

Loss of EGFR expression in oral squamous cell carcinoma is associated with invasiveness and epithelial-mesenchymal transition.

Iyo Kimura, Hiroko Kitahara, Kazuhiro Ooi, Koroku Kato, Natuyo Noguchi, Kunio Yoshizawa, Hiroyuki Nakamura, Shuichi Kawashiri

Department of Oral and Maxillofacial Surgery, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan

Correspondence to: Hiroyuki Nakamura, D.D.S., Ph.D., Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan.

E-mail: hnak@me.com

Running title: KIMURA et al: LOSS OF EGFR EXPRESSION IN OSCC.

Key words: Oral squamous cell carcinoma, EGFR, cetuximab, EMT, invasiveness.

Abstract

In recent years, inhibition of EGFR signaling has emerged as new treatment strategy for oral squamous cell carcinoma (OSCC). The EGFR-directed inhibitor cetuximab is the only approved targeted therapy for the treatment of OSCC. The EGFR status may influence the response to cetuximab treatments. In this study, by analyzing the immunomarker for EGFR, we found that it indicated positivity in 58.3% of all cases we analyzed, and the invasiveness was inversely correlated with the expression of EGFR. We quantified the expression level of EGFR and analyzed the correlation between EGFR expression and cetuximab sensitivity using three different grade invasive human OSCC lines. The EGFR expression in high grade invasive cells was significantly down-regulated compared to low grade invasive cells. There was no significant anti-proliferative effect in high grade invasive cells treated with various concentrations of cetuximab. The EMT-associated genes N-cadherin, vimentin and Snail were up-regulated in high grade invasive cells. Low grade invasive cells displayed characteristics of typical epithelial cells, including expression of E-cadherin and absence of N-cadherin, vimentin and Snail. TGF-beta induced this low grade invasive cell to undergo an EMT-associated gene switch that results in low EGFR expression. These data suggested that loss of EGFR expression in OSCC was associated with EMT, and might have functional implications in tumor invasion and resistance to cetuximab treatments.

Introduction

The epidermal growth factor receptor (EGFR/ErbB1/HER1) is overexpressed in a majority of OSCC tumors, and high expression levels have been associated with a more aggressive phenotype, poor prognosis, and resistance to cancer therapy (1). Multiple anti-EGFR antibodies (e.g., cetuximab and panitumumab) and small molecule tyrosine kinase inhibitors (e.g., gefitinib and erlotinib) targeting EGFR have been developed. However, so far, only the monoclonal antibody cetuximab (Erbix®) has been approved for the treatment of advanced OSCC (2). Cetuximab treatment has been proven to be effective in recurrent or metastatic OSCC both as a first-line treatment in combination with platinum-based chemotherapy (3-6) and as a second-line treatment in patients

with platinum-refractory disease (7-9). In clinical practice, EGFR expression is evaluated using a standardized immunohistochemistry (IHC) assay, designated to assess cell membrane staining. Recent IHC studies demonstrated EGFR overexpression in over three quarters of the analyzed OSCC cases, with values of 73.42% (10), 87.5% (11) and 72% (12). However, the cetuximab response rate is generally not higher than 20% (13), so the role of EGFR expression as a predictor of response to EGFR targeting agents remains unclear.

The sensitivity of some tumors to cetuximab can be explained by the presence of mutations in the EGFR tyrosine kinase domain (14, 15). However, such mutations are rare in OSCC (16). Thus, there is a need to elucidate the mechanisms underlying the differential drug response of cancer cells with wild type EGFR in order to identify those patients who could respond and clinically benefit from cetuximab and to develop new therapeutic strategies to circumvent the *de novo* or acquired resistance of tumors to cetuximab. Ideally, these resistance mechanisms would also be targetable and would thus provide a possibility to increase the efficacy of cetuximab treatment. The tumors are often therapy-resistant and may have their origin in tumor cells with an epithelial-mesenchymal transition (EMT) phenotype. EMT is a process whereby epithelial cells lose their polarity and cell-cell contact and acquire a migratory mesenchymal phenotype. EMT has also been shown to promote stem cell properties. Furthermore, the *in vitro* sensitivity of head and neck cancer cell lines to anticancer agents has been shown to be influenced by the expression of EMT-associated genes (17, 18).

In the present study, we evaluated the expression of EGFR in human OSCC tissues and cell lines and investigated their relationship to clinicopathological factors, and analyzed with regard to their EMT characteristics. OSCC cell lines were also examined for their intrinsic sensitivity to cetuximab treatments.

Materials and methods

Tissue samples. The present study was approved by the ethics committee of the Kanazawa University Graduate School of Medical Science and written informed consent had been obtained. The subjects were 24 patients with

primary OSCC who underwent surgical resection at the Department of Oral and Maxillofacial Surgery at Kanazawa University Hospital between 1998 and 2008. The patients with carcinoma ranged from 43 years to 89 years old (64.4 ± 11.2 years old, mean \pm SD). The tumors' TNM categories were classified according to the UICC (Union for International Cancer Control) system. The grade of tumor differentiation was determined according to the criteria proposed by the World Health Organization. The mode of tumor invasion was assessed according to the classification by Yamamoto et al. (19).

Immunohistochemistry. Each specimen was fixed in 10% buffered formalin and then embedded in paraffin to prepare serial sections. Immunohistochemical staining was performed using EGFR pharmDx™ Kit (K1492, DAKO). The specificities of the staining were confirmed by using Universal Negative Control for IS-Series Rabbit Primary Antibodies (IS600, DAKO) instead of the primary antibody as a negative control. Three regions were image-captured under a light microscope at 100 \times magnification, and the images were evaluated based on number of stained tumor cells, following the method described by Bernardes et al. (20). EGFR expression was evaluated on the basis of extent of immunolabeling in tumor cell membranes and was classified on a four-point scale: 0 (no labeling, or labeling in < 10% of tumor cells); 1 (weak labeling, homogeneous or patchy, in > 10% of tumor cells); 2 (moderate labeling, homogeneous or patchy, in > 10% of tumor cells); 3 (intense labeling, homogeneous or patchy, in > 10% of tumor cells). These scores were subsequently grouped into two categories: low expression (0 or 1) and high expression (2 or 3).

Cell lines. Three human oral squamous cell carcinoma cell lines established from tumor biopsies with different grade of invasive abilities were used: OSC-20 (low grade invasive cells), OSC-19 (low grade invasive cells) and HOC313 (high grade invasive cells). OSC-20 is a cell line derived a 58-year-old female with

tongue cancer metastatic to the cervical lymph nodes (21). OSC-19 was derived from a 61-year-old male with tongue cancer metastatic to the cervical lymph nodes (22). HOC313 was derived from a 51-year-old female with squamous cell carcinoma (involving the mandibular gingiva and oral floor) metastatic to the cervical lymph nodes (23). In addition, normal human dermal fibroblast cells (NHDF) were used as a control.

Proliferation assay and cetuximab sensitivity. We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to test the ability of cetuximab to inhibit the proliferation of all three OSCC lines in vitro. First, 2500 cells per well were grown in DMEM medium (D7440, SIGMA) supplemented with 10% FBS in 96-well tissue culture plates. After 24 hours, the cells were treated with various concentrations of cetuximab (Erbixax®, Merck) (0, 0.005 and 0.01 µg/ml) diluted in DMEM medium supplemented with 2% FBS. To measure the number of metabolically active cells after 24, 48 and 72 hour incubation, we used an MTT and quantified the results according to the manufacturer's protocols. The optical density at 450 nm was measured using a microplate reader (iMark microplate reader, BioRad).

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR). The mRNA expression levels of EGFR, E-cadherin, Vimentin, Twist, and Snail1 were analyzed using a Rotor-Gene Q 2plex System (No.9001560, QIAGEN) with FAM/ZEN/IBFQ probes (DNA sequence was not opened, Integrated DNA Technologies, INC.). Total RNA was extracted with the RNeasy Protect Mini Kit (No.74124, Qiagen), and cDNA was obtained using PrimeScript 1st strand cDNA Synthesis Kit (code.6110A, TAKARA). All reactions and conditions followed the manufacturer's instructions. We amplified 18S rRNA as an internal standard using HEX/ZEN/IBFQ probes (DNA sequence was not opened, Integrated DNA Technologies, INC.). Data were calculated using the comparative CT method, which presents the data as a fold difference in expression level relative to a calibrator sample; in this case, the mean expression of the three NHDFs.

Enzyme-linked immunosorbent assay (ELISA). For enzyme-linked immunosorbent assay analysis of EGFR and phosphorylated EGFR, we cultured three human oral cancer cell lines (OSC-20, OSC-19 and HOC313). Cells were seeded into a 96-well tissue culture plate and fixed for 5 min by 4% formaldehyde solution in PBS after incubation for 24 hours. The expression of EGFR and phosphorylated EGFR was quantified using human EGFR In-Cell ELISA kit (ab126419, Abcam) according to the manufacturer's protocols. The data are presented as a fold difference in expression level relative to the mean expression of the three NHDFs.

Epithelial-mesenchymal transition (EMT) induction. To induce EMT, OSC-20 and OSC-19 cells were seeded at 70% confluence and cultured for 48 h and 72 h in Dulbecco's modified Eagle's medium with 0.5% fetal bovine serum. Recombinant human TGF-beta1 (240-B, R&D) was added to a final concentration of 5 ng/ml.

Statistical analysis. Statistical analyses were conducted using JMP® 9.0 (SAS Institute, Inc., Cary, NC, USA). The relationships between the expression of EGFR proteins and clinicopathological parameters were examined by the Pearson's chi-square test. The data of ELISA and MTT are presented as the mean values \pm SEM. The differences between groups were tested for statistical significance using the two-tailed Mann-Whitney U test. Differences were considered significant at $p < 0.05$.

Results

Association between EGFR expression and clinicopathological features. EGFR were detected in the cell membrane and cytoplasm of tumor cells (Figure 1). According to the criteria for EGFR immunohistochemical evaluation (20), 58.3% (14/24) of all cases were positive. Table 1 displays the correlations between EGFR expression and the clinicopathological factors. The EGFR expression on the high grade invasive OSCC (4D in mode of invasion) was significantly down-regulated compared to low grade invasive OSCC (3 and 4C in mode of invasion) ($p=0.039$). On the other hand, EGFR expression was not

correlated with tumor size ($p=0.991$), lymph node metastasis ($p=0.456$), local recurrence ($p=0.071$) or histological differentiation ($p=0.137$).

EGFR expression in three different grade invasive OSCC cell lines. As demonstrated by immunohistochemical analysis, only invasiveness (mode of invasion) was inversely correlated with the expression of EGFR. Therefore, EGFR expression on three different grade invasive human OSCC lines (OSC-19, OSC-20 and HOC313) was analyzed at both the mRNA and the protein level by using quantitative real-time PCR and ELISA, respectively; data are presented as a fold difference relative to the mean of three NHDFs (normal human dermal fibroblast cells) (Figure 2). Among the three OSCC cell lines (OSC-19, OSC-20 and HOC313), the EGFR mRNA expression level ranged from 3.7 to 0.74-fold higher compared to NHDF (Figure 2A). The expression of EGFR mRNA in two low grade invasive cells (OSC-20 and OSC-19) was 3.7 times and 2.3 times higher compared to NHDF, respectively. The EGFR mRNA expression in the high grade invasive cells (HOC313) was very low and almost similar to that of control normal cells. On the other hand, the two low grade invasive cells (OSC-20 and OSC-19) had an EGFR protein expression level 3.5-fold and 2.1-fold higher than those of NHDF, respectively (Figure 2B). High grade invasive cells (HOC313) exhibited the lowest EGFR protein expression. The amounts of phosphorylated EGFR were assessed to determine the activation status of EGFR. Expression levels of phosphorylated EGFR ranged from 2.2 to 0.87-fold higher compared to NHDF (Figure 2C). Expression patterns of phosphorylated EGFR were similar to patterns of mRNA and of EGFR protein.

Cetuximab sensitivity of three different grade invasive OSCC cell lines. To evaluate the importance of EGFR status on cetuximab sensitivity, we further evaluated the cetuximab sensitivity of three human OSCC lines (OSC-19, OSC-20 and HOC313) with different grade of invasive abilities. Cells were seeded at clonal density, and the anti-proliferative effect of cetuximab was determined by MTT assay (Figure 3). Anti-proliferative effects of cetuximab were observed in OSC-20 and OSC-19 cell lines. In OSC-20 and OSC-19 cells (both are low grade invasive OSCC), after incubation for 48 and 72 hours in cetuximab

0.005 µg/ml and 0.01 µg/ml, the proliferation was significantly inhibited compared to non-treated cells. However, there was no significant anti-proliferative effect in HOC313 cell lines (high grade invasive OSCC) treated with various concentrations of cetuximab.

EMT phenotype on high grade invasive OSCC cell lines. To evaluate whether EGFR expression is associated with features of EMT, we tested levels of E-cadherin, N-cadherin, vimentin and Snail in three different grade invasive human OSCC lines (OSC-19, OSC-20 and HOC313) (Figure 4A). The expression levels of these EMT associated gene in the OSC-19 and HOC313 are presented as fold differences relative to the OSC-20. The HOC313 (high grade invasive cells and loss of EGFR expression cells) displayed a mesenchymal phenotype manifested by loss of E-cadherin and acquisition of N-cadherin, vimentin and Snail compared to the OSC-20 and OSC-19 (Figure 4A). To examine whether TGF-beta changes expression of the EMT associated gene and EGFR, two low grade invasive cells (OSC-20 and OSC-19) were exposed to TGF-beta (Figure 4B). The expression levels are presented as fold differences relative to the control vehicle at 48 and 72 hours, respectively. Serial examination of EMT markers (loss of E-cadherin and upregulation of N-cadherin, vimentin and Snail) in a time course (48 and 72 hours) showed that TGF-beta treatment resulted in EMT on OSC-20 (Figure 4B). On the other hand, the TGF-beta was not effective in EMT induction on OSC-19 cells. Strikingly, total mRNA of EGFR of OSC-20 and OSC-19 cells was reduced after EMT induction by TGF-beta treatment.

Discussion

The EGFR is an oncogene (a tyrosine-kinase receptor from the erbB family) identified in more than one malignancy, including breast cancer, prostate cancer, pulmonary cancer, bladder cancer and head and neck cancers (24). EGFR is overexpressed and appears to be the dominant control factor of the malignant phenotype in head and neck squamous cell carcinomas, through the adjustment of the molecules involved in invasive angiogenic and lymphangiogenic processes (10). By analyzing the immunomarker for EGFR in

this study, we found that it indicated positivity in 58.3% of all cases analyzed. This data were consistent with previous reports (10-12). And, we demonstrated for the first time that invasiveness (mode of invasion) was inversely correlated with the expression of EGFR on OSCC patients. We also have shown loss of EGFR expression in high grade invasive OSCC line (HOC313 cell). These data suggested that loss of EGFR expression was associated with invasive phenotype of OSCC. This potential association will be investigated in future studies.

We analyzed the correlation between expression of EGFR and cetuximab sensitivity using three human OSCC lines (OSC-19, OSC-20 and HOC313) with different grade of invasive abilities. We found variability in the response of different cell lines to cetuximab treatment. Cetuximab suppressed cell growth by approximately 23% in the most sensitive cell line (OSC-20), but it had no effect on HOC313 cell lines. The OSC-20 cell line showed a substantial increase in EGFR mRNA and protein compared to HOC313 cell lines. Conversely, the HOC313 cell line, which is not effected by cetuximab treatment, was the one that had the lowest EGFR expression. When evaluating the influence of the EGFR expression on the cetuximab treatment response, there was a significant difference in EGFR expression between sensitive cells (OSC-20) and resistant cells (HOC313) (Figures 2 and 3). Resistant cell lines had lower expression levels of EGFR, suggesting that the cetuximab treatment response may depend on the expression status of EGFR. In addition, a high expression level of phosphorylated EGFR tended to correlate with cetuximab sensitivity, indicating a role for activated EGFR in cetuximab response. Indeed, several studies have suggested that cells are sensitive to EGFR inhibition only if they are dependent on EGFR activation, cell survival, and growth (25). In concordance with these results, the level of phosphorylated EGFR has been to predict gefitinib (an EGFR tyrosine kinase inhibitor) sensitivity in OSCC.

The response to EGFR-targeted agents is inversely correlated with epithelial mesenchymal transition (EMT) in multiple types of tumors without known EGFR mutations, including NSCLC, bladder, colorectal, pancreas and breast carcinomas (26-30). In this study, the EMT associated genes, N-cadherin, vimentin and Snail, were up-regulated in cetuximab resistant OSCC tumor cells

(HOC313). We also demonstrated that TGF-beta induced low grade invasive OSCC cell lines (OSC-20) to undergo an EMT-associated gene switch that results in their loss of EGFR expression. These results indicated that the net effect of TGF-beta signaling was the loss of EGFR activity with a concomitant EMT-associated gene switch of tumor cells, thereby leading to a migratory mesenchymal phenotype. Further studies are necessary to elucidate the precise molecular mechanisms underlying this association between EMT and regulation of EGFR expression.

In this study, we demonstrated that EGFR status influenced the response to cetuximab treatments, and invasiveness and an EMT phenotype could be useful to predict EGFR status. EMT related changes in gene expression, e. g., upregulation of N-cadherin, vimentin and Snail have also been proposed as potential biomarkers for EGFR status and prediction of response to cetuximab treatment. Further *in vivo* studies are necessary to confirm these result using clinical samples.

Acknowledgements

We thank American Journal Experts (AJE) for English language editing.

References

1. Ang KK, Berkey BA, Tu X, et al.: Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 62: 7350-7356, 2002.
2. Loeffler-Ragg J, Schwentner I, Sprinzl GM and Zwierzina H: EGFR inhibition as a therapy for head and neck squamous cell carcinoma. *Expert Opin Investig Drugs* 17: 1517-1531, 2008.
3. Bourhis J, Rivera F, Mesia R, et al.: Phase I/II study of cetuximab in combination with cisplatin or carboplatin and fluorouracil in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* 24: 2866-2872, 2006.
4. Burtness B, Goldwasser MA, Flood W, Mattar B and Forastiere AA: Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus

cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. *J Clin Oncol* 23: 8646-8654, 2005.

5. Shin DM, Donato NJ, Perez-Soler R, et al.: Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. *Clin Cancer Res* 7: 1204-1213, 2001.

6. Vermorken JB, Mesia R, Rivera F, et al.: Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med* 359: 1116-1127, 2008.

7. Baselga J, Trigo JM, Bourhis J, et al.: Phase II multicenter study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck. *J Clin Oncol* 23: 5568-5577, 2005.

8. Herbst RS, Arquette M, Shin DM, et al.: Phase II multicenter study of the epidermal growth factor receptor antibody cetuximab and cisplatin for recurrent and refractory squamous cell carcinoma of the head and neck. *J Clin Oncol* 23: 5578-5587, 2005.

9. Vermorken JB, Trigo J, Hitt R, et al.: Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. *J Clin Oncol* 25: 2171-2177, 2007.

10. P Oc, Rhys-Evans PH, Modjtahedi H and Eccles SA: The role of c-erbB receptors and ligands in head and neck squamous cell carcinoma. *Oral Oncol* 38: 627-640, 2002.

11. Sarkis SA, Abdullah BH, Abdul Majeed BA and Talabani NG: Immunohistochemical expression of epidermal growth factor receptor (EGFR) in oral squamous cell carcinoma in relation to proliferation, apoptosis, angiogenesis and lymphangiogenesis. *Head Neck Oncol* 2: 13, 2010.

12. Ryott M, Wangsa D, Heselmeyer-Haddad K, et al.: EGFR protein overexpression and gene copy number increases in oral tongue squamous cell carcinoma. *Eur J Cancer* 45: 1700-1708, 2009.

13. Bonner JA, Harari PM, Giralt J, et al.: Radiotherapy plus cetuximab for

squamous-cell carcinoma of the head and neck. *N Engl J Med* 354: 567-578, 2006.

14. Lynch TJ, Bell DW, Sordella R, et al.: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129-2139, 2004.

15. Paez JG, Janne PA, Lee JC, et al.: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500, 2004.

16. Lemos-Gonzalez Y, Paez de la Cadena M, Rodriguez-Berrocal FJ, Rodriguez-Pineiro AM, Pallas E and Valverde D: Absence of activating mutations in the EGFR kinase domain in Spanish head and neck cancer patients. *Tumour Biol* 28: 273-279, 2007.

17. Hsu DS, Lan HY, Huang CH, et al.: Regulation of excision repair cross-complementation group 1 by Snail contributes to cisplatin resistance in head and neck cancer. *Clin Cancer Res* 16: 4561-4571, 2010.

18. Skvortsova I, Skvortsov S, Raju U, et al.: Epithelial-to-mesenchymal transition and c-myc expression are the determinants of cetuximab-induced enhancement of squamous cell carcinoma radioresponse. *Radiother Oncol* 96: 108-115, 2010.

19. Yamamoto E, Kohama G, Sunakawa H, Iwai M and Hiratsuka H: Mode of invasion, bleomycin sensitivity, and clinical course in squamous cell carcinoma of the oral cavity. *Cancer* 51: 2175-2180, 1983.

20. Bernardes VF, Gleber-Netto FO, Sousa SF, Silva TA and Aguiar MC: Clinical significance of EGFR, Her-2 and EGF in oral squamous cell carcinoma: a case control study. *J Exp Clin Cancer Res* 29: 40, 2010.

21. Yokoi T, Hirata S, Nishimura F, Miyakawa A, Odajima T and Kohama G: Some properties of a newly established human cell line derived from an oral squamous carcinoma. *Tumor Res* 25: 93-91-93, 1990.

22. Yokoi T, Homma H and Odajima T: Establishment and characterization of OSC-19 cell line in serum and protein free culture. *Tumor Res* 24: 1-17, 1988.

23. Ishisaki A, Oida S, Momose F, et al.: Identification and characterization of autocrine-motility-factor-like activity in oral squamous-cell-carcinoma cells. *Int J Cancer* 59: 783-788, 1994.

24. Oliveira S, van Bergen en Henegouwen PM, Storm G and Schiffelers RM: Molecular biology of epidermal growth factor receptor inhibition for cancer therapy. *Expert Opin Biol Ther* 6: 605-617, 2006.
25. Pernas FG, Allen CT, Winters ME, et al.: Proteomic signatures of epidermal growth factor receptor and survival signal pathways correspond to gefitinib sensitivity in head and neck cancer. *Clin Cancer Res* 15: 2361-2372, 2009.
26. Yauch RL, Januario T, Eberhard DA, et al.: Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients. *Clin Cancer Res* 11: 8686-8698, 2005.
27. Thomson S, Petti F, Sujka-Kwok I, Epstein D and Haley JD: Kinase switching in mesenchymal-like non-small cell lung cancer lines contributes to EGFR inhibitor resistance through pathway redundancy. *Clin Exp Metastasis* 25: 843-854, 2008.
28. Thomson S, Buck E, Petti F, et al.: Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res* 65: 9455-9462, 2005.
29. Adam L, Zhong M, Choi W, et al.: miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. *Clin Cancer Res* 15: 5060-5072, 2009.
30. Barr S, Thomson S, Buck E, et al.: Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. *Clin Exp Metastasis* 25: 685-693, 2008.

Figure Legends

Figure 1. Immunohistochemistry stain of EGFR in human OSCC tissues. (A) Positive immunoexpression of EGFR in OSCC. (B) The specificities of the staining were confirmed by using non-immune antibody instead of the primary antibody as a negative control. Scale bar, 100 μ m.

Figure 2. EGFR expression on three different grade invasive human OSCC lines. The OSC-19, OSC-20 and HOC313 cell lines were evaluated for differences regarding (A) EGFR mRNA, (B) EGFR protein and (C) phosphorylated EGFR expression. The data are presented as a fold difference in expression level relative to the mean expression of the three NHDFs. Horizontal lines represent median values.

Figure 3. The cetuximab sensitivity of three different grade invasive human OSCC lines. OSC-20, OSC-19 and HOC313 cells were treated with various concentrations of cetuximab as indicated and the anti-proliferative effect of cetuximab was determined by MTT assay. Each data point represents the mean of three independent experiments. The vertical bars show standard deviations. Error bars correspond to SEM. *P<0.05.

Figure 4. EGFR and EMT-associated genes expression on three different grade invasive human OSCC lines. (A) The high grade invasive OSCC cell line (HOC313) showed up-regulation of EMT-associated genes. Relative mRNA expression levels of EMT-associated genes: E-cadherin, N-cadherin, vimentin and Snail in OSC-20, OSC-19 and HOC313 cell lines. The expression levels in the OSC-19 and HOC313 are displayed here as fold differences relative to the OSC-20. (B) TGF-beta induced EMT-associated genes in low grade invasive OSCC cell line (OSC-20 and OSC-19). The cells were treated with human TGF-beta or control vehicle for 48 and 72 hours. Relative mRNA expression levels of EGFR and EMT-associated genes: E-cadherin, N-cadherin, vimentin and Snail in OSC-20 and OSC-19 cell lines. The expression levels are displayed here as fold differences relative to the control vehicle at 48 and 72 hours, respectively.

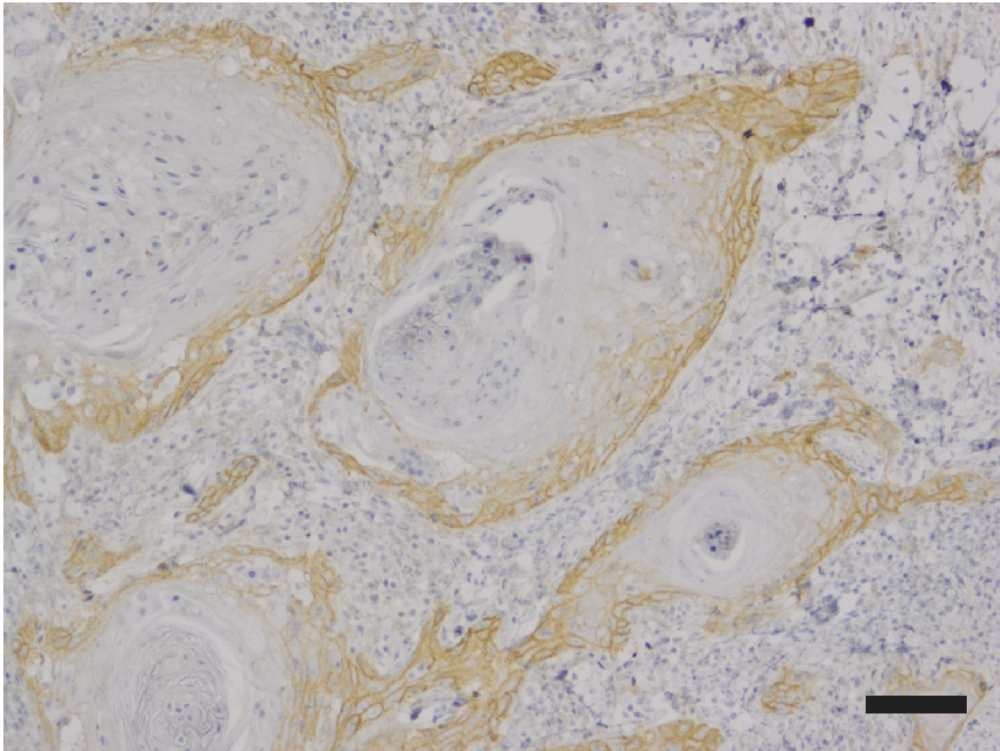
Table 1. Correlation between EGFR expression and the OSCC patients' clinicopathological factors

Factor		EGFR expression		P value
		High (n=14)	Low (n=10)	
T category	T1	3	2	0.991
	T2	8	6	
	T3	1	0	
	T4	2	2	
N category	negative	13	7	0.456
	positive	1	3	
Local recurrence	negative	10	4	0.071
	positive	4	6	
Differentiation	Well	10	7	0.137
	Moderately	3	0	
	Poorly	1	3	
Mode of invasion	Grade 3	7	1	*0.039
	Grade 4C	5	3	
	Grade 4D	2	6	

*P< 0.05

Figure 1.

A



B

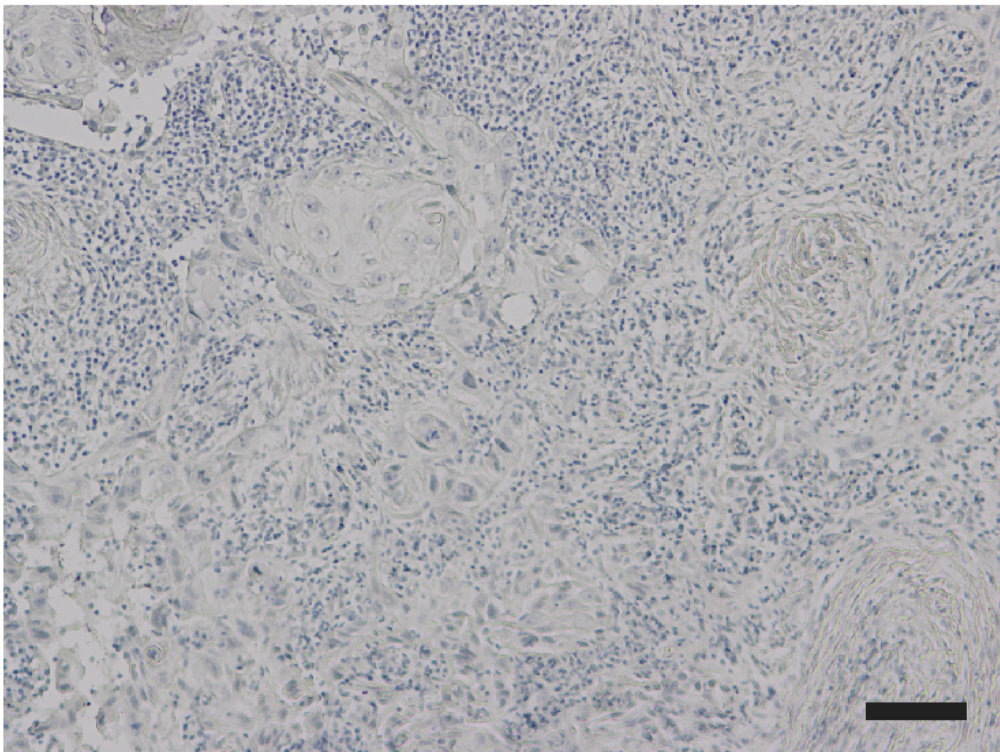


Figure 2.

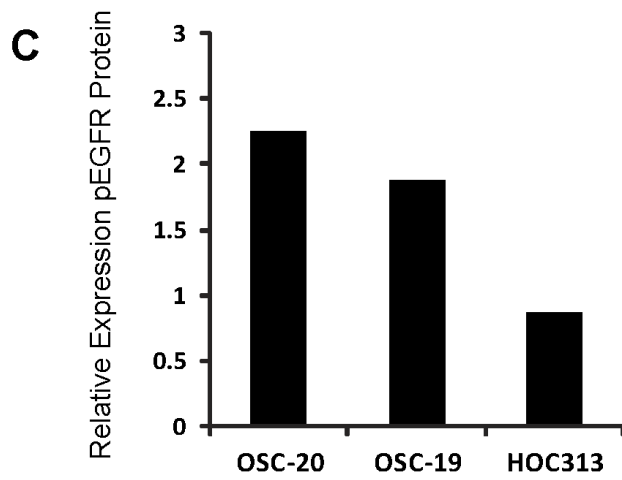
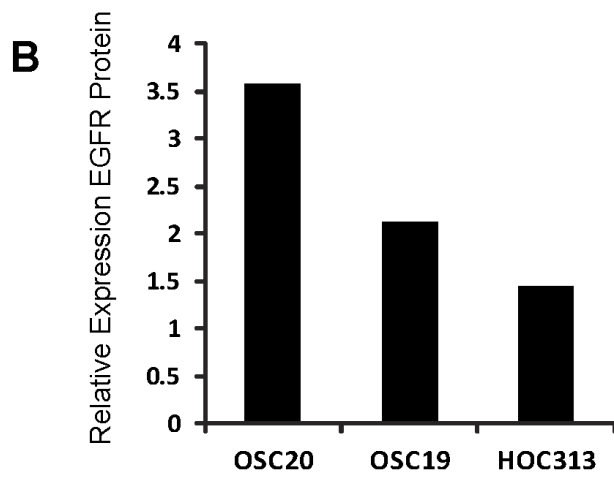
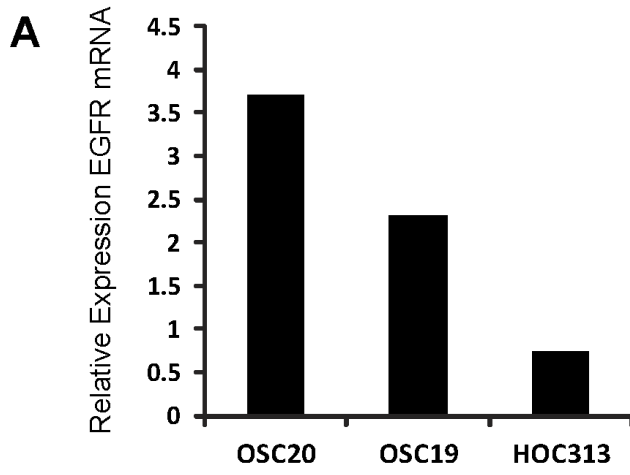


Figure 3.

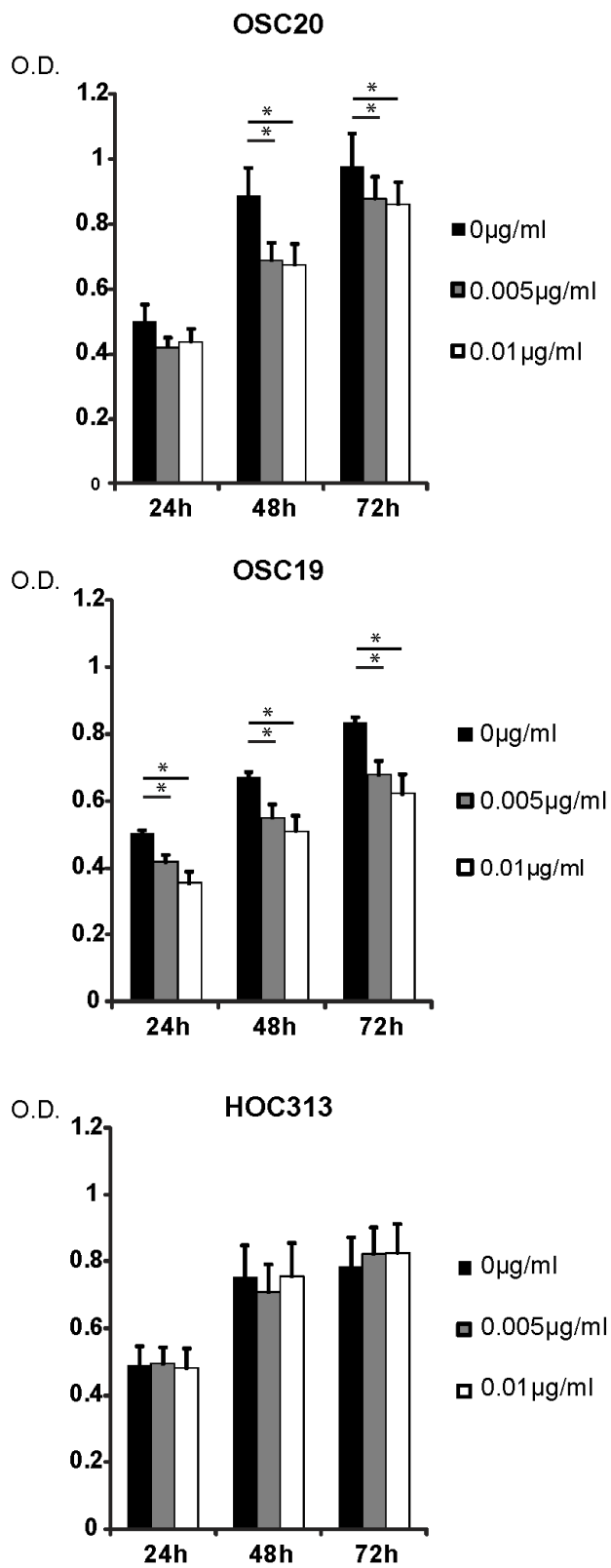


Figure 4.

