Hedgehog Signaling Alters Reliance on EGF Receptor Signaling and Mediates Anti-EGFR Therapeutic Resistance in Head and Neck Cancer

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Abstract

The EGF receptor (EGFR)-directed monoclonal antibody cetuximab is the only targeted therapy approved for the treatment of squamous cell carcinoma of the head and neck (HNSCC) but is only effective in a minority of patients. Epithelial-to-mesenchymal transition (EMT) has been implicated as a drug resistance mechanism in multiple cancers, and the EGFR and Hedgehog pathways (HhP) are relevant to this process, but the interplay between the two pathways has not been defined in HNSCC. Here, we show that HNSCC cells that were naturally sensitive to EGFR inhibition over time developed increased expression of the HhP transcription factor GLI1 as they became resistant after long-term EGFR inhibitor exposure. This robustly correlated with an increase in vimentin expression. Conversely, the HhP negatively regulated an EGFR-dependent, EMT-like state in HNSCC cells, and pharmacologic or genetic inhibition of HhP signaling pushed cells further into an EGFR-dependent phenotype, increasing expression of ZEB1 and VIM. In vivo treatment with cetuximab resulted in tumor shrinkage in four of six HNSCC patient-derived xenografts; however, they eventually regrew. Cetuximab in combination with the HhP inhibitor IPI-926 eliminated tumors in two cases and significantly delayed regrowth in the other two cases. Expression of EMT genes TWIST and ZEB2 was increased in sensitive xenografts, suggesting a possible resistant mesenchymal population. In summary, we report that EGFR-dependent HNSCC cells can undergo both EGFR-dependent and -independent EMT and HhP signaling is a regulator in both processes. Cetuximab plus IPI-926 forces tumor cells into an EGFR-dependent state, delaying or completely blocking tumor recurrence.

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Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is a devastating disease afflicting more than 45,000 people in the United States each year (1). The EGFR receptor (EGFR)-directed inhibitor cetuximab is the only approved targeted therapy for the treatment of HNSCC (2, 3). However, efficacy is low and patients that initially show a promising response often become refractory (4, 5). The EGFR and hedgehog pathways (HhP) have been implicated as key drivers of proliferation and survival of cancer cells. HhP signaling may also be a crucial pathway in the survival and characteristics of cancer stem cells and is a likely candidate for drug resistance (6, 7).

EGFR and HhP signaling converge and/or synergize upstream of GLI1 through the mitogen-activated protein/extracellular signal–regulated kinase (MEK)/extracellular signal–regulated kinase (ERK) signaling pathway in cancer cells and during keratinocyte oncogenic transformation (8–10). EGFR stimulates expression of GLI1 and target genes BCL2 and PTCH1 in gastric cancer (11), and the HhP ligand sonic hedgehog (SHH) signals through mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) to increase expression of HhP-specific targets in renal cancer (12).

Both pathways have been closely linked to epithelial-to-mesenchymal transition (EMT; refs. 13, 14). In this process, epithelial cells gain a more spindle- or fibroblast-like phenotype and become more mobile and invasive. Molecularly, EMT is characterized by expression of the pro-EMT ZEB, SNAIL, and TWIST transcription factors, loss of E-cadherin and increased levels of vimentin (Vim; ref. 15). The ability of cells to alter their morphology is often associated with drug resistance, allowing tumor cells to escape from cytotoxic and pathway-targeted therapies (16–18). Recently, reports have described an EGF-induced EMT-like state in EGFR-dependent HNSCC and...
prostate cancer cell lines (19, 20). On the other hand, chronic gefitinib treatment was found to generate a mesenchymal drug-resistant population in HNSCC cells independent of EGFR activation (21). The dichotomy of these EGFR-dependent and -resistant states and the role of HhP signaling have yet to be clarified in HNSCC.

The relationship between these pathways and their individual roles in EMT and drug resistance was previously investigated in immortalized keratinocytes or cancer cell lines (8, 11). We have generated and characterized a direct patient xenograft bank of HNSCC tumors implanted directly into mice with no time spent in culture. These in vivo tumor models may better mimic tumor heterogeneity and the relationship with the microenvironment (22).

We aimed to define the roles of EGFR and HhP signaling in early (EGFR-dependent) and late (EGFR-independent) EMT, migration/invasion, and anti-EGFR therapy susceptibility in HNSCC. We characterized the cross-talk between EGFR and HhP in HNSCC, and conducted combination studies targeting EGFR and HhP signaling in patient-derived xenografts.

Materials and Methods

Cell lines and in vitro drugs

HN11, Tu-167, FaDu, and 584 HNSCC cell lines were previously described (23–28) and grown in Dulbecco’s Modified Eagle Medium with 10% FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin. Low serum medium (LSM) contained 0.5% FBS. Erlotinib, AZD6244, and ZSTK474 were acquired commercially. IPI-926 was supplied by Infinity Pharmaceuticals Inc. To generate resistant cell lines, cells were continuously cultured in erlotinib (1, 5, 10, and 25 μM) or dimethyl sulfoxide (DMSO; control). Erlotinib concentration was increased when cultures proliferated at more than 50% of controls. Final selection at 50 μM/L erlotinib was completed 3× for 72 hours allowing growth in-between.

Gene silencing

siRNA experiments were completed in serum-free media (SFM) using 1 μM/L Dharmafect1 and 100 nmol/L siRNA (Thermo). GLI1 silencing was completed using doxycycline (0.5 μg/mL)-inducible pTRIPZ lentiviral constructs (RH5696-99636732; Open Biosystems) expressing short hairpin RNA (shRNA). Infection of cells with scramble or GLI1 sequences was conducted per the supplier’s instructions.

In vitro Matrigel invasion assay and colony formation

Cells were added to 6-well Matrigel-coated 8-μm pore inserts (BD Biosciences) and incubated for 24 hours. Invasion was quantified as cells per view, 6 fields per insert, repeated twice. Next, invading and noninvading cells were collected and seeded (300 cells/well). Cells were allowed to adhere (6–12 hours) before drug and incubated for 24 to 72 hours. Plates were incubated for 7 days. Resulting colonies (>50 cells) were fixed with 4% formalin and stained using 0.1% crystal violet.

Sulfonhodamine B colorimetric assay

Cells (2,500–5,000) were plated in 96-well plates and incubated overnight. Drug was added and plates were incubated for 96 hours. Cells were fixed with 50 μL of 10% TCA at 4°C (30 minutes), washed 5× with distilled water (dH2O), 70 μL/well Sulfonhodamine B (SBB) reagent was added, wells were washed 5× with 1% acetic acid, 200 μL/well 10 mmol/L Tris base was added, and absorbance was measured using a Synergy 2 microplate reader (BioTek).

Time-lapse imaging and cell tracking

Media on cells seeded on a 24-well plate was changed to LSM containing vehicle or drug for 24 hours before EGF treatment (100 ng/mL). Images were taken on a Zeiss Axiovert equipped with an environmental chamber and a Hamamatsu CCD camera. Images were taken every 10 to 15 minutes over the 48-hour time course. Acquisition and cell tracking were done using Volocity software (PerkinElmer). Three points were chosen per well, and 10 cells were tracked per point, for a total of 30 tracked cells per condition.

GLI1 promoter luciferase assay

A GLI1 promoter luciferase reporter construct was purchased (SwitchGear Genomics) containing approximately 1,000 bp DNA fragment upstream of GLI1 overlapping the transcribed region by 50 bp. Positive, negative, and GAPDH promoter controls were used. Cells were seeded (1,500/well in 100 μL medium) in white-walled 96-well plates and transfected as per the manufacturer’s instructions. Wells were treated with 1 μmol/L erlotinib for 24 hours, then plates were frozen at −80°C for more than 12 hours. Plates were thawed and 100 μL of luciferase substrate was added. Luminescence was measured on a Synergy 2 micro-plate reader (BioTek).

Human xenograft generation and in vivo studies

Briefly, fresh tumor tissue from patients with HNSCC consented at the University of Colorado Hospital (Aurora, CO) in accordance with the protocol approved by the Colorado Multiple Institutional Review Board (COMIRB # 08-0552) were collected. Prepared 3 mm × 3 mm × 3 mm tumor pieces were transplanted into Matrigel (BD Biosciences) and inserted into a “pocket” in both hind flanks of nude mice. Upon reaching 1,500 mm3, tumors were passed to a second colony of animals for therapeutic studies.

We tested cetuximab (acquired commercially), IPI-926, and the combination in 6 patient cases to generate efficacy data. For each case, we implanted 40 tumors in 20 mice. When tumors reached 200 mm3, mice were distributed into 4 groups (at least n = 8 tumors/group) and treated: control, cetuximab 40 mg/kg/2/week intraperitoneally, IPI-926 40 mg/kg 5/week per os, or cetuximab 40 mg/kg/2/week plus IPI-926 40 mg/kg/5/week for 4 weeks. Tumor size was evaluated twice weekly using the formula: volume = (length × width2)/2. Six hours after the last drug administration, tumors were extracted and portions were flash-frozen and embedded in paraffin. For shRNA studies, 100,000 cells transfected with scrambled or GLI1 constructs were injected subcutaneously in the flank of nude mice.

Immunohistochemistry

Immunohistochemical analyses were conducted on tissue arrays constructed using a manual Tissue Puncher (Beecher...
Incubated in 3% H2O2 for 10 minutes, followed by primary pS6K antibody (2211; Cell Signaling) and incubated for 60 minutes at room temperature. Staining was done in a Dako Autostainer. Slides were deparafed by standard techniques before antigen retrieval in alcohol by standard techniques before antigen retrieval in 4% to 12% Bis-Tris Midi Gel (Invitrogen) then processed. iBlot Gel Transfer Stack System (Invitrogen) then processed. Western blotting

Cell pellets were lysed in 30 to 100 μL radioimmunoprecipitation assay (RIPA) lysis buffer containing 5 μL/mL phenylmethylsulfonylfluoride (PMSF). Tissue sample (50 mg) portions were thawed in a 4°C for 20 minutes. Fixation was activated by erlotinib, whereas suppressed by EGF in both based luciferase assay, we confirmed that the GLI1 promoter was activated by erlotinib, whereas suppressed by EGF in both

Statistical analysis

Data are represented graphically as the mean ± SEM or SD for comparison between groups. P values less than 0.05 were considered significant.

Results

EGFR inhibition upregulates the HhP

Acquired resistance to EGFR inhibition is common. Previous reports synergistically linked EGFR and HhP through MEK/ERK (9), and we hypothesized that this relationship may provide a mechanism for EGFR resistance in HNSCC. Sensitivity to anti-EGFR therapy was previously correlated with c-FOS suppression in HNSCC cell lines (24). Following 1 μmol/L erlotinib treatment, c-FOS levels decreased by 6 hours and remained suppressed at 24 hours for the epithelial HN11 and Tu-167 cell lines, whereas levels remained unchanged in the mesenchymal 584 cells. GLI1 mRNA increased in HN11 and Tu-167 cells at both time points but remained unchanged in 584 cells (Fig. 1A). The induction of GLI1 corresponded with reduced levels c-FOS in HN11 and Tu-167, suggesting that GLI1 expression is only induced after treatment in EGFR-dependent cell lines.

Given that EGFR inhibition increased GLI1 mRNA in sensitive cells led us to identify the effects of EGFR activation. Serum-starved cells were treated with 100 ng/mL EGF, leading to increased levels of c-FOS within 1 hours. Twenty-four hours after EGFR activation, expression of GLI1 was significantly reduced in HN11 and Tu-167 cells but not in 584 (Fig. 1B). Suppression of GLI1 by EGFR activation was confirmed in HN11 and Tu-167 cells using the EGFR ligands TGF-α and amphiregulin (AR) (Supplementary Fig. S1A). Using a GLI1 promoter-based luciferase assay, we confirmed that the GLI1 promoter was activated by erlotinib, whereas suppressed by EGF in both

Immunocytochemistry

Cells grown in chamber wells were fixed in 4% paraformaldehyde for 10 minutes at room temperature then rinsed with PBS + Tween 20 (PBST) 3× for 5 minutes before permeabilizing in 0.2% Triton X-100 for 10 minutes, before blocking in 1% bovine serum albumin in PBS for 30 minutes at room temperature with shaking. Cells were incubated with E-cadherin (Cell Signaling, 4295) and/or vimentin (Cell Signaling, 9854) for 1.5 hours at room temperature at 1:50 and 1:200 dilutions, respectively. Alternatively, cells were incubated with isotype controls, including rabbit IgG conjugated with Alexa Fluor 488 (Cell Signaling, 2975) and rabbit IgG conjugated with Alexa Fluor 555 (Cell Signaling, 9569). Cells were rinsed in PBST 3× times, once with PBS and mounted with Prolong anti-fade with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were taken on an Olympus IX81 microscope equipped with a Hamamatsu CCD camera. Each experiment (200 cells analyzed) was repeated twice.

Analysis of vimentin-positive cells by flow cytometry

Cells (1 × 10⁶) were fixed in 2 mL 4% paraformaldehyde in PBS at 4°C for 15 minutes and then 100% cold ethanol was added drop-wise to a final concentration of 80%. Cells were resuspended in permeabilization buffer (0.25% Triton X-100 in PBS) for 30 minutes and washed 2× with cold PBS. Cells were suspended in anti-Vim-FITC antibody (Cell Signaling, 9454) and then 100% cold ethanol was added. The cells were centrifuged, washed and resuspended in permeabilization buffer (0.25% Triton X-100 in PBS) for 1.5 hours at room temperature with shaking. Cells were incubated with E-cadherin (Cell Signaling, 4295) and/or vimentin (Cell Signaling, 9854) for 60 minutes at room temperature. Staining was developed by: EnVision+ Dual Link System HRP (Dako) for 30 minutes and substrate-chromogen (DAB+) Solution (Dako) for 7 minutes. Slides were then counterstained with Automated Hematoxylin (Dako) for 5 minutes. The intensity (0, 1+, 2+, and 3+) and the percentage (0%–100%) of cells positive were interpreted blinded to the case and treatment.

RNA isolation and gene expression analysis

Samples (~30 mg) were placed in 300 μL of QiAzoL and homogenized using the MP Biomedicals Fast Prep 24. RNA was extracted using Qiagen kits according to the manufacturer’s instructions. RNA concentration and quality was measured using the NanoDrop. RNA was reverse-transcribed to cDNA in Oligo(dT) 18 primers using the Verso cDNA Synthesis Kit (Fisher Scientific). cDNA amplification was carried out using the Thermal Cycler (Applied Biosystems). TaqMan primer probes (Applied Biosystems), PCR amplification, and probe detection were accomplished using the StepOnePlus Real-Time PCR System (Applied Biosystems). All data are representative of experiments carried out at least 2 times in triplicate.

Western blotting

Cell pellets were lysed in 30 to 100 μL radioimmunoprecipitation assay (RIPA) lysis buffer containing 5 μL/mL phenylmethylsulfonylfluoride (PMSF). Tissue sample (50 mg) portions were thawed in a 4°C volume of RIPA buffer and homogenized using single-use plastic pestles. Protein was measured using the ELx800 absorbance microplate reader (BioTek) according to the manufacturer’s instructions. Thirty nanogram of protein was loaded per well into NuPage Novex 4% to 12% Bis–Tris Midi Gel (Invitrogen), transferred using the iBlot Gel Transfer Stack System (Invitrogen) then processed. Primary antibodies were purchased from Cell Signaling Technologies: 2236 phospho-EGFR (2211; Cell Signaling, 1H12) mouse monoclonal antibody (mAb), 4405 EGFR (15F8) rabbit mAb, 4060 phospho-Akt (Ser473; D9E) rabbit mAb, 4281 Akt (pan; 40D4) mouse mAb (biotinylated), 4094 phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; D13.14.4E) XP rabbit mAb (biotinylated), 9102 Pan-42 MAPK (Erk1/2) antibody, 2534 GLI1, and 4968 Pan-Actin and used as recommended. Secondary anti-rabbit immunoglobulin G (IgG; Immunoresearch) was used at a 1:50,000 dilution. The signal was visualized using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore).

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HN11 and Tu-167 cells with no change in 584 cells (Supplementary Fig. S1B). In contrast to previous reports (8), we found that neither erlotinib nor EGF treatment modulated GLI1 expression in human keratinocytes (Supplementary Fig. S1C and S1D). In HN11 and TU-167 cells, 72-hour EGF treatment suppressed GLI1 protein levels, whereas erlotinib increased GLI1 levels (Fig. 1C), with crosstalk occurring through MEK/ERK not PI3K/AKT signaling (Fig. 1D and 1E). Combined, these results suggest that EGFR signaling downregulates the HhP transcription factor GLI1 in EGFR-dependent HNSCC cells.

**EMT and anti-EGFR therapy resistance in HNSCC**

EMT and drug resistance are often related (18, 21), as observed in our HNSCC cell lines (Supplementary Fig. S2). We attempted to establish this connection by generating cell lines with acquired, high-level resistance to erlotinib (≥50 μmol/L). HN11 and Tu-167 cells continuously treated with 10 μmol/L erlotinib highly expressed the HhP transcription factor GLI1 and, to a lesser extent, GLI2 (Fig. 2A). Erlotinib-resistant (50 μmol/L) HN11 and Tu-167 cells released from erlotinib treatment had higher c-FOS, ZEB1, SNAIL, TWIST, and VIM expression when compared with parental cells (Fig. 2B), consistent with a mesenchymal phenotype. By flow cytometry, erlotinib-resistant cells also had significant increases in VIM-positive cell populations (Fig. 2C). Interestingly, cellular morphology did not differ dramatically from controls. GLI1 and GLI2 were upregulated in cells chronically treated with erlotinib; therefore, erlotinib-resistant cells were tested for sensitivity to HhP inhibition with the novel Smoothened (SMO) inhibitor IPI-926 (29). Erlotinib-resistant cells showed...
increased motility that was suppressed by erlotinib on time-lapse track analysis (Fig. 3B). EGF had no effect on motility in 584 cells (not shown). Interestingly, IPI-926 increased cellular velocity in both HN11 (P < 0.01) and Tu-167 cells after EGF treatment but this effect was not observed in combination with erlotinib (Fig. 3C). These results suggest that EGFR activation generates an EMT-like state that is not prevented by HhP inhibition, but that coinhibition of the EGFR and HhP suppresses EMT.

**EGFR signaling increases EMT expression**

Next, EMT-related gene expression was measured after EGF treatment with or without erlotinib, IPI-926, or in combination. Expression of the pro-EMT transcription factor ZEB1 was significantly increased by EGF treatment in HN11, Tu-167, and FaDu cells but not 584 cells (Fig. 3D and Supplementary Fig. S4C). In comparison, EGF did not substantially modulate the expression of ZEB2, SNAIL, and TWIST1 (not shown). VIM expression followed that of ZEB1, whereas E-cadherin was suppressed by EGFR activation. These effects were reversed by pretreatment with erlotinib, whereas pretreatment with IPI-926 before addition of EGF increased expression of both ZEB1 and VIM when compared with EGF alone. These data suggest that the HhP inhibition might contribute to EGFR-driven EMT.

**Figure 2.** Chronic erlotinib treatment generates resistant cells with a mesenchymal gene expression pattern. A, expression of GLI transcription factors are significantly increased in EGFR-dependent cell lines chronically treated with 10 μmol/L erlotinib. B, erlotinib-resistant cells highly express pro-EMT genes after removal from selection media. C, percentage of VIM-positive cells is significantly increased in erlotinib-resistant cell lines, measured by flow cytometry. D, erlotinib-resistant cells are not sensitized to HhP inhibition by IPI-926 but this effect was not observed in combination with erlotinib (Fig. 3C). These results suggest that chronic EGFR inhibition generates cells with EMT-like characteristics.

**EGFR activation induces EMT and motility in HNSCC cells**

Increased GLI1 expression after EGFR inhibition, as well as the generation of erlotinib-resistant cell lines, provided a rationale for studying of the relationship between the EGFR pathway, HhP, and EMT. HNSCC cell lines were treated with erlotinib and IPI-926 alone or in combination, with or without EGFR activation by addition of EGF ligand. As previously reported (19, 20), EGF (100 ng/mL) induced an EMT-like state, defined by spindle-like cellular morphology, in all 3 epithelial cell lines (Fig. 3A) but not in EGFR-independent 584 cells. It is important to note that 500 ng/mL SHH had no noticeable effect on the morphology of any cell line (not shown). As expected, erlotinib completely blocked the phenotypic transition but IPI-926 had no effect. EGF induces an EMT-like phenotype, with increased motility that was suppressed by erlotinib on time-
We used siRNAs to determine if ZEB1 is responsible for increased expression of VIM by EGFR activation. Both siRNAs suppressed expression of ZEB1 after EGF treatment that in turn led to significantly (P < 0.01) lowered levels of VIM mRNA (Supplementary Fig. S3A and S3B). However, silencing of ZEB1 did not dramatically change the generation of spindle-like cells by EGF but both siRNAs significantly reduced EGF induced invasion (not shown), indicating that ZEB1 may be a key transcriptional driver of the EGF-induced EMT-like state.

Flow cytometry and immunocytochemistry (ICC) confirmed modulation of Vim by EGF at the protein level. In normal culture, approximately 1% to 3% of HN11 and Tu-167 cells were Vim-positive (Fig. 4 and Supplementary Fig. S4), whereas 100% of 584 cells were Vim-positive. EGF treatment increased the percentage of Vim-positive cells 7- and 10-fold in Tu-167 and HN11 cells, respectively. Erlotinib completely blocked the EGF-driven increase in Vim-positive cells, whereas HhP inhibition slightly increased the Vim-positive population in Tu-167 cells, whereas remaining unchanged in HN11 cells (Supplementary Fig. S6A).

We next inquired if EGF could induce an EMT-like state in erlotinib-resistant cells. Unlike in parental cells, 1 μmol/L erlotinib did not increase GLI1 in resistant cells but instead seemed to suppress expression in HN11 and Tu-167 cells. However, EGF continued to suppress GLI1 expression slightly in Tu-167 cells (Supplementary Fig. S5A). Induction of ZEB1 and VIM by EGF was nearly completely suppressed in both HN11 and Tu-167 erlotinib-resistant cells. However, ZEB1 and VIM continued to be downregulated by erlotinib, whereas EGF continued to suppress E-cadherin.

Figure 3. Inhibition of the HhP may augment EGFR-driven EMT-like state in EGFR-dependent cell lines. A, EGFR signaling activation by the EGF ligand (100 ng/mL) generates an EMT-like phenotype in HN11 and Tu-167 cells that is completely blocked by erlotinib (1 μmol/L) but not IPI-926 (1 μmol/L). B and C, EGF increases cellular motility in EGFR-dependent cells, which was blocked by erlotinib but augmented by IPI-926. D, EGF increased expression of pro-EMT genes ZEB1 and VIM as well as suppressed E-cadherin generating an EMT-like gene expression profile in HN11 and Tu-167 cells but not 584 cells. However, E-cadherin expression was suppressed by erlotinib treatment in 584 cells. *, P < 0.05; **, P < 0.01.

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Increased invasion associated with EGFR-driven EMT

The EGFR induced EMT-like state increased invasion in EGFR-dependent cells. Increased invasion was blocked by erlotinib treatment (Fig. 5A). Consistent with our previous findings, IPI-926 augmented EGFR-driven invasion slightly in Tu-167 cells and significantly (P < 0.05) in the HN11 cell line (Fig. 5B). To confirm this was not an unintended effect of IPI-926, independent of HhP inhibition, we tested the invasiveness of HN11 and Tu-167 cells expressing shRNA against EGFR. Similar to IPI-926, silencing of GLI1 significantly (P < 0.05) increased the invasiveness of cells treated with saturating levels of EGF in both HN11 and Tu-167 cells (Fig. 5C and 5D). We next questioned whether invasiveness was indicative of highly proliferative state sensitizing cells to erlotinib. Non-invasive cells more readily formed colonies compared with invasive cells in HN11 and Tu-167, whereas the opposite was true for 584 (Fig. 5F). Finally, invasive EGFR-dependent cells did not show increased sensitivity to erlotinib but noninvasive HN11 cells had increased sensitivity when compared with their invasive counterparts (Fig. 5F). Combined, these findings indicate that though the EGFR-driven EMT-like state increases motility and invasiveness, proliferation is suppressed and cells are not sensitized to EGFR inhibition.

MEK/ERK and PI3K/AKT signaling are required for EGF-induced invasion

We next attempted to clarify what pathways downstream of EGFR regulate this EMT-like event. Before dosing with EGF, HN11 and Tu-167, cells were treated with 1 μmol/L of the MEK/ERK inhibitor AZD6244 or 1 μmol/L of the PI3K/AKT inhibitor ZSTK474. Similar to erlotinib, AZD6244 completely blocked phenotypic change and alone induced tightly associated cells and suppressed expression of ZEB1 and VIM. ZSTK474 did not affect the EMT induction and significantly increased (P < 0.05) expression of ZEB1 while further suppressing E-cadherin by EGF. However, unlike control cells that form spindle-like cells and dissociate, cells treated with ZSTK474 formed a distinct phenotype, elongated yet still tightly associated with adjacent cells (Supplementary Fig. S7A and S7B), as expected from previous reports. Previous reports support that MEK/ERK is primarily responsible for generating the mesenchymal phenotype but PI3K/AKT may be crucial to cell-to-cell adhesion and expression of matrix metalloproteinases (MMP; refs. 19, 30). Inhibition of MEK/ERK or PI3K/AKT signaling effectively blocked EGF-induced invasion (Supplementary Fig. S7C and S7D). Taken together, these results suggest that EGFR activation in EGFR-dependent cells generates a highly motile and invasive state that is decreased by MEK/ERK and PI3K/AKT signaling.
motile and invasive EMT-like state that is augmented by inhibition of the HhP via MEK/ERK signaling. Inhibition of EGFR and HhP signaling in HNSCC cell lines

The above findings lead us to hypothesize that by inhibiting the HhP we could achieve 2 objectives: (i) increase EGFR dependence and enhance anti-EGFR therapy efficacy, and (ii) prevent the EGFR inhibitor-driven EMT switch. These results also suggested that only dual targeting would achieve this goal. To this end, we conducted combination treatment experiments in vitro with erlotinib and IPI-926. IPI-926 has minimal activity in vitro at concentrations of 1 μmol/L or less and there was little additive effect with erlotinib. However, 250 μg/mL SHH ligand significantly reduced the antiproliferative effects of erlotinib (Supplementary Fig. S8), suggesting again that activation of HhP signaling may be a mechanism of resistance for anti-EGFR therapy.

Using in vitro systems, we had observed that suppression of the HhP increased EGFR-driven motility and invasion. To explore this in a more complex system, tumorigenicity of HNSCC cell lines expressing shGLI1 in immune-compromised mice was measured. Tumor formation was inhibited in both 584-shGlil1 (P = 0.16) and HN11-shGlil1 (P < 0.01; Fig. 6A). Resulting tumors were analyzed by flow cytometry for CD44 and turboRFP, expressed as part of the shRNA insert. Although CD44 expression was not altered dramatically, turboRFP expression was all but lost in shGlil1 tumors, suggesting that silencing of GLI1 inhibited tumor formation and the small resulting tumors may have arisen from cells that had lost the viral insert (Fig. 6B). Resulting tumor tissue was stained for E-cadherin and K14 and tumors expressing scramble sequence consisted primarily of cancer cells with interspersed stromal cells. However, cancer cells in shGlil1 tumors were found in smaller pockets within larger areas of mouse stromal cells possibly

Inhibition of EGFR and HhP signaling in HNSCC cell lines

The above findings lead us to hypothesize that by inhibiting the HhP we could achieve 2 objectives: (i) increase EGFR dependence and enhance anti-EGFR therapy efficacy, and (ii) prevent the EGFR inhibitor-driven EMT switch. These results also suggested that only dual targeting would achieve this goal. To this end, we conducted combination treatment experiments in vitro with erlotinib and IPI-926. IPI-926 has minimal activity in vitro at concentrations of 1 μmol/L or less and there was little additive effect with erlotinib. However, 250 μg/mL SHH ligand significantly reduced the antiproliferative effects of erlotinib (Supplementary Fig. S8), suggesting again that activation of HhP signaling may be a mechanism of resistance for anti-EGFR therapy.

Using in vitro systems, we had observed that suppression of the HhP increased EGFR-driven motility and invasion. To explore this in a more complex system, tumorigenicity of HNSCC cell lines expressing shGLI1 in immune-compromised mice was measured. Tumor formation was inhibited in both 584-shGlil1 (P = 0.16) and HN11-shGlil1 (P < 0.01; Fig. 6A). Resulting tumors were analyzed by flow cytometry for CD44 and turboRFP, expressed as part of the shRNA insert. Although CD44 expression was not altered dramatically, turboRFP expression was all but lost in shGlil1 tumors, suggesting that silencing of GLI1 inhibited tumor formation and the small resulting tumors may have arisen from cells that had lost the viral insert (Fig. 6B). Resulting tumor tissue was stained for E-cadherin and K14 and tumors expressing scramble sequence consisted primarily of cancer cells with interspersed stromal cells. However, cancer cells in shGlil1 tumors were found in smaller pockets within larger areas of mouse stromal cells possibly
indicating that silencing GLI1 leads to proliferation inhibition in vivo (Fig. 6C).

**Combination cetuximab and IPI-926 treatment in HNSCC patient xenografts**

To better mimic the true tumor environment, we next tested the efficacy of cetuximab, IPI-926, and the combination using patient-derived HNSCC xenografts. Four of 6 cases had tumor shrinkage after EGFR inhibition by cetuximab (Fig. 7A and B) but none regressed completely and 75% regrew to baseline during the study. IPI-926 showed modest single-agent efficacy with only moderate growth retardation in one xenograft (CUHN004). IPI-926 did not increase the rate of tumor regression in combination with cetuximab or dramatically decrease the T/C value at the end of treatment (Fig. 7B). However, after being released from therapy the regrowth kinetics for the cetuximab and combination arms of the study diverged dramatically for all 4 cetuximab sensitive cases. All tumors regressed completely and never regrew in the combination arm of 2 cases (CUHN002 and CUHN027), whereas regrowth was delayed by approximately 1 month in the other 2 cases (Supplementary Table S2), suggesting IPI-926 increases tumor control in combination with EGFR inhibition.

To unravel the molecular events underlying this dramatic efficacy, gene and protein expression were quantified for genes relevant to the EGFR pathway, GLI1, and EMT. As expected, expression of c-FOS decreased significantly in sensitive xenografts (CUHN002, CUHN004, CUHN013, and CUHN027) after cetuximab treatment, whereas remaining unchanged in resistant xenografts (CUHN014 and CUHN022; Supplementary Fig. S9A and S9B). IPI-926, cetuximab or combination treatment decreased expression of mouse GLI1 in both sensitive and resistant xenografts, whereas human GLI1 was relatively unchanged in sensitive xenografts. GLI1 increased in the combination arm of resistant cases, again confirming that the mouse stroma compartment may be target of HhP inhibition. Also, pro-EMT factors ZEB2 and TWIST1 and SNAIL were found to be upregulated after cetuximab and combination treatment in sensitive cases while remaining unchanged in the resistant group. Increased expression of pro-EMT transcription factors was observed for the cetuximab-sensitive xenografts and resembled the expression profile of erlotinib-resistant HNSCC cell lines. These findings suggest that after a 1 week pharmacodynamic (PD) study tumor cells may have undergone an EMT or selected for cells in an EMT state; these are likely the cells responsible for the repopulation of the tumor, and thus need to be specifically targeted to achieve a durable antitumor effect.

Protein analysis revealed that cetuximab reduced pEGFR in all cases except CUHN027, with a further reduction of total and pEGFR in combination therapy. Total EGFR was also reduced in CUHN004 and interestingly in the resistant CUHN014 and CUHN022 cases, suggesting that intrinsic dependence rather than the pharmacodynamic effect (that is necessary but not sufficient) determines susceptibility to EGFR inhibitors.
Downstream assessment was more informative: pMAPK and pAKT were suppressed in all sensitive cases treated with cetuximab or in combination. IPI-926 increased pMAPK in all sensitive cases, pAKT in both CUHN004 and CUHN027 xenografts after treatment. Treatment with IPI-926 decreased E-cadherin, pushing cells into an EGFR-driven EMT-like state (Supplementary Fig. S9C). Also, IPI-926 treatment increased pS6K in both CUHN004 and CUHN013 xenografts. Similarly, pS6K were suppressed in both cetuximab and combination treatment arms in sensitive xenografts while remaining unchanged in resistant cases by immunohistochemistry (Supplementary Fig. S9D). Finally, IPI-926 decreased E-cadherin in cetuximab-sensitive cases following our findings in vitro suggesting that HhP inhibition allows cells to enter an EGFR-dependent EMT-like state (Fig. 7C).

These results suggest that cetuximab significantly suppresses MEK/ERK and PI3K/AKT signaling in turn reducing expression of the downstream factor c-FOS in sensitive xenografts. Also, the HhP and EGFR signaling pathways may negatively regulate each other indicated by increased levels of pEGFR, pMAPK, pAKT, and pS6K after IPI-926 treatment, pushing tumor cells into a pro-EGFR state. Again, this confirms that while HhP inhibition is ineffective as a single agent, it can push tumor cells further into an EGFR-dependent state, leading to increased killing by anti-EGFR therapy resulting in tumor obliteration.

Discussion

Preventing intrinsic and acquired resistance to EGFR inhibition with cetuximab would be of benefit to patients suffering from HNSCC (2, 3), so we attempted to define the basis of EGFR resistance. Contrary to previous studies using keratinocytes (8), we identified increased expression of the HhP transcription factor GLI1 after EGFR inhibition as a unique characteristic of EGFR-dependent HNSCC cells. GLI1 has been shown to be a key driver of tumor growth and metastasis in multiple cancers (31, 32). As previously described (9), we found that “cross-talk” between the EGFR and HhP occurs through the MEK/ERK cascade, but that HhP inhibition may make cells more EGFR dependent.

Chronic erlotinib treatment in HN11 and Tu-167 cells dramatically increased GLI1 expression compared with parental controls and may signify the upregulation of a resistance pathway. Erlotinib-resistant cells were next generated to determine whether the HhP was a key driver of survival and proliferation when EGFR signaling is suppressed. Both strains had characteristics indicating that they had undergone an EMT event including erlotinib resistance, increased expression of pro-EMT factors ZEB1, ZEB2, TWIST, and SNAIL, and in Vimp-positive cell populations. Similarly generated gefitinib-resistant HNSCC cells lines had also undergone an EMT event (21).
However, resistant cells no longer overexpressed GLI1, indicating upregulation only occurred during the transition to the mesenchymal state, as has previously been reported (13, 33). These results confirm that EMT is at least an attribute, if not a direct mechanism, of drug resistance. Combined, these results generated a strong rationale for combination therapy inhibiting the EGFR and HhP in xenograft models.

Next, we defined how activation and inhibition of the EGFR and HhP, alone, and in combination, regulate signaling and may increase sensitivity to EGFR inhibition in HNSCC cell lines. From these studies, and as observed earlier, we established that there are 2 distinct mechanisms of EMT in HNSCC cells. As previously described in epithelial cancer cell lines (19, 20), activation of the EGFR pathway generates an EMT-like state in EGFR-dependent HNSCC cells. This “early” EMT-like event is defined by increased motility and mesenchymal morphology with increased expression of EMT signature genes and a Vim-positive population leading to invasiveness. Interestingly, this phenotype does not increase sensitivity or resistance to EGFR or HhP inhibition. Although this EMT-like state does not lead to drug resistance, it does grant cancer cells invasive potential, and is a potentially valuable in vitro model of tumor cell migration and invasion. Also, inhibition of the HhP, both pharmacologically and genetically, augmented the EGFR-driven phenotype by increasing activation of both MEK/ERK and PI3K/AKT. On the contrary, HhP activation by SHH increased resistance to erlotinib, confirming that the HhP negatively regulates EGFR signaling, whereas inhibiting HhP signaling allows cells to enter a state of greater EGFR dependence. Again, these findings support the rationale for combined inhibition of EGFR and HhP signaling as a means of enhancing anti-EGFR efficacy. We characterized 2 distinct EMT-like events in HNSCC cells, both EGFR-dependent and -independent and that both states are mutually exclusive as seen in 584 and erlotinib-resistant cells. Finally, HhP signaling may help initiate EGFR-independent EMT, whereas suppressing the EGFR driven EMT-like state.

The use of combination therapy in vitro was relatively uninformative, and our experiments highlighted the risks of artificial, growth factor saturated models. To clarify whether HhP signaling was a genuine target in HNSCC, in vivo GLI1 was silenced by shRNA in 2 cell lines and found GLI1 suppression inhibited tumor formation. Subsequently, and in an optimal use of an advanced patient-derived animal model, direct HNSCC patient xenografts were treated with cetuximab and IPI-926 alone and in combination. Cetuximab treatment induced tumor shrinkage in 4 of 6 cases but IPI-926 showed little response in this in vivo model. However, cetuximab-treated xenografts regrew to baseline after the end of treatment, whereas combination therapy with IPI-926 delayed regrowth by a month in 2 cases and all tumors were ablated in the other 2 xenografts. As expected, cetuximab blocked signaling through MEK/ERK and/or PI3K/AKT and combination treatment enhanced this effect, whereas IPI-926 increased pMAPK, pAKT, and pS6K in EGFR-dependent cases. It is important to note that IPI-926 also targets the stromal compartment as mouse GLI1 was suppressed after treatment and was previously observed in multiple carcinoma models (34, 35). However, our results do not rule out inhibition of the HhP in tumor cells, given that GLI1 shRNA abrogated tumor growth in vivo, an effect that cannot be explained by stromal effects.

These findings support the hypothesis that inhibition of HhP signaling pushes HNSCC cells into an even more EGFR-dependent state, sensitizing them to anti-EGFR therapy while blocking a relevant resistance mechanism. As for the efficacy of the novel therapeutic IPI-296, these findings support the use of a more complex animal model that better mimics the human environment. These provoking results provided the rationale for the clinical translation of this novel combinatory paradigm (NCT01255800).

Disclosure of Potential Conflicts of Interest
A. Jimeno has a commercial research grant from Infinity Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References
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