Effect of erlotinib on epidermal growth factor receptor and downstream signaling in oral cavity squamous cell carcinoma

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ABSTRACT: *Background.* The purpose of this study was to determine if there are differences in biomarker modulation and epidermal growth factor receptor (EGFR) degradation between the tumor and the normal mucosa after 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 every day for 7 days before surgical resection. Tumor and normal mucosa biopsies were obtained both pre-erlotinib and post-erlotinib. Changes in known markers of EGFR activity (phospho, AKT, STAT3) were measured by immunoblotting, whereas changes in tissue distribution were analyzed by immunohistochemical analysis.

Results. Twelve patients were enrolled; 7 had evaluable paired tumors and normal mucosa biopsies pretreatment and posttreatment. Expression of EGFR was higher in tumors compared to the normal mucosa ($p = .005$). Erlotinib administration was associated with marked inhibition of phosphorylated epidermal growth factor receptor (pEGFR) and reduction in total EGFR protein ($p = .004$, $p = .007$) in tumors, whereas there was heterogeneity in EGFR inhibition in the normal mucosa ($p = .10$ [pEGFR], and $p = .07$ [EGFR]). Reduced levels of pSrc and pSTAT3 and enhanced p27 levels were noted in tumors after 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 tumors 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. © 2012 Wiley Periodicals, Inc. *Head Neck* **35:** 1323–1330, 2013

KEY WORDS: erlotinib, degradation, primary oral cavity cancer, normal mucosa, biomarker

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% to 100% of head and neck squamous cell carcinomas are noted to have overexpression of EGFR, the clinical response rate produced by an EGFR inhibitor alone is only 10% to 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 patients with head and neck cancer (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 degradation could play an even greater role in predicting response.[19,22–26] For instance, knockdown of EGFR with 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 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

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of response. A first step in testing this hypothesis, and the primary purpose 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 purpose 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 patients with head and neck treated with concurrent chemoradiation.[28,29] 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.

MATERIALS AND METHODS

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 the 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 this study.

Treatment plan

All patients underwent a physical examination, medical history, laboratory evaluation, and CT imaging at baseline. Toxicities were graded using the National Cancer Institute common toxicity criteria version 3.0. Patients were instructed to start oral erlotinib 150 mg prescribed orally every day, 7 days before surgical resection. The final erlotinib dose was taken at least 8 hours before 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 prescribed orally every day. 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 $3 \times 3 \times 3$mm sample from both the tumor and the uninvolved contralateral oral mucosa were obtained. These samples were collected in ice-cold saline containing a cocktail of protease (Roche Diagnostic, Indianapolis, IN) and phosphatase inhibitors (Sigma, St. Louis, MO). After a 7-day course of EGFR tyrosine kinase inhibitor erlotinib, repeat core tumor and contralateral normal-appearing mucosa biopsies were obtained at the time of surgical resection in similar regions as the initial baseline biopsies. Biopsies were divided into 2 parts; the first was 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 (N.D'S.).

High-throughput immunoblotting

Tissue samples were lysed in the sample extraction buffer and immunoblotting was performed using a protocol previously described.[30] 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 membrane. A Miniblotter 28 dual system (Immunetics, Cambridge, MA) was used to probe all the antibodies). After incubating the membrane with different antibodies overnight, membranes were washed for 30 minutes with TRIS-buffered saline–Tween-20 (TBS-T) and probed with horseradish peroxidase conjugated immunoglobulin G (Cell Signaling Technology, Beverly, MA), diluted 1:10,000 in TBS-T for 1 hour 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 National Institutes of Health ImageJ software. Unless otherwise indicated, the relative protein levels shown represent a comparison to untreated controls.

Immunohistochemistry

Immunohistochemical staining was performed as described previously.[30] The 4- to 5-μm sections were prepared using a microtome and placed onto slides. Sections were subjected to heat-mediated antigen retrieval in citrate buffer (10 m*M* citric acid, pH 6.0). After blocking with 5% donkey serum for 1 hour at room temperature, sections were incubated with primary antibody overnight at 4°C in a humidified chamber. After 3 phosphate-buffered saline washes, horseradish peroxidase-conjugated second antibody was used to form a complex with the primary antibody, and unbound antibodies were removed by washing in TBS-T 3 times. Finally, this complex was visualized using the ECL Peroxidase Substrate Kit (Vector Labs, Burlingame, 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, Billerica, MA). Slides were evaluated by 2 investigators, and 3 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 2-tailed t test was used to evaluate immunohistochemistry results pretreatment and posttreatment for paired biopsies. The results of statistical tests were considered statistically significant at $p < .05$.

RESULTS

Patient characteristics

Twelve patients were enrolled in the study. Baseline characteristics are shown in Table 1. Median age was 72

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	
Floor of mouth	3
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.

years (range, 21–86 years). There were 7 women and 5 men. Median Zubrod score was 0 (range, 0–1). Primary oral cavity tumors were included; floor of mouth ($n = 3$), tongue ($n = 8$), and retromolar trigone ($n = 1$). The majority (7 of 12) of the tumors were American Joint Committee on Cancer T3 or T4 classification. Six patients also had nodal disease. Smoking status was available in all patients: current smokers ($n = 5$), former smokers ($n = 4$), and never smokers ($n = 3$). All 12 patients had pretreatment tumor biopsies. One patient underwent repeat positron emission tomography scan after enrollment and was noted to have newly diagnosed metastatic lung disease and therefore did not undergo surgical resection as planned. Tumor specimens were nonevaluable due to technical issues in 2 cases. In 4 cases, baseline normal tissue biopsies at postprocessing were nonevaluable due to the small size of the biopsies. Therefore, a total of 9 paired tumors 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-eosin stain, PAN-cytokeratin, and Ki-67 staining (Figure 1). PAN-cytokeratin and hematoxylin-eosin staining were used to confirm the presence of tumor cells. After 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 the tumor, whereas minimal effects were noted in the normal mucosa. Additional qualitative analysis using immunohistochemistry of EGFR confirmed strong staining in the pretreatment tumor biopsies. There was a dramatic reduction of EGFR staining after 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 (Figure 1 and 2). We also wanted to determine if erlotinib could cause tumor cell death after a 1-week course of treatment. We noted a modest increase in the number of apoptotic cells upon immunostaining in the tumor tissue posttreatment.

Immunoblotting analyses

In order to quantify the effects of erlotinib treatment on both the tumor and normal tissue, we performed immunoblotting for both the paired tumor ($n = 9$) and normal tissue ($n = 7$) biopsies. Erlotinib treatment led to a marked decrease of both phosphorylated epidermal growth factor receptor (pEGFR; both the Y845 site and the Y1173 site) and total EGFR protein ($p = .004$ and $p = .007$, respectively) in the tumor biopsies (Figure 2A and B). In contrast, EGFR inhibition in the normal mucosa was more heterogeneous showing a weak and nonsignificant decreasing trend after erlotinib for both pEGFR ($p = .10$) and total EGFR protein ($p = .07$; Figure 2C and D). We also analyzed the effects of erlotinib on other key downstream signaling molecules of EGFR. We compared pre-erlotinib and post-erlotinib matched tumor samples and found that levels of pSrc, pSTAT3, pERK, and pAKT were significantly reduced after 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 after erlotinib treatment (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 (Figures 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 G_1-phase.[31] 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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HSP90, neither of which showed any significant change in levels in response to erlotinib treatment (Figures 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 versus 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 Figure 1B in 2 tumors 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 cell lines were also treated with erlotinib for comparison (Figure 3E and F), which caused an increase in Bcl2. This suggests that erlotinib might protect normal cells from apoptosis. Other key signaling molecules were minimally altered.

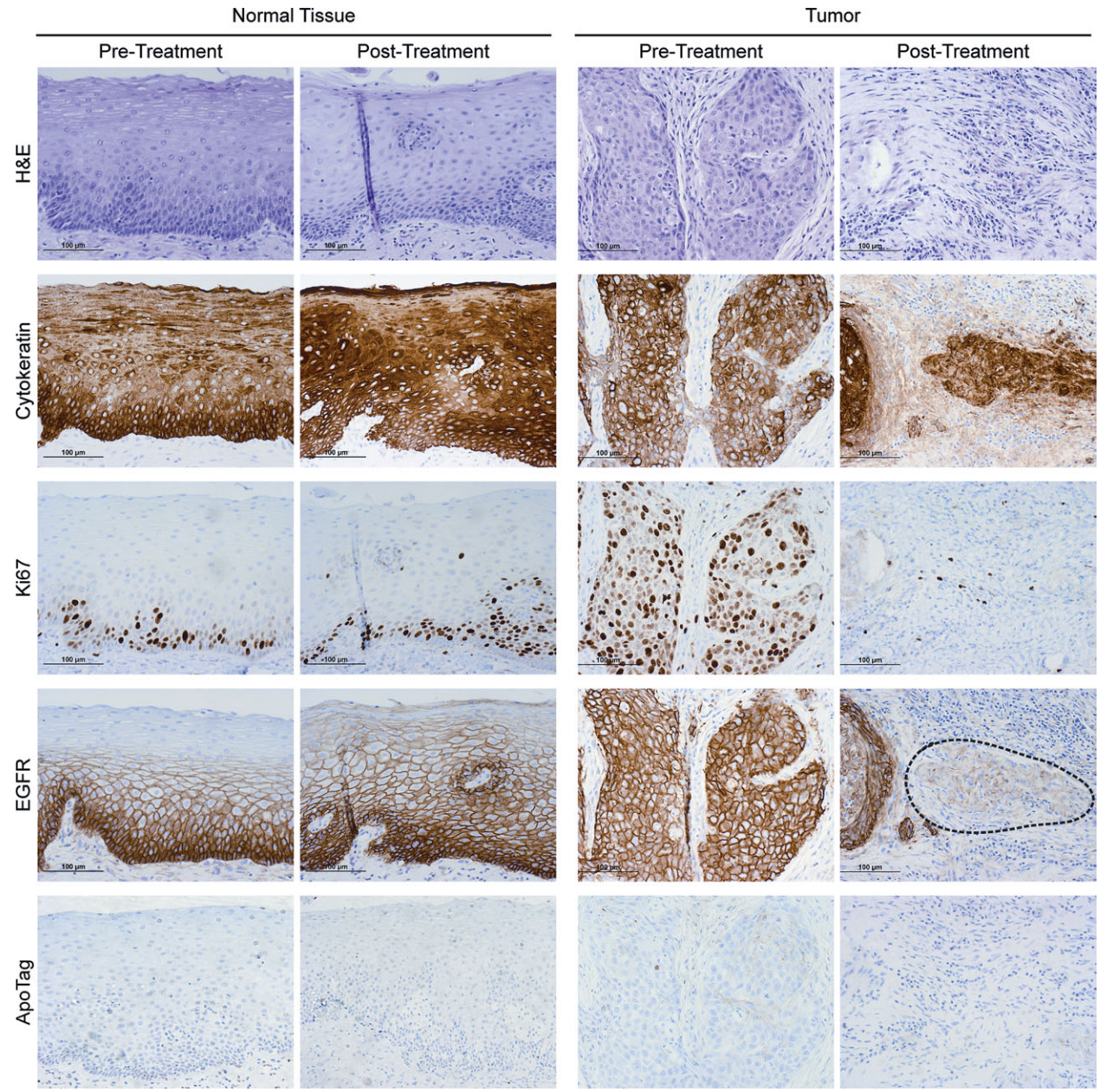

FIGURE 1. Assessment of epidermal growth factor receptor (EGFR) after erlotinib therapy in patients with head and neck cancer. Pre-erlotinib and post-erlotinib treated (oral, 150 mg/day × 7 days) tumor specimens were collected and analyzed for EGFR and other downstream molecules. In patient #1, EGFR staining in the tumor (shown inside the dotted line) appears reduced in the posttreatment biopsy compared to the pretreatment biopsy with unaffected staining noted in the adjacent normal gland. Tumor cells were confirmed by PAN-cytokeratin and hematoxylin-eosin staining. In the posttreatment tumor biopsy, there is a decrease in Ki67 staining and pan-cytokeratin staining along with altered cellular architecture as shown by hematoxylin-eosin stain. Sections were stained with ApopTag to assess if the mode of cell death was apoptosis. Increased ApopTag staining is seen in the posttreatment tumor biopsy compared to the pretreatment and normal biopsy specimens indicating an increase in apoptotic tumor cells after treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

This pilot study confirms that pEGFR, total EGFR, and key downstream signaling molecules are consistently decreased in head and neck tumors after a 1-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 patients with head and neck cancer is novel and is consistent with our prior preclinical findings.[19] Our data suggest 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.

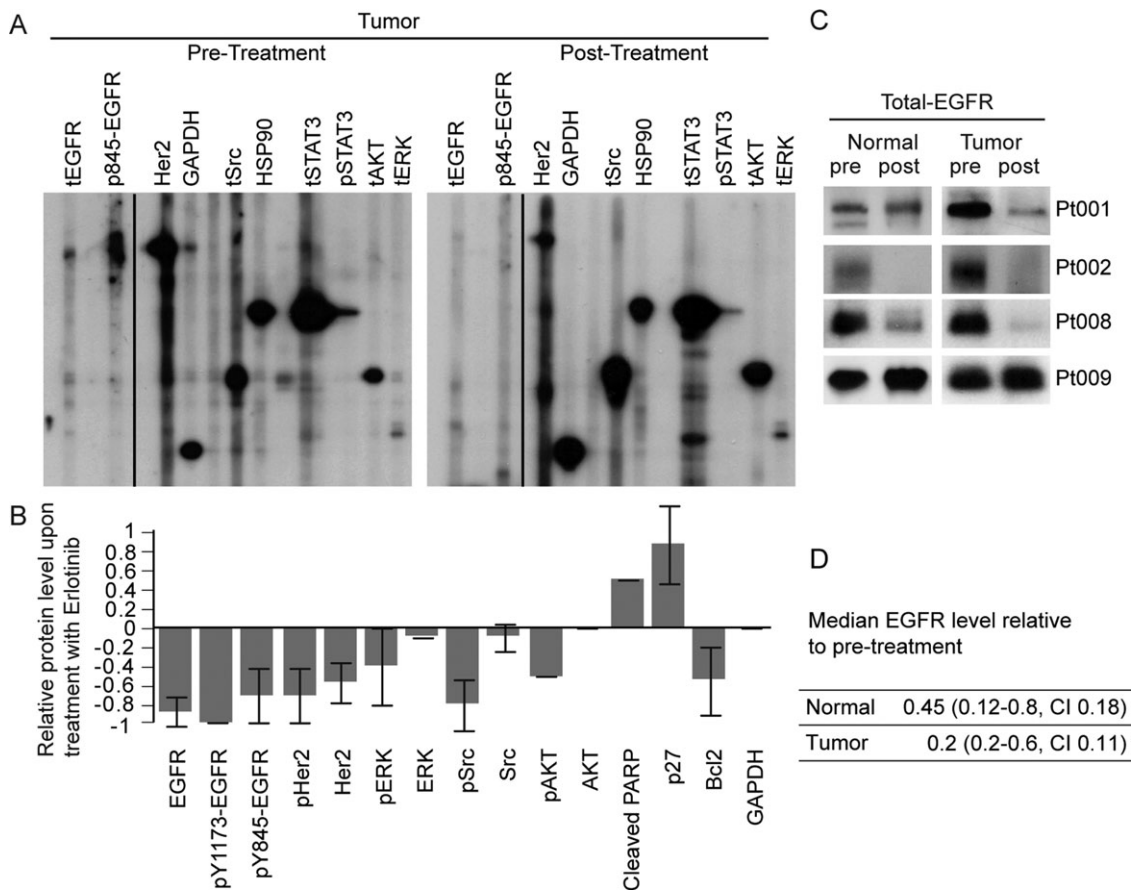

FIGURE 2. Analysis of changes in epidermal growth factor receptor (EGFR) levels and associated signaling mediators using multi-immunoblotting after erlotinib therapy. (A) Pretreatment and posttreatment specimens were resolved on 2D gels and multi-immunoblotting was carried out. Protein samples were probed with various antibodies against EGFR signaling mediators and several constitutively expressed proteins. Changes in signaling mediators pre-erlotinib and post-erlotinib treatment were analyzed by comparing film exposures showing similar levels of constitutively expressed proteins. Representative pre-erlotinib and post-erlotinib treatment multiblots (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. (B) Signaling mediators of EGFR pathway including downstream mediators were resolved, and changes after erlotinib treatment (normalizing with glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) are plotted, with reduced levels of pSTAT3, pAKT, pERK, BCL-2, and increase in p27. (C) The effect of erlotinib treatment on EGFR expression in tumor and normal tissue in 4 representative patient samples. The changes in EGFR levels in the tumor and normal tissue were quantified, and the median EGFR levels relative to pretreatment are expressed in (D) obtained from 9 paired tumor biopsies and 7 paired normal mucosa biopsies.

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 chemoradiation.[32–34] 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 chemoradiation with cisplatin.[35] After a median follow-up of 2.4 years, there was no difference in progression-free survival 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 arm.[35] Thus, it is unclear which patients may benefit from the addition of EGFR inhibitors to concurrent chemoradiation. Identification of potential molecular biomarkers predictive of response would be beneficial in selecting patients most likely to respond to targeted therapies not only in regard 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 human papillomavirus-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 seems to be an important mechanism of cell death in head and neck tumors that are dependent on EGFR

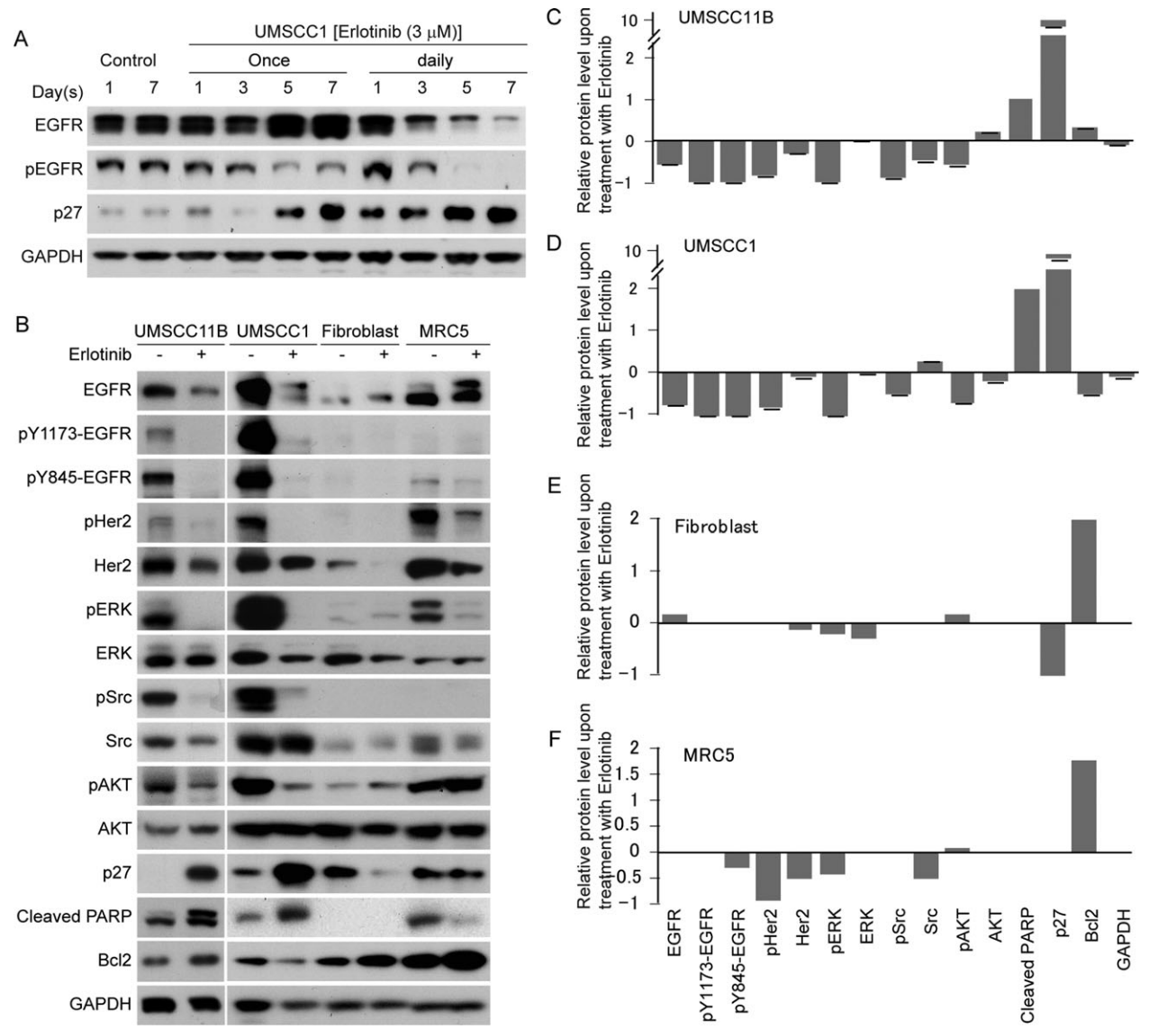

FIGURE 3. Effects of single versus daily exposure to erlotinib on epidermal growth factor receptor (EGFR) expression and downstream signaling in head and neck cancer cell lines. (A) UMSCC1 cells were treated with erlotinib (3 μM) either once or daily for 7 days. Cells were harvested, and immunoblotting was performed to detect EGFR, pY845EGFR, p27, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) The effects of a 1-week treatment with erlotinib on EGFR and other signaling molecules that were assessed in the patients' samples were analyzed in UMSCC1 and UMSCC11B cells. A decrease in total EGFR levels was observed in both cell lines. Treatment with erlotinib did not alter human epidermal receptor 2 (HER2) levels. Erlotinib treatment led to a decrease in pERK1/2, pAKT, and pSrc levels, and p27 accumulation in both cell lines. (C–F) The change upon erlotinib treatment (normalizing with GAPDH) are shown for UMSCC1 and UMSC11B (C and D) as well as normal fibroblasts and the MRC5 normal cell line (E and 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 after erlotinib treatment.

signaling. As EGFR plays a role in DNA repair,[30] EGFR degradation may decrease the ability of cells to repair damaged DNA, leading, therefore, to increased cytotoxicity. We have demonstrated that decreased EGFR levels after gemcitabine[26] or cisplatin[24] 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.[36] EGFR degradation occurs along a pathway similar to EGF-induced receptor downregulation. The loss of EGFR can lead to the downregulation 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.[37–39] A pilot study in patients with HNSCC used neoadjuvant erlotinib for 3 weeks before 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 in patients. Their results suggested that baseline p21(waf) expression seemed to positively correlate with clinical response to erlotinib, whereas the EGFR copy number did not.[37] Additional biomarker studies have analyzed the effects of erlotinib on 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 and a partial response, respectively. There was a trend for patients with more severe skin toxicity to have a more pronounced antitumor effect. Erlotinib therapy was associated with a decrease in pEGFR expression in 4 of 6 tumors (66%) and 7 of 20 sampled skin biopsies (35%). P-27 upregulation after erlotinib therapy was also noted in 11 of 19 evaluable skin biopsies (59%).[40] In a similar study, the effect of gefitinib on EGFR signaling was assessed after a 1-week course of gefitinib before combined gefitinib, paclitaxel, and radiation in locally advanced head and neck cancer.[41] Tumor biopsies were obtained before and 7 days after 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 the response to gefitinib combined with chemoradiation.

In this study, although the nuclear EGFR levels were significantly reduced in 3 of 7 patients, the authors did not find any correlation between molecular changes and final response to treatment.

To date, there are no reliable molecular markers identified that correlate with the 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 seem 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 after EGFR-targeted therapy is a potential molecular biomarker of clinical response in patients receiving cetuximab and radiation in patients with human papillomavirus–positive primary oropharyngeal cancer.

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