368-8375/$ - see front matter
doi:10.1016/j.oraloncology.2006.02.009

Introduction

Squamous cell carcinoma of the oral cavity and oropharynx, as a subgroup of head and neck cancers, accounts world wide for about 4% of all carcinomas in men and 2% in women, with geographically varying frequency. A high incidence of oral squamous cell carcinoma is present in India,[1] where chronic consumption of tobacco in combination with alcohol abuse are essential etiologic factors for this pathology.[2] Alcohol favours higher permeability of the oral mucosa, which in turn enhances the carcinogenic effects of nitrosamines and polycyclic hydrogen contained in tobacco. Insufficient oral hygiene, chronic pressure caused by dental prostheses, infection by papilloma virus and chronic diseases (such as Plummer-Vinson-syndrome or Lichen planus) are described to be further possible etiologic factors.[3] The prognosis of oral squamous cell carcinoma is based on clinical stage (according to TNM classification) and tumour grading.[4] Over the past years, various strategies adding chemotherapy to radiotherapy have been developed to improve treatment outcome.[5]

Epidermal growth factor receptor (EGFR) is a 170-kilodalton (kD) transmembrane cell-surface receptor. It is a tyrosine kinase (TK) receptor,[6] that is commonly altered in epithelial tumours. This protein is encoded by 28 exons spanning nearly 190,000 nucleotides on chromosome 7p12. It belongs to a subfamily of four similar receptors: HER-1 (ErbB1), HER-2/(neu/ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). Each of these transmembrane proteins consist of an external ligand-binding, a transmembrane lipophilic segment and a cytoplasmatic domain.[6] Different polypeptide growth factors, predominantly EGF and TGFα,[7] but also amphiregulin, betacellulin, heparin-binding protein, epiregulin and vaccinia virus growth factor[8] bind to the extracellular region of EGFR. The tyrosine kinase domain[9] represents the essential part of its effector function. Ligand binding to the EGFR causes conformational changes that facilitate homo-or heterodimerization. Growth factor-induced receptor dimerization of EGFR is followed by intermolecular autophosphorylation[8] of tyrosine kinase domains, which leads to activation of different downstream effectors such as ras/raf-1/mitogen-activated protein kinase, the phosphatidylinisotol-3-kinase, phospholipase C and Stat proteins.[10] Activation of these cascades leads to transcription of genes responsible for cell growth, angiogenesis, inhibition of apoptosis, cell adhesion, cell motility, and invasion, and thus might enhance the malignant potential of epithelial tissues overexperessing EGFR.[11] In line with these results, EGFR overexpression was reported to be correlated with poor prognosis in patients with head and neck cancer.[12]

Overexpression of EGFR[13] results from gene amplification and consequent increased transcriptional activity. Furthermore, receptor mutants that are constitutively active without ligand binding have been described.[14] At least seven classes of mutants (vI—vVII) have been identified in tumours such as gliomas, the most common mutant being EGFRvIII, which has been described to have lost the amino acids 6—273. Finally, activation of EGFR receptors by autocrine overproduction of ligands such as EGF and TGFα appears to be a common event in human carcinomas.[15]

The need to inhibit the tumour promoting function of the EGFR receptor has lead to the development of specific tyrosine-kinase inhibitors[9] (i.e., Gefitinib, Erlotinib) and monoclonal antibodies (Cetuximab).[16] These inhibitors are potential new anticancer drugs for a wide range of epithelial cancers.[17] Targeting EGFR with monoclonal antibodies, such as cetuximab is effective by inducing antibody dependent cellular cytotoxicity (ADCC)[16] or by impairing ligand binding.[18] By contrast, tyrosine kinase inhibitors act by physical interaction with the intracellular tyrosine kinase domain blocking intracellular signal transduction. Preclinical data show marked enhancement of the antitumour activity of conventional chemotherapy when tyrosine-kinases inhibitors are added.[19] In addition, the alteration of the EGFR signalling pathway in the EGFR can strongly influence the sensitivity and the resistance to radiation therapy.[20]

The search for prognostic and predictive factors of oral squamous cell carcinoma reflects the need for an improved risk assessment to customize therapeutic approaches. In order to elucidate whether EGFR is a clinically relevant determinant of the malignant phenotype of oral squamous cell carcinoma, this retrospective study has been performed.

Patients and methods

Patients

This retrospective study was based on a total number of 109 patients. These patient series represent about half of all cases with invasive squamous cell carcinoma of the oral cavity and oropharynx, which were operated at the Department of Maxillofacial Surgery, Innsbruck University Hospital, from 1980 to 1997. Fifty-five patients received adjuvant chemotherapy and/or radiation. All cases, for which paraffin-embedded tissue samples were retrievable from the local pathology repository, and for which clinical follow-up data were available, were included.

Histopathology

All tumour samples were formalin-fixed, embedded in paraffin wax and stored at the local pathology repository for varying periods of time. Of each tumour a haematoxylin- and eosin-stained slide was prepared, using routine methods, and then examined by light microscopy. The tumour grade was assessed by two co-authors in a blinded fashion using standard pathology criteria. A low, moderate and high differentiation grade was observed in 23%, 52% and 25% of patients, respectively.

Tissue micro array (TMA)

For TMA construction, the pathologist used the H& E-stainedslide from each tumour block to select a morphologically representative tumour area. Tissue cylinders with a diameter of 2 mm were punched from the marked tumour areas of each block (=donor block) and brought into a recipient paraffinblock (=recipient block) using a precision instrument (GX-BI-MPK1 — Motorized positioner, dual axis). Five different TMAs were constructed, each containing about 30 punches of oral squamous cell carcinoma in a specific arraying pattern. Sections from this block were cut with a microtome and mounted on standard slides, which were used for the immunohistochemistry analysis.

Immunohistochemistry

The expression of EGFR was determined by immunohistochemistry using the murine monoclonal EGFR antibody (Dako Cytomation, Denmark). 5-μm sections were cut from recipient paraffin blocks, mounted on microtissue array specific adhesive-coated glass slides, deparaffinized and rehydrated. Endogenous peroxidase was blocked with methanol containing hydrogen peroxide 3% for 20 min. Finally, a peroxidase-conjugated goat anti-mouse antibody ready for use (EnVisionTM, DAKO, Vienna, Austria) was added over 60 min for immunostaining. Slides were then placed into the chromogen which consisted of diaminobenzidine solution containing hydrogen peroxide 30%. Finally, slides were counterstained with Mayer's Hemalum solution and rinsed with water. Positive and negative controls were included in each run.

Evaluation of slides

One assessor (P.O.) who was unaware of clinical outcome evaluated EGFR expression using light microscopy. Antigen expression was defined as the presence of specific staining on the surface membrane of tumour cells. To quantify EGFR expression a total immunostaining score was calculated, using the product of a proportion score and an intensity score. The proportion score described the estimated fraction of positive stained tumour cells (0 = none; 1 = <10%; 2 = 10—50%; 3 = 50—80%; 4 = >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. Tumours were arbitrarily categorized into samples with high EGFR, low EGFR and no EGFR expression using a cut-off value: 0 = 0, 1—3 = low and 4—12 = high (Fig. 1).

Statistical methods

All calculations and the statistical analysis were performed using the statistical software program SPSS for Windows™. Differences between groups were examined for statistical significance using the χ^2 test. Survival curves were calculated according to the method of Kaplan and Meier. With this method *p*-values were evaluated using the log-rank test for censored survival data. Follow-up time was censored if the patient was lost to follow-up. The significance of various parameters for survival was analysed by the Cox proportional hazard model in the multivariate analysis. For all analyses a *p*-value of 0.05 was defined as statistically significant.

Results

Eighty-three (76.1%) of the patients were male and 26 (23.9%) female. The median age of the patients was 63.2 years (range 25.6—86.8 years). At the time of primary diagnosis 57.4% of the patients presented with a tumour diameter of 2—4 cm, 42.6% with a tumour diameter of more than 4 cm and 16.2% with a tumour diameter of less than 3 cm. Fifty-seven percent of the patients presented with lymph

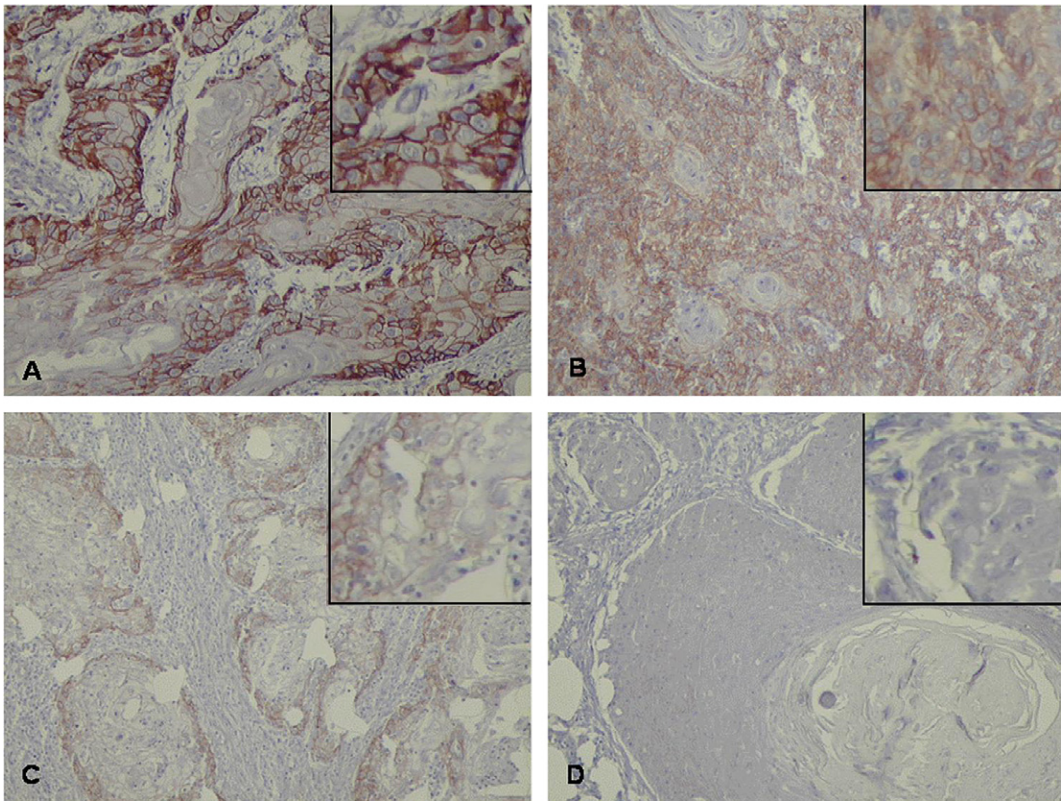

Figure 1 (A) Strong EGFR-expression of Oral squamous cell carcinoma (score 3). A higher power magnification of staining is shown in the upper right corner. (B) Oral squamous cell carcinoma with moderate EGFR staining (score 2). (C) Oral squamous cell carcinoma with weak EGFR — expression (score 1). (D) EGFR — negative Oral squamous cell carcinoma (score 0).

node positive disease at primary diagnosis. Fifty-eight percent had clinical stage IV, 21% stage III, 12% stage II and 9% stage I. Thirty-one of the patients experienced a relapse of their disease after a median time of 11.5 months (range 1—71 months). The median overall survival was 26.6 months (range 1—245 months). EGFR-antigen was found to be highly expressed in 73.4% ($n = 80$) of the analysed tumour samples. High expression of EGFR correlated significantly ($p = 0.05$) with poor survival in the whole patient group. The prognostic power of EGFR overexpression was pronounced in the subgroup of untreated patients ($p = 0.032$, Figs. 2 and 3). As expected, conventional prognostic parameters such as tumour grading ($p = 0.01$) and clinical stage ($p = 0.003$) were correlated with overall survival. Multivariate analysis including clinical stage, locoregional lymph node metastasis, grade and EGFR overexpression identified EGFR overexpression as independent prognostic marker ($p = 0.02$, Table 1). As shown in Table 2 and in accordance with its independence as a prognostic marker, there was no significant correlation of EGFR-overexpression with grading ($p = 0.24$), clinical stage ($p = 0.17$) and lymph node metastasis ($p = 0.6$).

Discussion

Identification of prognostic factors allows an accurate selection for assignation of patients to optimal therapy. In our study, we found frequent expression of EGFR in patients

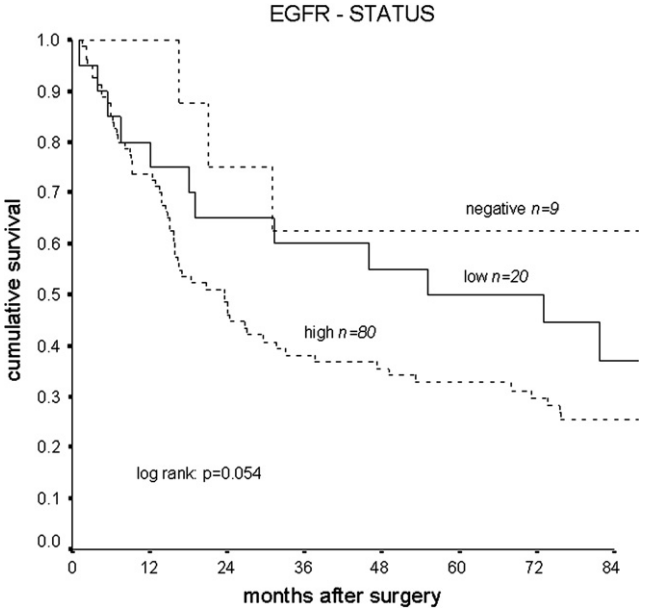

Figure 2 Prognostic significance of EGFR expression in 109 patients with oral squamous cell carcinoma regarding overall survival. Patients with high ($n = 80$), low EGFR ($n = 20$) and without EGFR expression ($n = 9$).

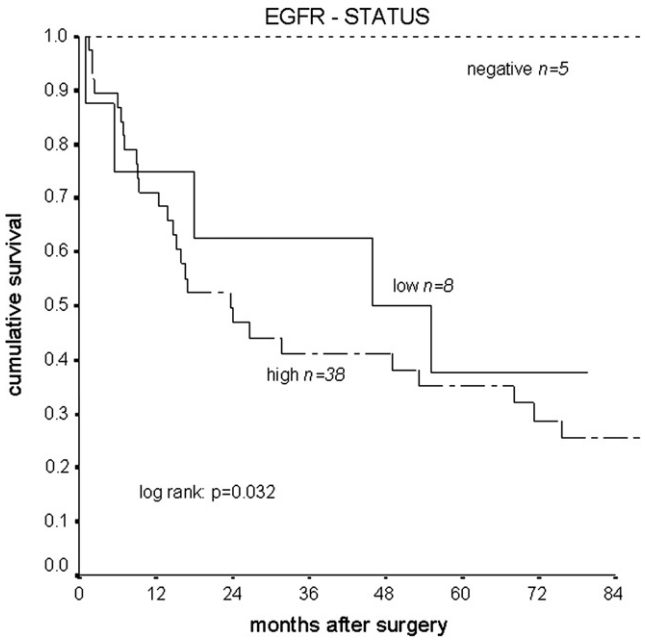

Figure 3 Prognostic significance of EGFR expression in 51 patients with oral squamous cell carcinoma regarding overall survival. These patients underwent surgery without adjuvant treatment. Tumours with high ($n = 38$), low ($n = 8$) without EGFR expression ($n = 5$).

Table 1 Multivariate analysis (Cox regression) of different prognostic parameters in patients with oral squamous cell carcinoma

	OS		
	p	RR[a]	95% CI[b]
Clinical stage (I,II vs III,IV)	0.013	2.4	1.2—4.8
Grade	0.017		
1 vs 2	0.025	2.1	1.09—4.01
1 vs 3	0.004	2.7	1.36—5.31
Lymph node metastasis	Ns[c]		
EGFR expression	0.024		
0 vs low	0.033	3.1	1.09—8.59
0 vs high	0.007	3.6	1.43—9.18

[a] Relative risk.
[b] Confidence Interval.
[c] Not significant.

Table 2 Correlation of EGFR expression with conventional clinicopathological parameters of 109 patients with oral squamous cell carcinoma

Characteristics	Patients	EGFR-expression			
		No	Low	High	*p*
Grading	*109*				
I	24	0 (0%)	7 (29.2%)	17 (70.8%)	$p = 0.24$
II	56	7 (12.5%)	9 (16.1%)	40 (71.4%)	
III	29	2 (6.9%)	4 (13.8%)	23 (79.3%)	
Clinical stage	*109*				
I + II	22	1 (4.5%)	7 (31.8%)	14 (63.6%)	$p = 0.17$
III + IV	87	8 (9.2%)	13 (14.9%	66 (75.9%)	
Lymph node metastasis	*109*				
No	46	3 (6.5%)	10 (21.7%)	33 (71.7%)	$p = 0.66$
Yes	63	6 (9.5%)	10 (15.9%)	47 (74.6%)	

with squamous cell carcinoma of the oral cavity and oropharynx. High EGFR expression correlates with poor overall survival, notably in patients that did not receive adjuvant therapy.

The more aggressive phenotype of EGFR expressing carcinomas may be attributable to the activation of different signalling pathways, such as the Ras-Raf mitogen-activated protein kinase, the phosphatidyl inositol 3′ kinase and Akt pathway. These pathways control diverse biologic processes, which sustain and promote the malignant behaviour of tumour cells, such as cellular proliferation, tumour cell motility, adhesion, inhibition of apoptosis[18] and angiogenesis.[21] Activated EGFR tyrosine kinase is involved in the progression of cells through G1 phase into S phase.[22]

Stimulation of EGFR leads to higher motility of cancer cells.[23] There are different downstream signalling pathways that can be linked to cell motility including activation of small GTP-binding proteins of the Rho subfamily,[24] PI3K and PLCg. This activation leads to morphogenic and motogenic changes and to alterations in adhesive interactions between tumour cells and extracellular matrix. Thus, tumour cell adhesion to extracellular matrix components can be modulated by EGFR activation.[25] Further, EGFR activation leads to activation of vascular endothelial growth factor, which is the primary inducer of angiogenesis.[26,27]

To sustain invasiveness and metastasis, cancer cells need proteolytic enzymes to invade extracellular matrix and penetrate lymphatic or blood vessel walls.[28] It has been shown, that collagenases, stromelysins, matrilysin and gelatinase B (MMP-9) can be upregulated by EGFR ligands. A direct correlation of EGFR expression and up-regulation of matrix metallo proteinases (MMP) has been described.[28]

The high expression of EGFR in oral squamous cell carcinoma makes this antigen an attractive target for target-specific therapeutic strategies in this tumour entity. Primarily, two different targeting strategies have been developed to inhibit the tumour promoting role of EGFR: monoclonal antibodies directed against the extracellular receptor domain and low molecular weight compounds that interfere with intracellular EGFR tyrosine kinase activity. What is the predictive value of EGFR immunohistochemistry for response to EGFR-specific treatment strategies? So far a correlation between the intensity of immunohistochemical staining of the tumour for EGFR and the presence or absence of a response to gefitinib has not been observed.[29] However, monoclonal antibodies are reported to have enhanced activity in colorectal cancer patients with high EGFR IHC status.[30] Thus, it can be assumed, that already few EGFR molecules per tumour cell are sufficient for specific tyrosine-kinase inhibitors to deploy their antitumour activity. By contrast, the antitumoural activity of monoclonal antibodies supposedly depends on the total amount of the target antigen on the cell membrane. It remains a matter of speculation, whether in particular the immune-mediated antitumour activity of monoclonal antibodies depends on the antigen density in tumour tissue.

The proper evaluation of the immunohistochemical EGFR status is still a matter of debate. The correlation between EGFR overexpression and clinicopathological parameters in head and neck squamous cell carcinoma (HNSCC) has been investigated in several studies. In some of these studies a correlation of EGFR expression with tumour grade was reported.[31,32] Most of these studies revealed a prognostic impact of EGFR expression in these patients.[12] However, varying sensitivity and specificity of antibody reagents and different dilutions of the antibodies may have caused discrepant results in defining antigen positivity and density. Thus, the cut-off for the number of positive cells defining an EGFR-positive tumour is mostly arbitrarily chosen and varies between research groups. Hence, a standardized method for EGFR staining and optimal thresholds need to be defined, to validate the immunohistochemical results for prospective trials.

This is the first study demonstrating the prognostic value of EGFR expression on a tissue microarray based immunohistochemical analysis. This technique enables same staining conditions for the whole tumour samples, reducing methodological errors. Besides the amount of EGFR antigen genetic abnormalities can also affect the efficacy of EGFR blockers.

Recent data in patients with non-small-cell lung cancer demonstrate a correlation between the clinical responsiveness to the tyrosine kinase inhibitor gefitinib and specific mutations in the EGFR gene.[33] Future studies have to clarify whether such somatic mutations of the EGFR gene are

specific for adenocarcinoma of the lung or if they can be found in a subset of oral squamous cell carcinomas as well.

In summary, this study confirms EGFR expression to be an important prognostic marker in patients with oral squamous cell carcinoma. Ongoing studies investigate the predictive value of EGFR expression for the response to EGFR specific therapeutics.

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