Published in final edited form as:

Head Neck. 2013 September ; 35(9): 1323–1330. doi:10.1002/hed.23128.

The effect of erlotinib on EGFR and downstream signaling in oral cavity squamous cell carcinoma

Christina I. Tsien^{1,*}, Mukesh K. Nyati^{1,*}, Aarif Ahsan¹, Susmita G Ramanand¹, Douglas B. Chepeha², Francis P. Worden³, Joseph I. Helman⁴, Nisha D'Silva⁵, Carol R. Bradford², Gregory T. Wolf², Theodore S. Lawrence¹, and Avraham Eisbruch¹

¹University of Michigan, Department of Radiation Oncology

²University of Michigan, Department of Otolaryngology

³University of Michigan, Department of Internal Medicine, Division of Hematology-Oncology

⁴University of Michigan, Department of Oral and Maxillofacial Surgery

⁵Department of Oral Medicine/Pathology and Oncology, School of Dentistry

Abstract

Purpose—To determine if there are differences in biomarker modulation and EGFR degradation between tumor and the normal mucosa following treatment with an EGFR inhibitor, erlotinib, in head and neck cancer.

Methods—Patients with primary oral cavity squamous cell cancers received a course of erlotinib, 150 mg qd for 7 days prior to surgical resection. Tumor and normal mucosa biopsies were obtained both pre and post erlotinib. Changes in known markers of EGFR activity (phospho, AKT, STAT3) were measured by immunoblotting, while changes in tissue distribution were analyzed by immunohistochemical analysis.

Results—Twelve patients were enrolled; seven had evaluable paired tumor and normal mucosa biopsies pre and post treatment. Expression of EGFR was higher in tumor compared to the normal mucosa ($p=0.005$). Erlotinib administration was associated with marked inhibition of pEGFR and reduction in total EGFR protein ($p=0.004$, $p=0.007$) in tumors while there was heterogeneity in EGFR inhibition in the normal mucosa ($p=0.1$ (pEGFR), and $p=0.07$ (EGFR). Reduced levels of pSrc and pSTAT3 and enhanced p27 levels were noted in tumors following erlotinib. Cell culture studies confirmed that EGFR is degraded in tumor cells after prolonged treatment with erlotinib.

Conclusion—Our results show that EGFR inhibition by erlotinib led to a marked reduction in EGFR protein levels in patients. Differential effects of erlotinib on tumor compared to the normal mucosa suggest there may be individual patient heterogeneity. These preliminary data suggest EGFR degradation should be further analyzed as a potential biomarker in selecting patients likely to benefit from EGFR inhibitors.

Corresponding author: Christina Tsien, M.D., University of Michigan Medical Center, Dept. of Radiation Oncology, 1500 E. Medical Center Drive, Ann Arbor, Michigan 48109, Tel: 734-936-4288, Fax: 734-763-7370, ctsien@umich.edu.

*These authors equally contributed to this study.

Presented in part at the 7th International Conference on Head & Neck Cancer, San Francisco, CA 2008 and annual ASCO meeting, Orlando, Florida, 2009

Introduction

Epidermal growth factor receptor (EGFR) represents a promising molecular target that regulates both the growth and potential spread of squamous cell carcinomas of the head and neck (1–4). Although 85–100% of head and neck squamous cell carcinomas are noted to have over-expression of EGFR, the clinical response rate produced by an EGFR inhibitor alone is only 10–15%. There has been no direct correlation noted between EGFR overexpression and clinical response (5–7). Other molecular predictors of response are needed to select patients most likely to benefit from targeted therapies (8). Unfortunately, although EGFR gene mutations predict response to EGFR tyrosine kinase inhibitors, such as erlotinib (9–11), in lung adenocarcinoma, there is no evidence of activating EGFR mutations in head and neck cancer (12–15). Similarly, neither EGFR gene amplification, polysomy, nor truncation (EGFRvIII) predicts response to EGFR inhibitors in head and neck cancer patients (although they do carry prognostic value) (12, 16–18).

Phosphorylation is a key factor in predicting response to EGFR inhibitors in preclinical studies (19–21). However, there is increasing preclinical evidence that EGFR receptor degradation could play an even greater role in predicting response (19, 22–26). For instance, knockdown of EGFR with small interfering ribonucleic acid (siRNA) can induce autophagic cell death independent of receptor tyrosine kinase activity (27). We have also found that EGFR degradation is an important mechanism that regulates chemotherapy-induced cytotoxicity (24, 26). These findings suggest that EGFR receptor degradation may be more effective in producing cytotoxicity of EGFR driven tumors than inhibition of EGFR activity alone.

We hypothesized that inhibition of EGFR signaling and/or EGFR degradation may be an important predictor of response. A first step in testing this hypothesis, and the primary aim of this pilot study, was to determine if erlotinib could produce inhibition of downstream EGFR signaling and EGFR degradation in patients with head and neck cancer. A secondary aim of this study was to determine if there were differences in EGFR levels as well as other possible biomarkers between tumor and the normal mucosa. Acute and late pharyngeal toxicities are the major cause of morbidity in head and neck patients treated with concurrent chemo-radiation (27–28). Although targeted therapies are anticipated to have less toxicity compared to chemotherapy due to selective cell kill, the differential effects of EGFR inhibition in tumor compared to normal tissue have not yet been studied.

Methods and Materials

Patient Characteristics

Patients eligible for this study had histologically confirmed head and neck squamous cell carcinoma (HNSCC) that required primary surgical resection. Eligibility criteria included age greater than 18 years, Zubrod score of 2, and ability to provide written consent. Exclusion criteria included prior EGFR antibody or tyrosine kinase inhibitor therapy, known malabsorption syndrome or any other condition that would impair absorption of study drug, and concurrent serious infections or coexisting medical problems that would limit study

compliance. Acceptable hematologic, renal, and liver function was required. Pregnant and lactating women were excluded from study.

Treatment Plan

All patients underwent a physical examination, medical history, laboratory evaluation and CT imaging at baseline. Toxicities were graded using the NCI common toxicity criteria (CTC) version 3.0. Patients were instructed to start oral erlotinib 150 mg po qd, seven days prior to surgical resection. The final erlotinib dose was taken at least 8 hours prior to surgical resection. In the event of a grade 2 or greater diarrhea or skin rash, the drug was withheld until resolution and then restarted at 100 mg po qd. The numbers of pills the patient had taken and the time at which the final dose was taken were recorded at the follow-up visit.

Tissue Biopsies

Baseline tissue core biopsies of a minimum of 3 mm × 3 mm sample from both the tumor and the uninvolved contra-lateral oral mucosa were obtained. These samples were collected in ice-cold saline containing a cocktail of protease (Roche Diagnostic Co., Indianapolis, IN) and phosphatase inhibitors (Sigma, St. Louis, MO). Following a 7-day course of EGFR tyrosine kinase inhibitor erlotinib, repeat core tumor and contralateral normal appearing mucosa biopsies were obtained at time of surgical resection in similar regions as the initial baseline biopsies. Biopsies were divided into two parts with one used in high-throughput and traditional immunoblot analysis, and the second was fixed in formalin for immunohistochemical analysis. EGFR phosphorylation, total EGFR and associated downstream signaling pathways were analyzed by immunoblotting and immunostaining as described below. All tumor and normal tissue biopsy specimens were also reviewed by an experienced head and neck pathologist (NDS).

High-throughput immunoblotting

Tissue samples were lysed in the sample extraction buffer, and immunoblotting was performed using a protocol previously described (29). A total of 100 µg protein was subjected to electrophoresis on a 2D 4 to 12% bis-tris precast gel (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene fluoride (PVDF) membrane. A Miniblotter 28 dual system (Immunelect, Cambridge, MA) was used to probe all the antibodies (Figure 2A). After incubating the membrane with different antibodies overnight, membranes were washed for 30 minutes with TBS-T and probed with horseradish peroxidase conjugated IgG (Cell Signaling Technology, Beverly, MA), diluted 1:10,000 in TBST for 1 hr at room temperature; the antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL-Plus; Amersham Biosciences, Piscataway, NJ). For quantification of relative protein levels, immunoblot films were scanned and analyzed using NIH ImageJ software. Unless otherwise indicated, the relative protein levels shown represent a comparison to untreated controls.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (28). 4–5 µm sections were prepared using a microtome and placed onto slides. Sections were subjected to heat-mediated antigen retrieval in citrate buffer (10 mM citric acid, pH 6.0). After blocking with 5% donkey serum for 1h at room temperature, sections were incubated with primary antibody overnight at 4° C in a humidified chamber. After three PBS washes, HRP-conjugated second antibody was used to form a complex with the primary antibody, and unbound anti-bodies were removed by washing in PBS-T three times. Finally, this complex was visualized using the ECL Peroxidase Substrate Kit (Vector Labs, CA), and sections were counterstained with hematoxylin, washed and mounted. Images were acquired using a DP70 camera fitted on an Olympus 1X-71 microscope. Apoptotic cell death was assessed using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Milipore, Massachusetts, USA). Slides were evaluated by two investigators, and three areas from each section were analyzed.

Statistics

Analysis was performed using SPSS for Windows statistical software package (SPSS version 11; SPSS, Chicago, IL). A pairwise two-tailed Student's t test was used to evaluate immunohistochemistry results pre and post treatment for paired biopsies. The results of statistical tests were considered statistically significant at $p < 0.05$.

Results

Patient Characteristics

Twelve patients were enrolled in the study. Baseline characteristics are shown in Table 1. Median age was 72 (range: 21–86) years. Seven were female and 5 male. Median Zubrod score was 0 (range: 0–1). Primary oral cavity tumors were included; floor of mouth (3), tongue (8), and retromolar trigone (1). The majority (7 of 12) of the tumors were AJCC T3 or T4. Six patients also had nodal disease. Smoking status was available in all patients: current smokers (5), former smokers (4) and never smokers (3). All 12 patients had pre-treatment tumor biopsies. One patient underwent repeat positron emission tomography (PET) scan following enrollment and was noted to have newly diagnosed metastatic lung disease and therefore did not undergo surgical resection as planned. Tumor specimens were non-evaluable due to technical issues in 2 cases. In 4 cases, baseline normal tissue biopsies at post-processing were non-evaluable due to the small size of the biopsies. Therefore, a total of 9 paired tumor and 7 paired normal tissue biopsies were available for analysis.

Pathologic and Immunohistochemistry Analyses

An initial qualitative analysis of the normal oral mucosa versus tumor cells was obtained using hematoxylin and eosin (H&E), PAN-cytokeratin, and Ki-67 staining (Figure 1). PAN-cytokeratin and H&E staining were utilized to confirm the presence of tumor cells. Following erlotinib treatment, there was a decrease in PAN-cytokeratin staining along with altered cellular architecture. Likewise, Ki67, a marker for proliferation, was markedly suppressed by erlotinib in tumor, while minimal effects were noted in the normal mucosa.

Additional qualitative analysis using immunohistochemistry (IHC) of EGFR confirmed strong staining in the pre-treatment tumor biopsies. There was a dramatic reduction of EGFR staining following a 7-day course of erlotinib. Normal mucosa biopsies showed a more variable response and did not consistently demonstrate a similar decrease in EGFR staining (see Figure 1 and 2). We also wanted to determine if erlotinib could cause tumor cell death following a one-week course of treatment. We noted a modest increase in the number of apoptotic cells upon immunostaining in the tumor tissue post treatment.

Immunoblotting Analyses

In order to quantify the effects of erlotinib treatment on both tumor and normal tissue, we performed immunoblotting (IB) for both the paired tumor (9) and normal tissue (7) biopsies. Erlotinib treatment led to a marked decrease of both pEGFR (both the Y845 site and the Y1173 site) and total EGFR protein ($p=0.004$, $p=0.007$, respectively) in the tumor biopsies (Figure 2A and B). In contrast, EGFR inhibition in the normal mucosa was more heterogeneous showing a weak and non-significant decreasing trend following erlotinib for both pEGFR ($p=0.1$) and total EGFR protein ($p=0.07$), see Figure 2C and D. We also analyzed the effects of erlotinib on other key downstream signaling molecules of EGFR. We compared pre and post erlotinib matched tumor samples and found that levels of pSrc, pSTAT3, pERK, and pAKT were significantly reduced following treatment with erlotinib. Total Src, STAT, ERK, and AKT were relatively unchanged, demonstrating that, among key signaling molecules, EGFR was specifically decreased. Our preclinical studies support that this is likely due to receptor degradation following erlotinib treatment (see Figure 3).

In addition to assessing the effect of 7 days of erlotinib treatment on kinases involved in growth and invasion, we also assessed the cell cycle checkpoint protein p27 (Figure 2A and 3B). As expected, we found a significant increase in p27 levels. In preclinical studies, it has been shown that this is associated with cell accumulation in G1-phase (30). We also assessed the effect of erlotinib treatment on B-cell CLL/lymphoma-2 (Bcl2) levels which is a known inhibitor of apoptotic cell death. We saw a decrease in Bcl2 levels in the patient tumor specimens, whereas in the normal tissue (not shown) or normal cells we observed a modest increase. Finally, we evaluated housekeeping proteins GAPDH and HSP90, neither of which showed any significant change in levels in response to erlotinib treatment (Figure 2 and 3). These key findings support the use of multiplex immunoblotting as a method for assessing multiple signal transduction molecules in prospective clinical studies.

Single Vs daily exposure to erlotinib in cultured cells

As erlotinib has chiefly been reported to block EGFR phosphorylation rather than cause EGFR degradation, we conducted cell culture studies to better understand these clinical observations. Whereas a single exposure to erlotinib only inhibited EGFR phosphorylation, continued exposure led to EGFR degradation and p27 accumulation (Figure 3A). We also determined the effect of EGFR inactivation on the downstream molecules shown in the Figure 1B in 2 tumor and 2 normal cell lines. Similar to patient data (Figure 2B), both of the tumor cell lines showed a decrease in total EGFR levels as well as downstream signaling molecules. Normal fibroblasts and the MRC-5 normal cells line were also treated with erlotinib for comparison (see Figure E, F), which caused an increase in Bcl2. This suggests

that erlotinib might protect normal cells from apoptosis. Other key signaling molecules were minimally altered.

Discussion

This pilot study confirms that pEGFR, total EGFR, and key downstream signaling molecules are consistently decreased in head and neck tumors following a one-week course of the tyrosine kinase EGFR inhibitor, erlotinib. In contrast, there was substantial heterogeneity in the effects of erlotinib on the normal mucosa in patients. The findings of a decrease in total EGFR in head and neck patients is novel and is consistent with our prior preclinical findings (19). Our data suggests a potential rationale for using molecular biomarkers to permit the selection of patients most likely to realize a therapeutic index with erlotinib in head and neck tumors. Given the small patient numbers, a larger prospective clinical trial is currently being conducted to confirm the finding of EGFR degradation as a potential important molecular biomarker of cytotoxicity.

The current standard of care for good performance patients with locally advanced head and neck cancer is chemoradiation, concurrent cetuximab and radiation, or primary surgery followed by adjuvant chemo-radiation (31–34). A Phase III randomized study showed that there is no further improvement in disease-free or overall survival from the addition of cetuximab in unselected patients receiving concurrent chemo-radiation with cisplatin (31). After a median follow-up of 2.4 years, there was no difference in progression-free survival (PFS) or 2-year overall survival; 82.6% in patients receiving cetuximab and 79.7% in patients treated without the monoclonal antibody. In this trial, patients in the cetuximab arm had a significantly higher incidence of severe (grade 3/4) mucositis (43% vs 33%; $P = .004$), in-field skin reactions (25% vs 15%; $P < .001$), and out-of-field skin reactions (19% vs 1%; $P < .001$). Late toxicity (> 90 days), including persistent dysphagia, occurred in a similar proportion of patients in each treatment arms (31). Thus, it is unclear which patients may benefit from the addition of EGFR inhibitors to concurrent chemo-radiation. Identification of potential molecular biomarkers predictive of response would be beneficial in selecting patients most likely to respond to targeted therapies not only in regards to efficacy but, potentially, in reducing normal tissue toxicity. Such information might also be useful in the national ongoing randomized Phase III trial for patients with favorable HPV-16 related oropharyngeal cancer to determine whether EGFR inhibition with radiation and weekly cetuximab is more efficacious than concurrent chemotherapy and radiation and also to confirm if the morbidity is reduced.

Our findings suggest that EGFR degradation is a potential biomarker of response to targeted therapies. Degradation appears to be an important mechanism of cell death in head and neck tumors that are dependent on EGFR signaling. As EGFR plays a role in DNA repair (35), EGFR degradation may decrease the ability of cells to repair damaged DNA, leading therefore to increased cytotoxicity. We have demonstrated that decreased EGFR levels following gemcitabine (26) or cisplatin chemotherapy correlate with decreased clonogenic survival. Furthermore, inhibiting EGFR degradation by treating with lysosomal or proteosomal inhibitors (26) or by introducing mutations that prevent c-Cbl from binding to EGFR (24), a key step in proteosomal degradation, decreases chemotherapy-induced

cytotoxicity (24). Cetuximab and erlotinib can also cause EGFR degradation (24). EGFR degradation occurs along a pathway similar to EGF-induced receptor downregulation. The loss of EGFR can lead to the down-regulation of pAKT causing apoptosis. Our *in vitro* studies presented in the current study confirm that EGFR degradation does not tend to occur after a single administration of erlotinib, which likely explains why degradation has not been detected by studies that have focused on shorter exposure periods.

Other potential molecular markers predictive of response to EGFR-targeted therapies have also been studied (36–38). A pilot study in patients with head and neck squamous cell carcinoma used neoadjuvant erlotinib for 3 weeks prior to surgical resection and noted a clinical response in 9 of 31 patients. Erlotinib led to a significant reduction in extracellular signal regulated kinases-1/2 (ERK1/2) in patients. Their results suggested that baseline p21(waf) expression appeared to positively correlate with clinical response to erlotinib while epidermal growth factor receptor copy number did not (36). Additional biomarker studies have analyzed the effects of erlotinib on epidermal growth factor receptor (EGFR)-related signaling in 7 paired tumor biopsies and 20 paired skin biopsies from patients with recurrent or metastatic HNSCC. Of 25 patients enrolled, 2 patients were noted to have a response; complete response (CR) and a partial response (PR), respectively. There was a trend for patients with more severe skin toxicity to have a more pronounced anti-tumor effect. Erlotinib therapy was associated with a decrease in p-EGFR expression in 4 of 6 tumors (66%) and 7 of 20 sampled skin biopsies (35%). P-27 up-regulation following erlotinib therapy was also noted in 11 of 19 evaluable skin biopsies (59%) (37). In a similar study, the effect of gefitinib on EGFR signaling was assessed following a one week course of gefitinib prior to combined gefitinib, paclitaxel and radiation in locally advanced head and neck cancer. (39) Tumor biopsies were obtained prior and 7 days following gefitinib. The main focus of this study was to assess the baseline membrane and nuclear EGFR and changes in the molecular profile using immunohistochemical analysis, to predict response to gefitinib combined with chemoradiation. In this study, although the nuclear EGFR levels was significantly reduced in 3 out of 7 patients, authors didn't find any correlation between molecular changes and final response to treatment. To date, there are no reliable molecular markers identified which correlate with response to EGFR inhibitors in head and neck cancers.

Clinical data continue to support EGFR as a key molecular target in head and neck cancer. In this study, we were able to confirm that erlotinib caused a marked reduction in EGFR protein levels consistent with our prior preclinical studies. We provided preliminary clinical data suggesting that the normal mucosa does not appear to be as consistently affected by EGFR-targeted therapy as the tumor, suggesting a differential effect of erlotinib in tumors compared to the normal mucosa. Therefore, we are currently conducting a larger prospective clinical trial to evaluate whether EGFR degradation following EGFR-targeted therapy is a potential molecular biomarker of clinical response in patients receiving cetuximab and radiation in HPV positive primary oropharynx patients.

Acknowledgments

Supported by: Conquer Cancer Foundation and Head and Neck SPORE P50 CA097248 and drug supplied by Genentech

References

1. Leemans CR, Braakhuis BJM, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011; 11:9–22. [PubMed: 21160525]
2. Perez-Ordóñez B, Beauchemin M, Jordan RCK. Molecular biology of squamous cell carcinoma of the head and neck. *Journal of Clinical Pathology*. 2006; 59:445–453. [PubMed: 16644882]
3. O-charoenrat P, Rhys-Evans PH, Modjtahedi H, Eccles SA. The role of c-erbB receptors and ligands in head and neck squamous cell carcinoma. *Oral Oncology*. 2002; 38:627–640. [PubMed: 12167415]
4. Khademi B, Shirazi FM, Vasei M, Doroudchi M, Gandomi B, Modjtahedi H, et al. The expression of p53, c-erbB-1 and c-erbB-2 molecules and their correlation with prognostic markers in patients with head and neck tumors. *Cancer Letters*. 2002; 184:223–230. [PubMed: 12127695]
5. Ciardiello F, Tortora G. Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. *Eur J Cancer*. 2003; 39:1348–1354. [PubMed: 12826036]
6. Scartozzi M, Bearzi I, Berardi R, Mandolesi A, Fabris G, Cascinu S. Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: Implications for treatment with EGFR-targeted monoclonal antibodies. *J Clin Oncol*. 2004; 22:4772–4778. [PubMed: 15570078]
7. Parra HS, Cavina R, Latteri F, Zucali PA, Campagnoli E, Morengi E, et al. Analysis of epidermal growth factor receptor expression as a predictive factor for response to gefitinib ('Iressa', ZD1839) in non-small-cell lung cancer. *Br J Cancer*. 2004; 91:208–212. [PubMed: 15187994]
8. Brugger W, Triller N, Blasinska-Morawiec M, Curescu S, Sakalauskas R, Manikhas GM, et al. Prospective Molecular Marker Analyses of EGFR and KRAS From a Randomized, Placebo-Controlled Study of Erlotinib Maintenance Therapy in Advanced Non-Small-Cell Lung Cancer. *J Clin Oncol*. 2011; 29:4113–4120. [PubMed: 21969500]
9. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *Journal of the National Cancer Institute*. 2005; 97:339–346. [PubMed: 15741570]
10. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *Journal of the National Cancer Institute*. 2005; 97:643–655. [PubMed: 15870435]
11. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science*. 2004; 304:1497–1500. [PubMed: 15118125]
12. Szabo B, Nelhubel GA, Karpatis A, Kenessey I, Jori B, Szekely C, et al. Clinical significance of genetic alterations and expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas. *Oral Oncology*. 2011; 47:487–496. [PubMed: 21498106]
13. Ali M, Gunduz M, Nagatsuka H, Gunduz E, Cengiz B, Fukushima K, et al. Expression and mutation analysis of epidermal growth factor receptor in head and neck squamous cell carcinoma. *Cancer Sci*. 2008; 99:1589–1594. [PubMed: 18754871]
14. Lee JW, Soung YH, Kim SY, Nam HK, Park WS, Nam SW, et al. Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clinical Cancer Research*. 2005; 11:2879–2882. [PubMed: 15837736]
15. Lemos-Gonzalez Y, de la Cadena MP, Rodriguez-Berrocal FJ, Rodriguez-Pineiro AM, Pallas E, Valverde D. Absence of activating mutations in the EGFR kinase domain in Spanish head and neck cancer patients. *Tumor Biol*. 2007; 28:273–279.

16. Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. *J Clin Oncol.* 2006; 24:4170–4176. [PubMed: 16943533]
17. Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res.* 2006; 12:5064–5073. [PubMed: 16951222]
18. Fukuoka M, Wu YL, Thongprasert S, Sunpaweravong P, Leong SS, Sriuranpong V, et al. Biomarker Analyses and Final Overall Survival Results From a Phase III, Randomized, Open-Label, First-Line Study of Gefitinib Versus Carboplatin/Paclitaxel in Clinically Selected Patients With Advanced Non-Small-Cell Lung Cancer in Asia (IPASS). *J Clin Oncol.* 2011; 29:2866–2874. [PubMed: 21670455]
19. Feng FY, Lopez CA, Normolle DP, Varambally S, Li X, Chun PY, et al. Effect of epidermal growth factor receptor inhibitor class in the treatment of head and neck cancer with concurrent radiochemotherapy in vivo. *Clin Cancer Res.* 2007; 13:2512–2518. [PubMed: 17438112]
20. Nyati MK, Lawrence TS. Hitting the target: measuring EGFR response to tyrosine kinase inhibitors. *Cancer Biol Ther.* 2005; 4:1387–1388. [PubMed: 16418570]
21. Nyati MK, Maheshwari D, Hanasoge S, Sreekumar A, Rynkiewicz SD, Chinnaiyan AM, et al. Radiosensitization by pan ErbB inhibitor CI-1033 in vitro and in vivo. *Clin Cancer Res.* 2004; 10:691–700. [PubMed: 14760092]
22. Ray D, Ahsan A, Helman A, Chen G, Hegde A, Gurjar SR, et al. Regulation of EGFR Protein Stability by the HECT-type Ubiquitin Ligase SMURF2. *Neoplasia.* 2011; 13:570–578. [PubMed: 21750651]
23. Argiris A, Duffy A, Kummar S, Simone NL, Arai Y, Kim S, et al. Early tumor progression associated with enhanced EGFR signaling with bortezomib, cetuximab, and radiotherapy for head and neck cancer. *Clin Cancer Res.* 2011
24. Ahsan A, Hiniker SM, Ramanand SG, Nyati S, Hegde A, Helman A, et al. Role of epidermal growth factor receptor degradation in cisplatin-induced cytotoxicity in head and neck cancer. *Cancer Res.* 2010; 70:2862–2869. [PubMed: 20215522]
25. Ahsan A, Hiniker SM, Davis MA, Lawrence TS, Nyati MK. Role of cell cycle in epidermal growth factor receptor inhibitor-mediated radiosensitization. *Cancer Res.* 2009; 69:5108–5114. [PubMed: 19509222]
26. Feng FY, Varambally S, Tomlins SA, Chun PY, Lopez CA, Li X, et al. Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity. *Oncogene.* 2007; 26:3431–3439. [PubMed: 17146438]
27. Rosenthal DI, Lewin JS, Eisbruch A. Prevention and treatment of dysphagia and aspiration after chemoradiation in head and neck cancer. *JCO.* 2006; 24(17):2636–2643.
28. Eisbruch A, Lyden T, Bradford CR. Objective assessment of swallowing dysfunction and aspiration after radiation concurrent with chemotherapy for head-and-neck cancer. *Int J Radiat Oncol Biol Phys.* 2002; 53(1):23–28. [PubMed: 12007937]
29. Weihua Z, Tsan R, Huang WC, Wu Q, Chiu CH, Fidler IJ, et al. Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer cell.* 2008; 13:385–393. [PubMed: 18455122]
30. Toyoshima H, Hunter T. P27, A NOVEL INHIBITOR OF G1 CYCLIN-CDK PROTEIN-KINASE ACTIVITY, IS RELATED TO P21. *Cell.* 1994; 78:67–74. [PubMed: 8033213]
31. Ang KK, Zhang QE, Rosenthal DI, et al. A randomized phase III trial (RTOG 0522) of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III-IV head and neck squamous cell carcinomas (HNC). *J Clin Oncol.* 2011; 29(suppl; # 5500)
32. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol.* 2010; 11(1):21–28. [PubMed: 19897418]
33. Cooper JS, Pajak TF, Forastiere AA, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med.* 2004; 350(19):1937–1944. [PubMed: 15128893]

34. Dittmann K, Mayer C, Rodemann HP. Inhibition of radiation-induced EGFR nuclear import by C225 (Cetuximab) suppresses DNA-PK activity. *Radiother Oncol.* 2005b; 76:157–161. [PubMed: 16024112]
35. Nyati MK, Feng FY, Maheshwari D, Varambally S, Zielske SP, Ahsan A, et al. Ataxia telangiectasia mutated down-regulates phospho-extracellular signal-regulated kinase 1/2 via activation of MKP-1 in response to radiation. *Cancer Res.* 2006; 66(24):11554–11559. 15; [PubMed: 17178844]
36. Thomas F, Rochaix P, Benlyazid A, et al. Pilot study of neoadjuvant treatment with erlotinib in nonmetastatic head and neck squamous cell carcinoma. *Clin Cancer Res.* 2007; 13(23):7086–7092. [PubMed: 18056187]
37. Agulnik M, da Cunha Santos G, Hedley D, et al. Predictive and pharmacodynamic biomarker studies in tumor and skin tissue samples of patients with recurrent or metastatic squamous cell carcinoma of the head and neck treated with erlotinib. *J Clin Oncol.* 2007; 25(16):2184–2190. [PubMed: 17538163]
38. Bentzen SM, Atasoy BM, Daley FM, et al. Epidermal Growth Factor Receptor Expression in Pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *JCO.* 2005; 23(24): 5560–5567.
39. Van Waes C, Allen CT, Citrin D, et al. Molecular and Clinical Response in a Pilot Study of Gefinitinib with Paclitaxel and Radiation in Locally Advanced Head and Neck Cancer. *IJROBP.* 2010; 77(2):447–454.

Figure 1. Assessment of EGFR following erlotinib therapy in head and neck cancer patients
 Pre and post erlotinib treated (oral, 150mg/day×7 days) tumor specimens were collected and analyzed for EGFR and other down-stream molecules. In patient 001, EGFR staining in the tumor (shown inside the dotted line) appears reduced in the post-treatment biopsy compared to the pre-treatment biopsy with unaffected staining noted in the adjacent normal gland.. Tumor cells were confirmed by PAN-cytokeratin and H&E staining. In the post-treatment tumor biopsy, there is a decrease in Ki67 staining and pan cytokeratin staining along with altered cellular architecture as shown by H&E. Sections were stained with ApopTag to

assess if the mode of cell death was apoptosis. Increased ApopTag staining is seen in the post treatment tumor biopsy compared to pretreatment and normal biopsy specimens indicating an increase in apoptotic tumor cells following treatment.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Analysis of changes in EGFR levels and associated signaling mediators using multi-immunoblotting following erlotinib therapy

Figure 2A: Pre and post treatment specimens were resolved on 2D gels and multi-immunoblotting was carried out. Protein samples were probed with various antibodies against EGFR signaling mediators and several constitutively expressed proteins. Changes in signaling mediators pre and post erlotinib treatment were analyzed by comparing film exposures showing similar levels of constitutively expressed proteins. Representative pre and post erlotinib treatment multi-blot (averaged data obtained from 3 patients) showed not only a decrease in EGFR and pY845EGFR as observed in traditional immunoblotting, but various other downstream and associated signaling mediators of EGFR were also analyzed simultaneously on a single platform. Figure 2B: Signaling mediators of EGFR pathway including downstream mediators were resolved, and changes following erlotinib treatment (normalizing with GAPDH) are plotted, with reduced levels of pSTAT3, pAKT, pERK, BCL-2, and increase in p27. Figure 2C shows the effect of erlotinib treatment on EGFR expression in tumor and normal tissue in 4 representative patient samples. The changes in EGFR levels in tumor and normal tissue were quantified, and the median EGFR levels

relative to pre-treatment are expressed in Figure 2D obtained from 9 paired tumor biopsies and 7 paired normal mucosa biopsies.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Effects of single Vs. daily exposure to erlotinib on EGFR expression and downstream signaling in head and neck cancer cell lines

Figure 3A: UMSSC1 cells were treated with erlotinib (3 μ M) either once or daily for seven days. Cells were harvested, and immunoblotting was performed to detect EGFR, pY845EGFR, p27 and GAPDH. Figure 3B: The effects of a one week treatment with erlotinib on EGFR and other signaling molecules that were assessed in the patients samples (Figure 2B) were analyzed in UMSSC1 and UMSSC11B cells. A decrease in total EGFR levels was observed in both cell lines. Treatment with erlotinib did not alter Her2 levels. Erlotinib treatment led to a decrease in pERK1/2, pAKT and pSrc levels and p27 accumulation in both cell lines. Figure 3C-F: The change upon erlotinib treatment (normalizing with GAPDH) are shown for UMSSC1 and UMSSC11B (Figure 3C,D) as well as normal fibroblasts and the MRC5 normal cell line (Figure 3E,F). Similar downstream

effects were noted in the cancer cell lines as were seen in the patient tumor samples. Normal cell lines showed an increase in the Bcl2 levels following erlotinib treatment.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Baseline characteristics.

Characteristic	No. of patients (N=12)
Median age, 72 y (range, 2–61 y)	
Male/female	5/7
Primary tumor site: oral cavity	3
Floor of mouth	
Tongue	8
Retromolar trigone	1
AJCC tumor stage	
I	2
II	1
III	3
IVA	5
IVC	1
Smoking history	
Never smokers	3
Former smokers	4
Current smokers	5

Abbreviation: AJCC, American Joint Committee on Cancer.