Targeting GPCR-Mediated p70S6K Activity May Improve Head and Neck Cancer Response to Cetuximab

Neil E. Bhola¹, Sufi M. Thomas², Maria Freilino², Sonali Joyce², Anirban Sahu², Jessica Maxwell², Athanassios Argiris³, Raja Seethala¹, and Jennifer R. Grandis¹,²

Abstract

Purpose: Epidermal growth factor receptor (EGFR) overexpression is correlated with decreased survival in head and neck cancer (HNC) where the addition of EGFR inhibition to standard chemoradiation approaches has improved treatment responses. However, the basis for the limited efficacy of EGFR inhibitors in HNC is incompletely understood. G-protein–coupled receptors (GPCR) have been shown to be overexpressed in HNC where GPCR activation induces HNC growth via both EGFR-dependent and -independent pathways. We hypothesized that targeting GPCR-induced EGFR-independent signaling would improve the efficacy of EGFR inhibition.

Experimental Design: Using a high-throughput phosphoproteome array, we identified proteins that were phosphorylated in HNC cells where EGFR expression was downmodulated by RNA interference (RNAi) in the presence or absence of a GPCR ligand. We confirmed the findings from the array by Western blotting followed by in vitro and in vivo phenotypic assays.

Results: p70S6K phosphorylation was elevated approximately sixfold in EGFR siRNA-transfected cells treated with a GPCR ligand. In addition to RNAi-mediated EGFR downmodulation, GPCR-mediated phosphorylation of p70S6K was modestly increased by EGFR inhibitor cetuximab approved by the Food and Drug Administration. Biopsies from cetuximab-treated patients also displayed increased phospho-p70S6K staining compared with pretreatment biopsies. HNC cells were growth inhibited by both genetic and pharmacologic p70S6K targeting strategies. Furthermore, p70S6K targeting in combination with cetuximab resulted in enhanced antitumor effects in both in vitro and in vivo HNC models.

Conclusions: These results indicate that increased phosphorylation of p70S6K in cetuximab-treated patients may be due to increased GPCR signaling. Therefore, the addition of p70S6K targeting strategies may improve treatment responses to EGFR inhibition. Clin Cancer Res; 17(15); 4996–5004. ©2011 AACR.

Introduction

Head and neck cancer (HNC) is characterized by overexpression of the epidermal growth factor receptor (EGFR). Increased expression of EGFR in HNC has been correlated with decreased patient survival, regardless of primary therapy (1). The addition of the EGFR monoclonal antibody cetuximab (C225, Erbitux) to radiation therapy improved survival leading to the Food and Drug Administration (FDA) approval of this agent for HNC in 2006 (2).

However, only a subset of HNC patients will experience a clinical response to cetuximab when administered as a primary treatment approach or in the setting of recurrent or metastatic disease (3). To date, no consistent association between response to EGFR inhibition and baseline expression of a specific biomarker, including EGFR, has been shown. In addition, there is a paucity of studies analyzing posttreatment tumors from patients treated with cetuximab, so that the effects of EGFR inhibitors on other signaling pathways in vivo are largely unexplored.

G-protein–coupled receptors (GPCR) are 7 transmembrane receptors that mediate cell growth, motility, and differentiation via stimulation by cognate agonists (4). The GPCR, bradykinin receptor 2 (B2R), which stimulates the upregulation of COX-2 and its downstream effector prostaglandin E2 (PGE2), another GPCR ligand, is overexpressed in HNC (5, 6). We and others have shown that GPCR ligands including PGE2, bradykinin (BK), gastrin-releasing peptide, and lysoosphosphatic acid mediate HNC proliferation and invasion via the autocrine release of EGFR ligands and the consequent activation of EGFR (7–9). Furthermore, combined inhibition of GPCRs and EGFR

Authors’ Affiliations: Departments of ¹Pharmacology and Chemical Biology, ²Otolaryngology, ³Medicine, and ⁴Pathology, University of Pittsburgh School of Medicine and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Jennifer Rubin Grandis, Eye and Ear Institute, Suite 500, 203 Lothrop Street, University of Pittsburgh, Pittsburgh, PA 15213. Phone: 412-647-5280; Fax: 412-647-0108; E-mail: grandisjr@upmc.edu
doi: 10.1158/1078-0432.CCR-10-3406
©2011 American Association for Cancer Research.
**Translational Relevance**

Epidermal growth factor receptor (EGFR) overexpression is correlated with poor prognosis in head and neck cancer (HNC) where, to date, efforts to target EGFR have been associated with limited clinical responses. Identification of alternative signaling mechanisms that promote tumor survival in the presence of EGFR blockade may identify new targets that when inhibited, can improve responses to anti-EGFR strategies. Here, we show that G-protein–coupled receptors (GPCR) activate p70S6K as a consequence of EGFR targeting. Furthermore, biopsies from cetuximab-treated patients displayed elevated p70S6K phosphorylation. Combined inhibition of p70S6K and EGFR was associated with improved antitumor effects in HNC preclinical models. These results indicate that inhibition of EGFR is associated with activation of collateral signaling pathways including p70S6K, which when targeted, can improve response to EGFR inhibitors. Clinical trials to test the efficacy of combined EGFR and p70S6K targeting in cancer patients are underway.

displayed enhanced antitumor effects, indicating that GPCRs can induce EGFR-independent pathways in addition to transactivation of EGFR (9, 10). Identification of the proteins induced in the absence of EGFR, or in the setting of EGFR blockade, may reveal new therapeutic targets, which can be inhibited to augment clinical responses in combination with cetuximab.

This study was carried out to elucidate “druggable” targets that contribute to GPCR-mediated HNC growth when EGFR is downregulated or inhibited. We used an antibody microarray to identify proteins that were activated by GPCRs under EGFR-downmodulated conditions. We then targeted the activated pathway by using genetic and pharmacologic approaches, alone and in combination with EGFR inhibitors, in HNC preclinical models in vitro and in vivo.

**Materials and Methods**

**Cell lines**

All HNC cell lines (PCI-37A, 1483, UM-22B, CAL133, and 686LN) were of human origin. 1483 cells were derived from an oropharyngeal tumor, UM-22B cell line was derived from metastatic lymph nodes, and PCI-37A was from a primary tumor in the epiglottis (11). CAL133, derived from oral squamous cell carcinoma cells (12), were a kind gift from Dr. Gerard Milano (Centre Antoine-Lacassagne, Nice, France). 686LN cell line was derived from the lymph node metastasis of a patient with oral squamous cell carcinoma and was a kind gift from Dr. Georgia Chen (Emory University, Atlanta, GA). Cells were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum (Invitrogen) at 37°C with 5% CO2.

**Reagents**

EGF and PGE2 were obtained from Calbiochem. Bradykinin was obtained from Bachem. RAD001 (everolimus) was provided by Novartis and C225 (cetuximab, Erbitux) was obtained from the pharmacy of University of Pittsburgh Cancer Institute.

**Phosphoprotein antibody array**

Phosphoprotein arrays were obtained from FullMoon Biosystems. PCI-37A cells were seeded in four 10-cm culture dishes: (a) nontargeting control (NTC) siRNA plus vehicle, (b) NTC siRNA plus PGE2, (c) EGFR siRNA plus vehicle, and (d) EGFR siRNA plus PGE2. Cells were transiently transfected with NTC siRNA or EGFR siRNA, serum-starved for 72 hours, and treated with vehicle or 10 nmol/L PGE2. Cells were lysed with the extraction buffer provided as described according to manufacturer’s instructions. Protein samples were biotinylated by using biotin reagent dissolved in N,N-dimethylformamide (DMF). Protein (10 μL) sample was mixed with labeling buffer (40 μL) followed by addition of biotin/DMF reagent at a 1:7 ratio. Biotin-labeled protein samples were conjugated to the cancer/apoptosis phospho-antibody array. Antibody array slides were incubated with blocking solution provided on a rotating shaker at room temperature. Slides were then rinsed 3 times in water and allowed to dry. Protein coupling mix was added over the array slide and incubated at 4°C overnight. Slides were washed twice for 10 minutes each with 1 × wash solution. Cy3-streptavidin solution was then added to the slides for 60 minutes at room temperature with shaking. Slides were scanned by the GenePix 4300 Array Scanner. Fold change in intensities from 6 replicates between PGE2-treated NTC siRNA and PGE2-treated EGFR siRNA cells was calculated and tabulated (Supplementary Table S1).

**Immunoblotting**

Cells were lysed with lysis buffer and quantitated as described previously. Lysates were resolved by 8% or 10% SDS-PAGE. After being transferred onto a nitrocellulose membrane, the membrane was blocked in 5% milk and blotted with various primary antibodies in 5% milk dissolved in TBS and Tween 20 (TBST) solution [0.6% dry milk powder, 0.9% NaCl, 0.5% Tween 20, and 50 mmol/L Tris (pH 7.4)]. After washing 3 times with TBST solution, the membrane was incubated with the secondary antibody (goat anti-rabbit/mouse IgG–horseradish peroxidase conjugate; Bio-Rad Laboratories) for 1 hour and washed 3 times for 10 minutes. Membranes were developed with Luminol Reagent (Santa Cruz Biotechnology) by autoradiography. Blots were stripped in Restore Western Blot Stripping buffer (Pierce) for 25 minutes at room temperature, blocked for 1 hour, and reprobed with primary antibodies.

**RNA interference studies**

Silencing RNA oligonucleotides targeting EGFR, p70S6K, were obtained from Dharmacon. EGFR siRNA was...
designed to target the following sequence: 5’-CUUUGAGGGAGAAAGAAA-3’. P70S6K siRNA was designed to target the following sequence: 5’-CCAAGGCUAUUGAAGACCA-3’. HNC cells (2 × 10^6) were seeded in 10-cm plates and allowed to incubate overnight at 37°C. Cells were transfected with siRNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. CAL133 cells were infected with lentiviral particles encoding p70S6K short hairpin RNA (shRNA; UPCI Vector Core Facility), cultured, and selected with puromycin for 2 weeks. Clones were selected with puromycin, cultured, and verified for protein knockdown of p70S6K.

**Proliferation assays**

HNC cells were seeded and incubated overnight at 37°C. Cells were treated with pharmacologic inhibitors or transfected with siRNA for different time points. For experiments involving p70S6K siRNA alone, MTT assay was carried out as described previously (10). For subsequent experiments involving cytostatic inhibitors cetuximab and RAD001, we observed no effect with MTT assay. Subsequent testing of different cell viability assays was conducted, and growth-inhibitory experiments involving cetuximab and RAD001 were determined by using the more sensitive fluorimetric resazurin–based CellTiter-Glo Assay (Promega) according to manufacturer’s instructions. Cells were read by using a Victor3 multilabel counter at 560ex/590em wavelengths. For BK-treated experiments, cells were trypsinized and stained with trypan blue solution before being transferred to a hemocytometer and viable cells were counted.

**Matrigel invasion assays**

*In vitro* invasion assays were carried out in the growth factor–reduced Matrigel-coated Transwell chambers (BD Biosciences). 1483 cells were plated in a 6-well plate. Twenty-four hours later, 4 wells were treated with vehicle, C225, RAD001, and C225 and RAD001 in serum-free media for 48 hours. For p70S6K siRNA studies, cells were seeded and transfected with control or p70S6K siRNA for 48 hours. Cells were trypsinized, counted, and plated in serum-free media into the Transwell chambers. For p70S6K siRNA experiments, cells used for invasion assay were plated in CellTiter-Glo Assay to determine survival. The lower well contained 10% serum-containing media, and cells were allowed to invade for 24 hours at 37°C and 5% CO₂. The cells on the insert were removed by gently wiping with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema3 Solution (Fisher Scientific). Invading cells in 5 representative fields were counted at 400× magnification by light microscopy. The percentage invasion was calculated and normalized to cell survival in the p70S6K siRNA experiments.

**Xenograft experiments**

All animal procedures and care was done under the guidelines instituted by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Adenocarcinoma nude mice were injected subcutaneously with 2 × 10^6 1483 or UM-22B cells in the right flank. Tumor-bearing mice were randomized into 4 groups: vehicle (saline), C225, RAD001, or C225 plus RAD001. C225 was administered by intraperitoneal (i.p.) injection at a dose of 0.8 mg/mouse twice weekly whereas RAD001 was administered 5 days a week at a dose of 5 mg/kg over a 4-week period. RAD001 was provided as a 2% microemulsion which was dissolved in fresh 5% glucose daily before administration. For UM-22B xenografts, mice were treated with 5 mg/kg placebo or RAD001 daily for 12 days immediately following randomization and the first tumor measurement.

**Immunohistochemical analysis and construction of tissue microarrays**

Tumor biopsies were obtained from HNC patients under a protocol approved by the Institutional Review Board at the University of Pittsburgh (IRB#9911206). Informed consent was obtained from all subjects. Paraffin "donor" blocks which contained the highest percentage of tumor were selected for each case by one of the authors (R.R. Seethala). Using a manual tissue arrayer (MTA-1; Beecher Instruments), 1.0-mm cores were transferred from each donor block to a blank recipient paraffin block and arrayed in duplicate along with normal tonsillar controls. The newly constructed array block was then warmed to 35°C to 37°C for 10 minutes to allow annealing of donor cores to the paraffin wax of the recipient block and minimize core loss. Donor cores ranged from 2 to 4 mm in length. Immunohistochemistry was carried out on formalin-fixed paraffin-embedded tissue microarray (TMA) sections by using antibodies against phospho-p70S6K (T421/S424) (1:75 dilution, 1:75 overnight 4°C incubation, Santa Cruz Biotechnology). Antigen retrieval was carried out for 15 minutes in 0.01 mol/L citrate buffer. TMAs were blocked and stained with primary antibodies. Following three 5-minute washes, TMAs were incubated with biotinylated anti-rabbit secondary antibody followed by treatment with avidin–biotin complex. Signal was developed with 3,3′-diaminobenzidine (DAB) substrate, modestly counterstained with hematoxylin, and slides were analyzed microscopically. Immunohistochemical staining was assessed semiquantitatively for each core. The percentage of immunoreactive cells was recorded and rounded to the nearest 10 percentile. Additionally, intensity was scored as follows: 0 (none), 1+ (weak), 2+ (moderate), and 3+ (strong). A composite score was derived from the product of the percentage and intensity of staining. The scores across replicate cores on slides were averaged. The average composite score for both antibodies were graphed by GraphPad Prism Software.

**Statistics**

The differences between treatment groups in biochemical, growth, and invasion assays were tested with the 2-tailed Student’s *t* test. The differences between treatment groups in xenograft experiments were tested with the exact
Wilcoxon–Mann–Whitney 1-sided test; \( P < 0.05 \) was considered to be statistically significant.

Results

**GPCR ligands activate p70S6K in the absence of EGFR**

We previously reported that in the presence of EGFR inhibition, the GPCR ligands PGE2 and BK induced extracellular signal–regulated kinase (Erk) activation (9, 10). We therefore wanted to identify GPCR-induced “drug-gable” pathways that mediated EGFR-independent growth in HNC. We conducted a phosphoprotein antibody array to identify phosphorylated proteins that were induced in HNC cells transfected with EGFR siRNA compared with control siRNA in the setting of PGE2 stimulation. We first verified the efficacy of EGFR siRNA and showed that it downmodulated EGFR expression 72 hours posttransfection (Supplementary Fig. S1). There was a 2-fold or greater increase in phosphorylation of 5 of the 155 proteins assessed in the array induced by EGFR knockdown/PGE2 treatment (Table 1). Of the 5 proteins identified, expression of phosphorylated p70S6K (Ser 424) was induced to the greatest degree (5.6-fold). We confirmed the increased expression of phosphorylated p70S6K in samples used in the array by immunoblotting for the Thr389 site of p70S6K (Supplementary Fig. S1), we observed 3- and 2-fold increases, respectively, in p70S6K phosphorylation by EGFR knockdown even without GPCR stimulation in UM-22B and 1483. These results suggest that p70S6K may be an EGFR-independent molecular target that is activated in the setting of EGFR blockade and further enhanced by GPCR signaling.

**BK-induced p70S6K phosphorylation is modestly induced in the presence of the EGFR inhibitor cetuximab**

We previously reported that PGE2 and BK activated MAPK activation in the presence of the EGFR tyrosine kinase inhibitor (TKI) AG1478 (9). To determine the pharmacologic significance of the effects observed with EGFR siRNA, we chose to investigate p70S6K signaling in the presence of the FDA-approved EGFR inhibitor cetuximab (C225). Cetuximab’s mode of action involves neutralization of the ligand-binding domain and internalization of EGFR. As shown in Figure 1C, BK-mediated induction of phosphorylated p70S6K was modestly increased in the presence of cetuximab. EGF stimulation was used as a control for C225 efficacy, as EGF-induced p70S6K phosphorylation was abrogated by C225 treatment (Fig. 1C, Supplementary Fig. S2). These results indicate that BK induces p70S6K phosphorylation independently of EGFR.

**Post-cetuximab HNC biopsies display elevated p70S6K phosphorylation levels**

Cetuximab (C225, Erbitux) is a monoclonal antibody that has been reported to mediate its action by down-regulating EGFR membrane expression and is approved by FDA for the treatment of HNC. The effect of cetuximab on phosphorylated p70S6K has not been reported in human cancer. To determine the role of p70S6K activation in the setting of cetuximab treatment, we evaluated a tissue microarray containing samples obtained before and/or after the administration of cetuximab in a cohort of HNC patients treated at the University of Pittsburgh. Tumor tissue was obtained from a total of 19 patients including 12 patients with tissue biopsies before cetuximab treatment, 5 patients with tissue biopsies after progression on cetuximab, and 2 patients with paired tumor sample biopsies before and after cetuximab exposure. All patients were previously cetuximab naïve. Patient characteristics are listed in Table 2. Immunohistochemical staining of the TMA with phospho-p70S6K (Thr421/Ser424) was carried out, and the composite scores were calculated (incorporating both the intensity of staining and the percentage of cells that stained positively). In the 7 tumor samples obtained following progression after cetuximab treatment, expression of phospho-p70S6K was elevated 2-fold compared with levels in the 15 tumors biopsies obtained prior to cetuximab administration (Fig. 2A), including the 2 paired biopsies (Fig. 2B). Although there were only 2 patients with paired tumor tissue for analysis, both progressed on cetuximab therapy suggesting that increased expression of phosphorylated p70S6K may be associated with poor response.

### Table 1. Fold increase in expression of phosphorylated proteins induced by PGE2 in EGFR siRNA-transfected cells compared with PGE2-stimulated control siRNA-transfected cells

<table>
<thead>
<tr>
<th>Phospho-protein</th>
<th>Fold induction vs. PGE2-stimulated NTC siRNA cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-p70S6K</td>
<td>5.6</td>
</tr>
<tr>
<td>Phospho-PKCδ (Ser645)</td>
<td>3.6</td>
</tr>
<tr>
<td>Phospho-PKCθ (Ser679)</td>
<td>3.1</td>
</tr>
<tr>
<td>Phospho-ikBα (Ser32)</td>
<td>2.9</td>
</tr>
<tr>
<td>Phospho-IRS1 (Ser312)</td>
<td>2.3</td>
</tr>
<tr>
<td>Phospho-MAPK (Thr202)</td>
<td>2.2</td>
</tr>
</tbody>
</table>
to cetuximab. The observations made in these limited patient samples suggest that p70S6K may be a possible therapeutic target that can be exploited to improve responses to EGFR-targeted therapy.

**Targeting p70S6K decreases HNC growth in vitro and in vivo**

We previously reported that combination of BK and PGE2 antagonists with EGFR inhibitors resulted in improved antiproliferative and antiinvasive effects in vitro (9). Therefore, we wanted to determine whether p70S6K inhibition in combination with C225 would lead to enhanced antitumor effects compared with each agent administered alone. First, we targeted p70S6K by using both RNA interference and the pharmacologic inhibitor RAD001. siRNA completely abrogated the protein expression of p70S6K in both UM-22B and 1483 cell lines 72 hours posttransfection in conjunction with significant growth inhibition compared with control siRNA-treated cells ($P < 0.001$; Fig. 3A and B). In addition to siRNA, we also showed that p70S6K shRNA decreased p70S6K expression and HNC growth (Supplementary Fig. S3). The mTOR inhibitor RAD001 (everolimus) was previously reported to abrogate p70S6K phosphorylation in ovarian cancer cells (13). We observed that 10 and 100 nmol/L of RAD001 completely abolished p70S6K phosphorylation in UM-22B and 1483 cells, respectively (Fig. 3C). Next, we determined the IC50 values for RAD001 on 1483 and UM-22B cells in vitro. In Figure 3D, RAD001 had a cytostatic effect and only induced 50% cell death at a concentration of 14 μmol/L. At 10 and 100 nmol/L concentrations, there was an approximately 20% decrease in HNC survival. To test the effects of p70S6K inhibition on tumor growth in vivo, HNC xenograft-bearing mice were treated with RAD001 (5 mg/kg daily) in conjunction with tumor volume determinations. As shown in Figure 3E, RAD001 significantly decreased HNC tumor growth in vivo compared with placebo ($P = 0.001$).
Targeting p70S6K enhances cetuximab-mediated antitumor effects

Having established that p70S6K inhibition was associated with antiproliferative effects, we next sought to determine the efficacy of combining p70S6K inhibition with EGFR blockade. We treated control and p70S6K siRNA-transfected HNC cells with the EGFR inhibitor cetuximab followed by cell viability determinations. Compared with cetuximab or p70S6K siRNA alone, we observed that downregulation of p70S6K increased the effects of cetuximab on HNC growth in both HNC cell lines tested (P < 0.05; Fig. 4A). Similar effects were observed with p70S6K shRNA cells treated with cetuximab (Supplementary Figs. S3 and S4). In the presence of BK, which has been previously shown to increase EGFR ligand production (9), the combination of cetuximab plus RAD001 decreased HNC growth to a greater degree compared with cetuximab or RAD001 alone (Fig. 4B; P < 0.05). In addition to proliferation, we investigated the combined effects of cetuximab plus RAD001 on HNC invasion. Cetuximab or RAD001 alone decreased HNC invasion by 50%. However, an enhanced 20% reduction of invasion was observed by using a combination of cetuximab plus RAD001 (Fig. 4C; P = 0.02). Cetuximab also enhanced the anti-invasive effects in p70S6K siRNA-transfected cells (Supplementary Fig. S5). Next, we treated HNC tumor-bearing mice with subtherapeutic doses of cetuximab (0.8 mg C225 i.p. twice weekly), RAD001 [5 mg/kg per os (p.o.) 5 d/wk], or combined treatment with cetuximab plus RAD001, or vehicle control. After 28 days, we observed that the combination of low dose cetuximab plus RAD001 significantly decreased tumor growth compared with cetuximab alone (Fig. 4D; P = 0.03). These observations show that targeting p70S6K activation can increase the efficacy of cetuximab treatment in HNC.

Discussion

The monoclonal EGFR antibody cetuximab (C225/Erbitux) was approved by FDA for the treatment of primary HNC in combination with radiation in 2006, making it the first new drug for this cancer in more than 45 years (2). Despite widespread EGFR expression, cetuximab is only effective in a subset of HNC patients. We previously reported that GPCR ligands can transactivate EGFR via autocrine release of EGFR ligands (9, 14). In addition, GPCR ligands can activate the mitogenic MAPK pathway in the presence of EGFR inhibitors and combined targeting of individual GPCRs and EGFR resulted in enhanced cytotoxicity. In this study, we showed that GPCR ligand BK can...
enhance the phosphorylation of p70S6K in the presence of EGFR siRNA or cetuximab. In addition, we observed that combined treatment with cetuximab and RAD001 improves antitumor response in both \textit{in vitro} and \textit{in vivo} HNC models.

GPCRs have been previously linked to the activation of p70S6K in different benign and malignant cell types. In bladder cancer, the GPCR ligand carbachol was shown to activate p70S6K in the presence of the EGFR TKI AG1478 (15). In smooth muscle airway cells, thrombin and EGF were reported to synergistically activate p70S6K (16). In addition, we identified that p70S6K phosphorylation was additively decreased by combined treatment with a gastrin-releasing peptide receptor inhibitor and EGFR TKI by reverse-phase protein array analysis (10). In this study, we show for the first time that in the presence of EGFR downmodulating agents, including cetuximab, GPCR ligands can induce p70S6K phosphorylation. We also observed that even in the absence of GPCR ligands, EGFR downmodulation results in increased p70S6K phosphorylation alone.
Furthermore, the increased expression of phosphorylated p70S6K in post-cetuximab–treated biopsies support p70S6K as plausible therapeutic target to overcome de novo and acquired resistance to EGFR inhibition.

Although studies to date have not identified p70S6K activation as a feedback mechanism in response to EGFR downmodulation, several reports show that inhibition of EGFR results in activation of the insulin-like growth factor (IGF) receptor pathway, which signals via the phosphoinositide 3-kinase (PI3K)/Akt/p70S6K pathway. IGF receptor downmodulation and increased p70S6K phosphorylation, at least in part, via increased insulin signaling.

Table 1 shows that phospho-IRS (Ser312) was increased 2-fold in EGFR siRNA-transfected cells and it has been reported that EGFR downmodulation in HNC leads to increased p70S6K phosphorylation, at least in part, via increased insulin signaling.

The effects of mTOR inhibitors have been shown to be promising in preclinical HNC models. However, rapalogs are allosteric inhibitors and their activity results in...
a feedback loop that induces Akt phosphorylation (22). However, dual PI3K/mTOR inhibitors (23) may be more potent in overcoming the limited clinical efficacy of cetuximab. Although the combination of C225 and RAD001 did not result in tumor regression, this may be due to use of the subtherapeutic concentrations of cetuximab used. Therefore, it is possible that a combination of therapeutic doses of cetuximab and RAD001 will be more potent and result in tumor regression. It is also important to note that RAD001 lacks specificity and also inhibits phosphorylation of 4EBP1, so it is not possible to distinguish precisely which target is most important by using this rapalog. The results of this study provide a biological basis for the ongoing clinical trial combining cetuximab and RAD001 in HNC patients (http://clinicaltrials.gov/ct2/show/NCT01009346). Because GPCR ligands including BK and PGE2 in HNC are ubiquitous in the tumor microenvironment, p70S6K represents a promising therapeutic target, particularly in combination with EGFR blockade.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Received December 22, 2010; revised May 4, 2011; accepted June 1, 2011; published OnlineFirst June 8, 2011.

References


Targeting GPCR-Mediated p70S6K Activity May Improve Head and Neck Cancer Response to Cetuximab

Neil E. Bhola, Sufi M. Thomas, Maria Freilino, et al.

Clin Cancer Res 2011;17:4996-5004. Published OnlineFirst June 8, 2011.

Updated version  
Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-3406

Supplementary Material  
Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/07/26/1078-0432.CCR-10-3406.DC1

Cited articles  
This article cites 23 articles, 13 of which you can access for free at: http://clincancerres.aacrjournals.org/content/17/15/4996.full.html#ref-list-1

Citing articles  
This article has been cited by 3 HighWire-hosted articles. Access the articles at: /content/17/15/4996.full.html#related-urls

E-mail alerts  
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.