Cancer Therapy: Preclinical

Dual Blockade of EGFR and c-Met Abrogates Redundant Signaling and Proliferation in Head and Neck Carcinoma Cells

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Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) is usually fatal, and innovative approaches targeting growth pathways are necessary to effectively treat this disease. Both the epidermal growth factor receptor (EGFR) and the hepatocyte growth factor (HGF)/c-Met pathways are overexpressed in HNSCC and initiate similar downstream signaling pathways. c-Met may act in consort with EGFR and/or be activated as a compensatory pathway in the presence of EGFR blockade.

Experimental Design: Expression levels of EGFR and c-Met were determined by Western analysis in HNSCC cell lines and correlated with antitumor responses to inhibitors of these pathways.

Results: Combining the c-Met inhibitor PF2341066 with the EGFR inhibitor gefitinib abrogated HNSCC cell proliferation, invasion, and wound healing significantly more than inhibition of each pathway alone in HNSCC cell lines. When both HGF and the EGFR ligand, TGF-α, were present in vitro, P-AKT and P-MAPK expression were maximally inhibited by targeting both EGFR and c-Met pathways, suggesting that c-Met or EGFR can compensate when phosphorylation of the other receptor is inhibited. We also showed that TGF-α can induce phosphorylation of c-Met over sixfold by 8 hours in the absence of HGF, supporting a ligand-independent mechanism. Combined targeting of c-Met and EGFR resulted in an enhanced inhibition of tumor volumes accompanied by a decreased number of proliferating cells and increased apoptosis compared with single agent treatment in vivo.

Conclusions: Together, these results suggest that dual blockade of c-Met and EGFR may be a promising clinical therapeutic strategy for treating HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) constitutes 90% of all head and neck cancers. Treatment for head and neck cancer includes surgery followed by concurrent chemoradiotherapy or chemoradiation alone. The overall 5-year survival rate for HNSCC is estimated to be 58% for males and 62% for females and has remained unchanged over the past several decades (1). Thus, head and neck cancer is in need of improved therapeutic approaches. Novel therapies targeting multiple growth factor pathways may be useful to effectively treat this disease.

The epidermal growth factor receptor (EGFR) is overexpressed in up to 90% of HNSCC tumors and is involved in HNSCC growth, invasion, metastasis, and angiogenesis (2). Elevated protein levels of EGFR and its ligand, TGF-α, in HNSCC correlates with poor prognosis (3). Functional deletion of EGFR in vitro or in vivo inhibits HNSCC proliferation without affecting the growth of normal mucosal epithelial cells, rendering EGFR an attractive therapeutic target (4). The phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways are 2 critical biological pathways activated by EGFR which play crucial roles in cell survival, proliferation, apoptosis, and migration (5–6). There are 2 main therapeutic strategies which have been implemented to inhibit EGFR: (a) monoclonal antibodies directed at the EGFR extracellular domain such as cetuximab and (b) small molecule adenosine triphosphate-competitive tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib (7). Single agents targeting EGFR have shown only modest activity in clinical trials for HNSCC which play crucial roles in cell survival, proliferation, apoptosis, and migration (5–6). There are 2 main therapeutic strategies which have been implemented to inhibit EGFR: (a) monoclonal antibodies directed at the EGFR extracellular domain such as cetuximab and (b) small molecule adenosine triphosphate-competitive tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib (7). Single agents targeting EGFR have shown only modest activity in clinical trials for HNSCC due to intrinsic and acquired resistance, resulting in a need to develop more effective strategies to improve EGFR-targeted therapy for HNSCC (8).

One receptor tyrosine kinase (RTK) that can activate many of the same downstream signaling pathways as EGFR...
Translational Relevance

More effective treatment strategies are necessary for patients with head and neck squamous cell cancer. This work examines the combined effect of targeting 2 intersecting oncogenic signaling pathways implicated in head and neck cancer: the epidermal growth factor receptor (EGFR) and c-Met pathways. We also provide the first evidence of EGFR ligand, TGF-α, inducing activation of the c-Met pathway in head and neck squamous cell cancer. This study provides an increased understanding of EGFR signaling with another oncogenic pathway, c-Met, and should facilitate the development of more effective molecular targeting strategies for head and neck cancer patients. Cotargeting of both the EGFR and c-Met pathways may be an effective treatment strategy for head and neck cancer patients and has the potential to improve current therapeutic approaches to head and neck cancer.

and has been shown to be involved in resistance to EGFR inhibitors is c-Met, the RTK for hepatocyte growth factor (HGF; ref. 9). The c-Met pathway is aberrantly activated in various epithelial carcinomas which leads to a variety of signals that mediate tumor growth, metastasis, and angiogenesis (10). We previously elucidated that ligand activation of c-Met by HGF increases proliferation of HNSCC cells and a clinically relevant c-Met TKI, PF2341066, inhibited HGF-induced phosphorylation of both MAPK and AKT, and decreased wound healing in vitro (11). Furthermore, decreased proliferation and increased apoptosis was observed in HNSCC xenografts in mice treated with PF2341066 (11). PF2341066 also inhibits the oncogenic fusion protein variants of anaplastic lymphoma kinase (ALK); however, these ALK fusion proteins are not expressed in head and neck cancer cells used in this study (not shown). We have also previously reported that both HGF and c-Met protein were increased in HNSCC tumor tissue compared with normal mucosa (11). EGFR ligand release has also been documented in HNSCC cell lines (12). As c-Met activation by HGF induces similar downstream pathways as EGFR in HNSCC, c-Met may either act in consort with EGFR or act as a compensatory signaling pathway in the setting of EGFR blockade or vice versa (13, 14). Thus, targeting EGFR in combination with a c-Met inhibitor may enhance the antitumor effect compared with targeting a single RTK alone and may possibly eliminate acquired resistance.

Integration of HGF/c-Met and EGFR signaling in cancer cells indicates that treatment regimens designed to target both receptor pathways may be efficacious. Here, we extend our studies to determine whether the addition of c-Met targeting can enhance the antitumor efficacy of EGFR TKIs in HNSCC cell lines and in an in vivo animal model. To explore the antitumor effects of combined targeting of EGFR and c-Met, we examined the effect of dual inhibition of both pathways on proliferation, invasion, and wound healing. We further determined whether the combined treatment could decrease expression of important downstream signaling molecules of both EGFR and HGF/c-Met pathways. Dual targeting resulted in significantly more inhibition of cell proliferation, invasion, wound healing, and downstream signaling. Furthermore, for the first time in HNSCC cells, we show that TGF-α induces the phosphorylation of c-Met in an HGF-independent manner. Finally, targeting these 2 pathways in an in vivo tumor xenograft model resulted in additive antitumor effects compared with single-agent treatment. These results provide preclinical support that targeting EGFR in combination with c-Met is a promising therapeutic strategy for HNSCC patients.

Materials and Methods

Cell lines and reagents

PCI-15B, UM-22A, UM-22B, and Cal-33 tumor cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Inc.) supplemented with 10% FBS, UM-SCC-1 cells were maintained in DMEM supplemented with 10% FBS plus 0.4 μg/mL hydrocortisone, OSC-19 cells were maintained in minimum essential medium (Invitrogen) supplemented with 10% FBS plus 1% nonessential amino acids. PCI-15B and OSC-19 cells were obtained from T. Whiteside (University of Pittsburgh), UM-22A, UM-22B, and UM-SCC-1 cells were obtained from T. Carey (University of Michigan). Cal-33 cells were obtained from Centre Antoine-Lacassagne (Nice, France). UM-22A, UM-SCC-1, and Cal-33 are all from primary tumors from therapy-naïve patients. UM-22B, PCI-15B, and OSC-19 cells were derived from metastases from HNSCC primaries; treatment status at the time of metastases excision is unknown. All cells were genotypically authenticated and verified to be mycoplasma free within 1 month of conducting the experiments. SU11274 was purchased from EMD Chemicals, Inc. PF2341066 was generously provided by Pfizer Inc. Gefitinib was purchased from Chemitek. Recombinant human HGF and TGF-α were purchased from R&D Systems. SU11274 and PF2341066 were dissolved at 10 mmol/L in dimethyl sulfoxide (DMSO) and stored at −20°C for use in vitro.

Cell proliferation assays

HNSCC cells were plated in 24-well plates at 3 \times 10^4 cells/well plate in complete growth media and allowed to attach overnight prior to experimental treatments. Cells were treated in triplicate for 72 hours with different concentrations of PF2341066, SU11274, and/or gefitinib as indicated in the figures. Cells were washed with PBS and 200 μL MTT solution (5 mg/mL) was added to each well followed by incubation for 20 to 50 minutes at 37°C. The media was removed and the dark crystals in each well were dissolved in 100 μL DMSO and transferred to a 96-well plate. Absorbance was measured with a microplate reader at 560 nm. The percentage of growth is shown relative to
untreated controls. Each experiment was conducted at least 3 times.

Combination indexes (CI) were calculated by the method of Chou and Talalay (15) to assess synergy by using CalcuSyn V2 software (BIOSOFT). CIs values less than 1.0 were considered evidence of synergism.

**Protein isolation and Western analysis**

Cells were lysed as described previously (11). Protein concentration was measured by Bradford Assay (Thermo Scientific). Protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed overnight at 4°C with anti-Met (1:250, sc-161; Santa Cruz Biotechnology), anti–P-Met (1:1,000, #9272; Cell Signaling Technology), anti-EGFR (1:1,000, #9101; Cell Signaling Technology), anti-AKT (1:1,000, #9271; Cell Signaling Technology), anti–P-AKT (1:1,000, #9272; Cell Signaling Technology), anti-EGFR (1:1,000, #8169; BD Transduction Laboratories), anti-p–P38 MAPK (1:1,000, #2234; Cell Signaling Technology), anti-α-tubulin (1:1,000, #2126; Cell Signaling Technology), anti-β-actin (1:1,000, #485B-5; Santa Cruz Biotechnology), or anti–P-60 kinase (1:1,000, #9102; Cell Signaling Technology). Protein isolation and Western analysis with 10 ng/mL TGF-α for 0 to 24 hours. Culture supernatant was removed and analyzed in triplicate by Quanti-kinine Human HGF ELISA (R&D Systems) according to the manufacturer’s instructions. Serum-free media with no cells and conditioned media from normal lung fibroblasts were used as negative and positive controls, respectively.

**Human HGF ELISA**

HNSCC cells (8 × 10^5) were plated in 6-cm dishes. Cells were serum deprived for 48 hours followed by treatment with 0.1 mg/mL TGF-α for 0 to 24 hours. Culture supernatant was removed and analyzed in triplicate by Quantikine Human HGF ELISA (R&D Systems) according to the manufacture’s instructions. Serum-free media with no cells and conditioned media from normal lung fibroblasts were used as negative and positive controls, respectively.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted by using the RNaseasy kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized by a single reverse transcription reaction. The primers and probes used for detection of HGF are as described previously (16). cDNA from normal lung fibroblasts was used as a positive control. cDNA from MCF7 cells was used as a negative control. Negative controls which did not include cDNA were performed for each reaction.

**In vivo tumor xenograft model**

Female C.B-17/IcrHsd-scid mice were purchased from Harlan. UM-22B tumor cells (3 × 10^6) were injected subcutaneously into the flank region of each mouse and allowed to grow until the tumor volume reached approximately 100 mm^3. The mice were randomized into 4 treatment groups: (a) placebo control, (b) PF2341066, (c) gefitinib, and (d) PF2341066 plus gefitinib. PF2341066 (12.5 mg/kg), placebo control (0.9% saline/1% Tween-80), and/or gefitinib (50 mg/kg) were administered daily by oral gavage for 3 weeks. Tumor size was measured once per week and reported as tumor volume (in mm^3). At the end of the treatment period, the animals were sacrificed and the tumors were removed and fixed in 10% buffered formalin for subsequent immunohistochemical analysis. The animal experiment was repeated once and the results of the 2 independent experiments were combined with 15 to 16 animals per group. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

**Immunohistochemical analysis**

Formalin-fixed tumors were paraffin-embedded, sliced, and mounted on slides. Paraffin was removed from the slides with xylene and slides were stained with hematoxylin and eosin to examine the tumor morphology. The number of apoptotic cells and Ki67 staining was determined as described previously (17). Brown staining was considered positive.

**Statistical analysis**

All values are expressed as the mean ± SEM. Concentration response curves were made by nonlinear curve
regression (GraphPad Prism 4). Treatment group comparisons in the tumor xenograft model compared untransformed tumor volumes on the final day of volume measurement. A 2-way ANOVA was conducted to test for interaction between PF2341066 and gefitinib. Tumor protein staining for Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) used cell count data which were square-root transformed as is appropriate for Poisson count data. An omnibus 1-way ANOVA test was conducted and if \( P \) value was less than 0.05, all pairwise comparisons were tested with Sidak-adjusted \( P \) values. Other comparisons were conducted with a \( t \) test on untransformed data without adjustment for multiple comparisons.

**Results**

**c-Met and EGFR protein are expressed in HNSCC tumor cells and cell proliferation is inhibited with c-Met or EGFR TKIs**

We first examined c-Met and EGFR protein expression in a panel of 6 HNSCC cell lines. Whole cell lysates were analyzed by Western blot from cells grown in complete media. All 6 cell lines examined expressed both EGFR and c-Met although to different extents as indicated by relative densitometry readings (Fig. 1A). There was no correlation between c-Met and EGFR expression levels.

Three cell lines (PCI-15B, UM-22A, UM-22B) with varying degrees of c-Met and EGFR expression were chosen for further characterization of the effect of inhibition of the c-Met or EGFR pathways on cell proliferation. Furthermore, UM-22A and UM-22B cells were chosen because these are isogenic cell lines where UM-22A represents the cells derived from a primary tumor and UM-22B cells are from a metastasis from the same patient (18). UM-22B cells also form fast-growing tumors when implanted as xenografts in immunocompromised mice.

We first determined relative cell survival concentration–response curves with 2 c-Met TKIs, PF2341066 and SU11274, and with the EGFR TKI, gefitinib, for all 3 cell lines (Fig. 1B–D). As shown in Figure 1B–D, all 3 TKIs inhibited HNSCC cell growth in a concentration-dependent manner. Of the 3 cell lines examined, UM-22B was the most sensitive to gefitinib, with an IC\(_{50}\) value of 0.158 μmol/L compared with IC\(_{50}\) values of 1.73 and 16.2 μmol/L for UM-22A and PCI-15B, respectively. This is over a 100-fold difference in sensitivity among the cell lines tested. The relative sensitivity to gefitinib for each cell line correlated with the relative protein expression of EGFR; UM-22B was the most sensitive and also had the highest EGFR expression level whereas PCI-15B was the least sensitive of the cells tested and had lower EGFR expression. In regards to c-Met inhibition, the cells were more sensitive to PF2341066 than to SU11274 with IC\(_{50}\) values 1.31 μmol/L versus 2.99 μmol/L in PCI-15B, 1.00 μmol/L versus 3.79 μmol/L in UM-22A, and 0.718 versus 2.44 μmol/L in UM-22B for PF2341066 and SU11274, respectively. The cell line with the highest c-Met expression (UM-22B) was the most sensitive to c-Met inhibition. In the isogenic cell lines, there is a shift in sensitivity based on the amount of c-Met. Nonisogenic cell lines such as PCI-15B may have other genetic or epigenetic differences that have an impact on how much the c-Met pathway is used, which could modulate sensitivity of c-Met inhibitors. Larger differences in c-Met sensitivity have been identified in cells with a c-Met amplification or mutation, but those genetic changes were not found in these cell lines.

**Combination of PF2341066 and gefitinib maximally inhibit HNSCC cell proliferation, invasion and wound healing compared with single-agent treatment in vitro**

The interaction of EGFR with cell surface proteins and other receptors, such as c-Met, may affect the efficacy and contribute to resistance to targeted therapies (19). We hypothesized that combined targeting of both the EGFR and HGF/c-Met pathways would enhance the antitumor efficacy in HNSCC cells. We used the more clinically relevant and more potent c-Met TKI, PF2341066, for further examination of the combined effect with gefitinib. As with each single-agent treatment shown in Figure 1, combined treatment also inhibited HNSCC cell growth in a concentration-dependent manner in PCI-15B, UM-22A, and UM-22B cells (Fig. 2A–C). All concentration combinations of the 2 inhibitors showed evidence of synergism with CIs of less than 1 in PCI-15B and UM-22A cells. In UM-22B cells which are more sensitive to both inhibitors, the CI was 1 or more suggesting only an additive effect.

Representative quantitation is shown in Figure 2D–F. As shown in Figure 2D, combined treatment of PF2341066 (5 μmol/L) and gefitinib (10 μmol/L) maximally inhibited the growth of PCI-15B cells by 95.1%, 88.1%, and 90.4% compared with control (\( P = 0.0002 \)), PF2341066 (\( P = 0.0004 \)), and gefitinib (\( P = 0.0002 \)), respectively. Similar results were also observed with UM-22A cells (Fig. 2E). Combined treatment of PF2341066 (1 μmol/L) and gefitinib (1 μmol/L) inhibited UM-22A cell growth by 79.7%, 43.5%, and 58.2% compared with control (\( P < 0.0001 \)), PF2341066 (\( P = 0.0024 \)), and gefitinib (\( P < 0.0001 \)), respectively. UM-22B cells were the most sensitive to both PF2341066 and gefitinib (Fig. 1D). Combined treatment of PF2341066 (0.5 μmol/L) and gefitinib (0.5 μmol/L) maximally inhibited UM-22B cell growth by 85.9%, 76.6%, and 54.4% compared with control (\( P < 0.0001 \)), PF2341066 (\( P = 0.0003 \)), and gefitinib (\( P < 0.0017 \)), respectively (Fig. 2F). Cal-33 and OSC-19 cells also showed a significantly greater inhibition of cell proliferation with the combination treatment of PF2341066 and gefitinib compared with single-agent treatment (Supplementary Fig. S1A and B).

To further characterize the antitumor effect of combined targeting with PF2341066 and gefitinib, wound healing assays were carried out in the 3 cell lines. In PCI-15B cells, wound closure was inhibited 37.6% (\( P < 0.0001 \)) by PF2341066 (5 μmol/L), 29.4% (\( P < 0.0001 \)) by gefitinib (10 μmol/L), and 70.6% (\( P < 0.0001 \)) by the combination compared with control (Fig. 3A). Combined treatment reduced the wound closure in PCI-15B cells by 52.9%...
(P < 0.0001) and 58.4% (P < 0.0001) compared with PF2341066 and gefitinib single-agent treatment, respectively. Similar results were observed for UM-22A cells with inhibition of wound closure of 36.9% (P < 0.0001) by PF2341066, 39.2% (P < 0.0001) by gefitinib, and 80.7% (P < 0.0001) by combination compared with control (Fig. 3B). Combination treatment decreased wound closure by 69.3% (P < 0.0001) and 68.2% (P < 0.0001) compared with PF2341066 and gefitinib alone, respectively, in UM-22A cells. In UM-22B cells, lower concentrations of gefitinib were used due to the high sensitivity of this cell line to gefitinib. Using a 10-fold lower concentration of gefitinib, the same effect was observed in wound closure with combination treatment with statistically
significant differences compared with single-agent treatment (Fig. 3C). Similar results for wound healing were observed by using the Cal-33 and OSC-19 cell lines (Supplementary Fig. S1C and D).

As both EGFR and c-Met initiate signaling cascades for invasive growth (20, 21), we next examined whether targeting both EGFR and c-Met pathways could achieve more inhibition of HNSCC invasion in vitro by using optimized concentrations for each cell line. In PCI-15B cells, the PF2341066 and gefitinib combination inhibited invasion by 79.8% (P = 0.003) and 96.7% (P = 0.003) compared with PF2341066 and gefitinib single-agent treatment.
respectively (Fig. 3D). UM-22A cells were not invasive in vitro even though the time for cells to invade was extended up to 72 hours (data not shown), so these could not be evaluated in this assay. In UM-22B cells, invasion was inhibited by 36.1% ($P = 0.0468$), 65.2% ($P = 0.0043$), and 97.7% ($P = 0.0003$) by PF2341066 alone, gefitinib alone, and combination, respectively, compared with control (Fig. 3E). The combination treatment significantly...
inhibited invasion by 96.5% and 93.3% compared with PF2341066 and gefitinib single-agent treatment. Similar results for invasion with the combination treatments were also observed in Cal-33 and OSC-19 cells (Supplementary Fig. S1E and F).

**P-MAPK and P-AKT are maximally inhibited by targeting both EGFR and c-Met in HNSCC**

As both EGFR and c-Met can activate important downstream signaling molecules such as AKT and MAPK which play critical roles in HNSCC growth, survival, and invasion (22, 23), we hypothesized that dual targeting of EGFR and c-Met would be necessary to maximally inhibit P-AKT and P-MAPK. We tested the effect of specific inhibitors of the c-Met and EGFR pathways alone and in combination in the presence of ligands for each pathway in HNSCC cells. UM-22A (Fig. 4A and B) and UM-22B (Fig. 4C and D) cells were pretreated with control, PF2341066 alone, gefitinib alone, or PF2341066 plus gefitinib followed by stimulation with both HGF and TGF-α. Optimal concentrations were chosen on the basis of previous results in wound healing and invasion assays as well as IC_{50} values of PF2341066 and gefitinib for each cell line. In both cell lines examined, P-Met and P-EGFR were selectively inhibited by PF2341066 and gefitinib, respectively, showing the specificity of each inhibitor alone (Fig. 4B and D). Partial inhibition of P-AKT and P-MAPK was observed with each inhibitor alone in the presence of both ligands. In UM-22A cells, P-AKT expression was decreased 23% (P < 0.05) by PF2341066, 19% (P < 0.05) by gefitinib, and 92.5% (P = 0.0001) by the combination compared with control (Fig. 4B). Dual targetinge reduced P-AKT by 90% (P = 0.005) and 91% (P = 0.055) compared with PF2341066 and gefitinib alone, respectively. Similar results were observed with P-MAPK inhibition. Compared with control, a 3% (P > 0.05) decrease by PF2341066 alone, a 48% (P = 0.038) decrease with gefitinib and an 89% (P = 0.002) decrease with the combination was observed. Compared with PF2341066 and gefitinib treatments, combination treatment inhibited P-MAPK by 88% (P = 0.028) and 78% (P = 0.134), respectively.

As shown in Figure 1D, UM-22B cells are the most sensitive to both PF2341066 and gefitinib. Concentrations were chosen on the basis of IC_{50} values of PF2341066 and gefitinib. As observed with UM-22A cells, inhibitors to both pathways were necessary to achieve full signaling inhibition of P-AKT and P-MAPK. P-AKT and P-MAPK were inhibited by 36% (P > 0.05) and 29% (P > 0.05) by PF2341066, 74% (P = 0.0002) and 34% (P > 0.05) by gefitinib, 96% (P < 0.0001) and 73% (P = 0.0025) by combination treatment, respectively, compared with control. Dual treatment resulted in an 86% to 94% inhibition compared with single-agent treatment for P-AKT expression and a 59% to 62% decrease for P-MAPK expression (Fig. 4D). Together, these results show that when ligands of both pathways are present, inhibitors of both pathways are necessary to achieve maximum downstream signaling inhibition of P-MAPK and P-AKT, showing that c-Met and EGFR signaling can substitute for each other.

**TGF-α induces c-Met phosphorylation through an HGF-independent pathway**

It has been previously reported in other model systems including human hepatocellular carcinoma cells and non–small cell lung cancer (NSCLC) that EGFR activation contributes to c-Met phosphorylation (16, 24, 25). Because HNSCC express both receptors, we determined whether the EGFR ligand, TGF-α, could induce c-Met phosphorylation in HNSCC cells. Serum-deprived UM-22B were treated with TGF-α for 0 to 24 hours and total cell lysates were analyzed for P-Met, T-Met, P-EGFR, and T-EGFR expression (Fig 5A). Cell culture media was also collected at these time points for analysis of HGF secretion (Fig. 5B). TGF-α stimulation induced both T-Met and P-Met expression. TGF-α induced a 1.4-fold increase in total c-Met protein levels by 8 hours (P = 0.0304) and 1.8-fold increase by 24 hours (P = 0.038; Fig. 5C). A maximum 6.6-fold increase of c-Met phosphorylation by TGF-α was observed at 8 hours (P = 0.041; Fig. 5D). When normalized to β-actin, a 4.1-fold increase in c-Met phosphorylation occurred by 4 hours with a maximal increase of 7.8-fold by 8 hours (P = 0.039) declining somewhat by 24 hours (Fig. 5E). This suggests that induction of T-Met protein only contributes partially to the sustained increased activation of c-Met initiated by TGF-α. In contrast, EGFR phosphorylation began at 5 minutes and was completed by 2 hours (Fig. 5A). TGF-α induced a prolonged c-Met phosphorylation in HNSCC that peaks at a later time point (8 hours), whereas our previous results showed that, as expected, HGF stimulated c-Met phosphorylation transiently, beginning at 5 minutes and terminating quickly (11). Delayed c-Met phosphorylation following TGF-α stimulation was also observed in the Cal-33 cell line (Supplementary Fig. S2).

HGF secreted in the tissue culture media of UM-22B cells treated with TGF-α was measured over time. HGF was not detected at any time point (Fig. 5B). Results were similar in Cal-33 cells (Supplementary Fig. S2B). Furthermore, we did not detect any HGF mRNA from the cells at the same time points by real time PCR (data not shown). These results show that HGF autocrine signaling is not responsible for the EGFR-induced c-Met phosphorylation that we observed and is consistent with what we reported previously in regards to HGF paracrine signaling in HNSCC (11). As depicted in the model in Figure 5F, this is a novel mode of regulation for HNSCC cells in which EGFR ligands can regulate activation of c-Met through prolonged c-Met tyrosine phosphorylation and increased c-Met protein that is not attributable to HGF-ligand autocrine production. c-Met and EGFR can compensate for each other because they both induce similar downstream signaling through activation of the same adaptor and/or signaling molecules such as Grb2, Gab1, SOS, Ras, AKT, and MAPK.
Figure 4. P-AKT and P-MAPK are only completely inhibited by targeting both EGFR and c-Met in HNSCC cells. UM-22A (A and B) and UM-22B (C and D) were grown to 75% confluency in 60-mm plates and serum deprived for 48 hours. The cells were pretreated for 2 hours with DMSO (control), PF2341066 (PF), and/or gefitinib (GEF) as indicated followed by stimulation with HGF and TGF-α for 5 minutes. A and C, representative Western blots; B and D, quantitation. The ratio of phosphorylated to total protein was calculated and expressed relative to control (set to 1). Values are the mean ± SE of 3 independent experiments. ns, not significant; *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \), unpaired Student’s t test.
Combined targeting of c-Met and EGFR has enhanced antitumor activity in a xenograft model of HNSCC

To address whether dual targeting of both the EGFR and c-Met pathways leads to enhanced antitumor effects in vivo, SCID mice bearing UM-22B flank tumors were treated with either vehicle control, gefitinib, PF2341066, or gefitinib plus PF2341066 for 5 days per week for 3 weeks. PF2341066 alone (12.5 mg/kg) and gefitinib alone (50 mg/kg) resulted in 23.7% (P = 0.048) and 37.1% (P = 0.0004) decreases of tumor volume compared with vehicle control. A, UM-22B cells were grown to 75% confluency in 60-mm plates and serum deprived for 48 hours. Cells were treated with TGF-α (10 ng/mL) for 0 to 24 hours. Whole cell lysates were extracted and total and phosphorylated c-Met and EGFR and β-actin were detected by Western blot. A representative experiment is shown. B, UM-22B cells were treated as described for (A) and the cell culture supernatant was collected from 0 to 24 hours after TGF-α treatment. HGF release was measured by ELISA assay. Serum-free media without cells and conditioned media from fibroblasts were used as negative and positive control, respectively. C, quantitation of T-Met/β-actin. D, quantitation of P-Met/T-Met. E, quantitation of P-Met/β-actin. Values are the mean ± SE of 2 independent experiments with control set to 1. F, model of cross-talk between EGFR and HGF/c-Met pathway in HNSCC. HGF/c-Met and EGFR pathway share many of the same critical adaptor molecules (Gab-1, Grb2, SOS, and Ras) and downstream cascades including PI3K/AKT and MAPK which induce growth, survival, and invasion, and TGF-α induced delayed phosphorylation of c-Met in HNSCC.
control, respectively (Fig. 6A). The combination treatment resulted in a greater decrease in tumor volume compared with control 54.8% ($P < 0.0001$). Tumor volume from tumors treated with PF2341066 alone was significantly different from combination treatment ($P = 0.0011$) whereas gefitinib alone compared with combination treatment was very nearly significant ($P = 0.0525$; Fig. 6A). Both main effects of gefitinib and PF2341066 were significant ($P = 0.005$ and $P = 0.022$, respectively) without interaction ($P = 0.930$), suggesting that the combination treatment resulted in an additive decrease in tumor volume compared with control (Fig. 6A).

The mean number of apoptotic cells in the tumors isolated at the end of the experiment as assessed by the
TUNEL assay was increased in all treatment groups (PF2341066: 15.7 ± 2.9; gefitinib: 11.7 ± 8.8; combination: 37.3 ± 7.8) compared with the mean number of positive cells in the control group (4.67 ± 8.9; Fig. 6B). The number of apoptotic cells revealed that the mean number of apoptotic cells in the combination group was significantly higher than the mean number of apoptotic cells in the PF2341066 (P < 0.05) and gefitinib (P < 0.01) treatment groups (Fig. 6B). Histogram results of the combination treatment group have much less tumor cellularity. Tumor cells are replaced by debris, areas of differentiation, and infiltration of stroma (Fig. 6B), suggesting the antitumor effect may be underestimated by volume measurements. Similar results were observed with the number of proliferating cells in the tumors as assessed by Ki67 staining. The mean number of Ki67 positive cells in each treatment group (PF2341066: 70.3 ± 2.4 P < 0.01; gefitinib: 85.0 ± 3.1, P < 0.01; combination: 52.7 ± 6.1, P < 0.005) was statistically lower than the number of cells in control tumors (125.7 ± 8.2; Fig. 6B). The mean number of proliferating cells in the combination treatment group was lower than either the mean number of positive cells in the PF2341066 (nonsignificant P value) or gefitinib (P < 0.05) treated groups alone (Fig. 6B).

Discussion

EGFR amplification, activating mutations within the EGFR kinase domain, and high EGFR protein expression have all been shown to associate with sensitivity to EGFR TKIs in NSCLC (26). Although EGFR-activating mutations are rare in HNSCC (27), extensive EGFR overexpression as well as an autocrine loop with TGF-α and aberrant amplification, factors which are associated with poor outcome in HNSCC, have provided a rationale for investigation of EGFR targeted strategies in HNSCC (28–30). However, EGFR inhibitors as single agents have yielded low response rates of 4% to 13% in clinical practice for treatment of head and neck squamous cell tumors (31, 32).

Aberrant activation of the HGF/c-Met pathway has been implicated in the development and progression of various epithelial carcinomas and correlated with invasive growth (33–35). Numerous antagonists and c-Met TKIs have been developed to target the HGF/c-Met signaling pathway over recent years. One c-Met TKI which is currently in phase II/III clinical testing, PF2341066, showed antitumor activity by inhibiting angiogenesis, proliferation, and metastasis while increasing apoptosis in preclinical animal models of ovarian, breast, and gastric carcinomas (36–38). We and others have previously identified the HGF/c-Met pathway as a potential therapeutic target for HNSCC with similar responses as described earlier, using PF2341066 in vitro and in vivo in HNSCC models (11, 39). However, accumulating evidence indicates that cooperation of multiple RTKs, including EGFR and HGF/c-Met, is common and accounts for resistance to either single targeting therapy (9, 13, 40). Recently, Seiwert and colleagues have shown that the c-Met TKI, SU11274, could synergize with either erlotinib or cisplatin in HNSCC in vitro, suggesting that targeting the EGFR with c-Met may be critically important in HNSCC (39). Improving the clinical response rate and eliminating resistance to EGFR inhibition may be circumvented by combining EGFR inhibitors together with other treatment modalities.

We observed coexpression of EGFR and c-Met in all HNSCC cell lines that we examined, although to different extents, which suggests that EGFR and c-Met may show variation in the extent to which they cooperate to drive cells to survive, grow, and migrate. We also provide evidence of antitumor effects of dual EGFR and c-Met targeting strategies in HNSCC using gefitinib and PF2341066. Combined targeting of these pathways in vitro showed enhanced inhibition of cell proliferation compared with either inhibitor alone. In vivo, combination treatment showed an additive effect that was highly suggestive of increased inhibition compared with single treatment based on tumor volume, and histologic changes were consistent with reduced tumor cellularity. The reduction in tumor burden may result from increased apoptosis and decreased expression of Ki67 which was also found in the xenograft model. EGFR/c-Met combination therapy has shown enhanced antitumor effects in many other cancer models that display EGFR and c-Met cross-talk including lung, breast, kidney, colon, stomach, and pancreas (41).

EGFR and c-Met both utilize similar adaptor molecules and activate important downstream signaling molecules such as AKT and MAPK which are implicated in various biological activities including cell growth, survival, and invasion (2, 22, 23, 42, 43). Inhibition of PI3K/Akt and MAPK by blocking either EGFR or c-Met may be compensated for by activation of alternate pathways, which may cause failure of single pathway targeting. Here, we showed that P-AKT and P-MAPK formation in response to the combination of an EGFR and c-Met ligand could not be completely inhibited by gefitinib or PF2341066 alone, whereas the TKIs in combination maximally reduced both P-AKT and P-MAPK. Decreased activation of Akt and MAPK brought about by targeting both pathways prevents HNSCC cells from switching to a compensatory pathway. Our results show the redundancy of these pathways and provide further rationale for a dual targeting strategy in HNSCC, when both signaling pathways are active. Expression of multiple EGFR ligands as well as HGF has been well documented in HNSCC tissues, suggesting these tumors often display compensatory signaling (2, 3, 11). Recent proteomic results reported in lung cancer also showed extensive overlap between the HGF and EGF signaling networks (44).

Cross-talk between EGFR and other signaling pathways such as G protein–coupled receptor, estrogen receptor, and COX-2 has often been observed, and combined targeting of EGFR with these signaling pathways also produced promising antitumor efficacy in HNSCC (12, 45, 46). c-Met can be activated through HGF autocrine action which occurs in osteosarcomas and glioblastomas or through paracrine activation as found in breast, prostate, and lung.
c-Met can also be activated in a ligand-independent manner through protein overexpression or mutation (47). We recently identified another intrinsic mechanism of c-Met activation in lung cancer cells in which EGFR activation induces prolonged phosphorylation and increased c-Met protein levels in NSCLC cells (16). In NSCLC, total c-Met upregulation by EGFR ligands is due to new c-Met transcription (16) whereas in neuroblastoma this increase is thought to occur via EGFR activation of the hypoxia-inducible factor 1α pathway (48). Here, for the first time, we report that a similar mechanism also occurs in HNSCC involving delayed c-Met phosphorylation and increased total c-Met protein expression by TGF-α stimulation, which was independent of HGF. C-Src has been shown to be the critical signaling intermediate linking EGFR to c-Met in lung cancer (16), and because c-Src has been previously shown to be activated downstream of EGFR in HNSCC, it is a likely candidate (49). Preliminary data suggest that pretreatment of HNSCC cells with the pan-src inhibitor, PP2, leads to inhibition of TGF-α–induced P-Met expression, suggesting that c-src does play a role (data not shown); however, the exact mechanism in HNSCC is yet to be determined.

Consistent with recent observations of extensive EGFR and c-Met downstream signaling overlap (20, 21, 50), we provide evidence that targeting both pathways yielded maximum antitumorigenic effects compared with single-agent therapy in HNSCC both in vitro and in a preclinical xenograft model. We also present a novel mechanism for HNSCC by which c-Met can be activated by EGFR through a c-Met ligand-independent pathway, suggesting that c-Met can serve as a downstream mediator of EGFR action in addition to signaling in response to HGF. Future studies will seek to clarify what potential signaling intermediates are active in the EGFR/c-Met lateral signaling pathway and what the functional significance of lateral signaling is. The clinical utility of EGFR inhibition for head and neck cancer may be enhanced by addition of a c-Met antagonist.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Dual Blockade of EGFR and c-Met Abrogates Redundant Signaling and Proliferation in Head and Neck Carcinoma Cells

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