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Pathway-Specific Genome Editing of PI3K/mTOR Tumor Suppressor Genes Reveals that PTEN Loss Contributes to Cetuximab Resistance in Head and Neck Cancer

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ABSTRACT

Cetuximab, an mAb targeting EGFR, is a standard of care for the treatment for locally advanced or metastatic head and neck squamous cell carcinoma (HNSCC). However, despite overexpression of EGFR in more than 90% of HNSCC lesions, most patients with HNSCC fail to respond to cetuximab treatment. In addition, there are no available biomarkers to predict sensitivity or resistance to cetuximab in the clinic. Here, we sought to advance precision medicine approaches for HNSCC by identifying PI3K/mTOR signaling network–specific cetuximab resistance mechanisms. We first analyzed the frequency of genomic alterations in genes involved in the PI3K/mTOR signaling circuitry in the HNSCC TCGA dataset. Experimentally, we took advantage of CRISPR/Cas9 genome editing approaches to systematically explore the contribution of genomic alterations in each tumor suppressor gene (TSG) controlling the PI3K–mTOR pathway to cetuximab resistance in HNSCC cases that do not exhibit PIK3CA mutations. Remarkably, we found that many HNSCC cases exhibit pathway-specific gene copy number loss of multiple TSGs that normally restrain PI3K/mTOR signaling. Among them, we found that both engineered and endogenous PTEN gene deletions can mediate resistance to cetuximab. Our findings suggest that PTEN gene copy number loss, which is highly prevalent in HNSCC, may result in sustained PI3K/mTOR signaling independent of EGFR, thereby representing a promising mechanistic biomarker predictive of cetuximab resistance in this cancer type. Further prospective studies are needed to investigate the impact of PTEN loss on cetuximab efficacy in the clinic.

Introduction

Head and neck squamous cell carcinomas (HNSCC), which include cancers of the oral cavity, oropharynx, and larynx, are among the top 10 most common cancers worldwide, with over 65,000 estimated new cases per year in the United States alone, and accounting for about 15,000 estimated cancer deaths (1). Despite aggressive multimodality therapies and recent advances in treatment, the prognosis of patients with HNSCC is still poor, with 5-year survival estimates of approximately 65% (1), which are even lower if the cancers are detected at advanced stages. Thus, there is an urgent unmet need to develop new therapeutic options to treat patients with HNSCC. The recent deep sequencing of HNSCC has revealed that this malignancy harbors a remarkable multiplicity and diversity of genomic alterations, with particular emphasis on aberrant activation of the EGFR and PI3K/Akt/mTOR signaling pathways (2–5).

Over 90% of HNSCC lesions overexpress EGFR, one of the upstream molecules of PI3K/mTOR signaling (6, 7), and EGFR expression is associated with poorer outcomes of patients with HNSCC (8, 9). Cetuximab, a chimeric IgG1 mAb against the EGFR extracellular domain, was approved in 2006 by the FDA for the treatment of patients with HNSCC based on the results of seminal clinical trials (10–12). However, the overall response rates of cetuximab as a single agent or the increased response rates observed when cetuximab is added to radiation or chemotherapy are less than 10% to 20% (10–12), much lower than initially expected considering the high level of EGFR expression in HNSCC. In addition, recent phase III clinical trials showed that cetuximab-based chemoradiation therapy (CRT) demonstrated inferior efficacy to cisplatin-based CRT in HPV-positive oropharyngeal cancer (13, 14).

EGFR-targeted therapies have demonstrated improvement in clinical outcomes in several cancer types, including non–small cell lung cancer and colorectal cancer (15–18), where, unlike HNSCC, molecular biomarkers have been identified to determine which patients are most likely to benefit from these agents. In the case of HNSCC, there are no biomarkers available in the clinic to predict sensitivity or resistance to cetuximab despite the recent characterization of the molecular alterations of this malignancy. The upregulation and activation of multiple receptor tyrosine kinases (RTK), including HER3, c-MET, and AXL, have been reported to mediate intrinsic or acquired resistance to cetuximab in HNSCC (19–22). In addition, we have shown that mutation of the PIK3CA gene, the most commonly mutated oncogene in HNSCC (5), as well as mutant RAS gene can confer cetuximab resistance in HNSCC experimental models (23). However, ~75% of the HNSCC lesions lack PIK3CA or RAS mutations. Additional biomarkers predictive of cetuximab resistance or sensitivity are warranted to further advance precision medicine in HNSCC.

In this study, we took advantage of the CRISPR/Cas9 genome editing approaches to systematically explore the contribution of genomic alteration in the PI3K/mTOR signaling network to cetuximab resistance in HNSCC cases that do not exhibit PIK3CA mutations.

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Remarkably, we found that many HNSCC cases exhibit pathway-specific gene copy number loss of multiple tumor suppressor genes (TSG) involved in PI3K/mTOR signaling. Among them, we found that both engineered and endogenous PTEN loss can mediate resistance to cetuximab, due to sustained PI3K/mTOR signaling activity. Our findings suggest that PTEN gene copy number loss, which is highly prevalent in HNSCC, may represent a promising biomarker predictive of cetuximab resistance in this disease.

Materials and Methods

Antibodies and reagents
Antibodies against pEGFR (Tyr1180) (#2236), pERK1/2 (T202/Y204) (#4370), ERK1/2 (#4696), pS6 (S235/236) (#2211), S6 (#2217), α-tubulin (#3873), and GAPDH (#2118) were purchased from Cell Signaling Technology. Antibody against EGFR (sc-03) was purchased from Santa Cruz Biotechnology. Erlotinib was purchased from Selleck Chemical, and cetuximab was obtained from the pharmacy of UCSD Moores Cancer Center.

Cell lines, culture condition, and transfection
The human HNSCC cell lines HN12, CAL27, and Detroit 562 were genetically characterized as part of NIH/NIDCR Oral and Pharyngeal Cancer Branch cell collection, and have been previously genetically characterized as part of NIH/NIDCR Oral and Pharyngeal Cell lines, culture condition, and transfection (Genetica Laboratories, Inc.) prior to the described experiments to ensure consistency in cell identity. No presence of mycoplasma was found.

DNA laboratories, Inc.) prior to the described experiments to ensure the absence of mycoplasma. These oligos were cloned into lentiCRISPR v2 vector and packaged to obtain lentivirus. These lentivirus were used for transfection of the cells. After transfection, the cells were harvested and the expression of the gene was confirmed by Western blot analysis. The siRNA knockdown of each gene was validated by Western blot analysis. siRNA transfection was performed using the CRISPR/Cas9 system as described previously (26).

Western blot analysis and image quantifications
Cells and tissues were lysed on ice in RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1.0% NP-40) containing Halt Protease and Phosphatase Inhibitor Cocktail (#78440; Thermo Fisher Scientific). Protein concentrations were measured by Bio-Rad Protein Assay (Bio-Rad). Equal amounts of total proteins were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 3% BSA or 5% nonfat dry milk in TBS-T buffer (50 mM NaCl, 50 mM Tris/HCL, pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween-20) for 1 hour, and then incubated with primary antibodies in blocking buffer for 1 hour at room temperature. Detection was conducted by incubating the membranes with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Southern Biotechnologies) used at a dilution of 1:10,000 in 5% milk-TBS-T buffer for 1 hour at room temperature, and visualized with Immobilon Western Chemiluminescent HRP substrate (Millipore). The image quantifications were performed with ImageJ.

Cellular proliferation and viability assay
Cells were cultured in 96-well plates and treated with drugs for 72 hours, then incubated with AlamarBlue (Invitrogen) for 2 hours at 37°C. Absorbance was read at 570 nm, using 600 nm as a reference wavelength. Each experiment was repeated three times in triplicate.

Sphere formation assay
Cells were seeded in 96-well ultra-low attachment culture plates (Corning) at 100 cells per well. Medium consisted of serum-free DMEM/F12 Glutamax supplement medium (+10565042; Thermo Fisher Scientific), basis FGF (bFGF: 20 ng/mL, #13256029; Thermo Fisher Scientific), EGF (20 ng/mL, #PHG0313; Thermo Fisher Scientific), B-27 (1:50 dilution, #17504044; Thermo Fisher Scientific), and N2 supplement (1:100 dilution, #17502-048; Thermo Fisher Scientific). Ten days after seeding, photographs were obtained, and the sizes and numbers of sphere colonies on each well were counted using a microscope. Each experiment was repeated three times in triplicate.

Animal work
All studies in mice were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of California, San Diego (protocol #S15195) or the University of California, San Francisco (protocol #AN173372-02C). To establish tumor xenografts, 2.0 × 10^6 cells were transplanted into the both flanks of athymic nude mice (female, 4–6 weeks old; Charles River Laboratories), and when the tumor volume reached approximately 100 mm^3, the mice were randomized into groups and treated by intraperitoneal injection with cetuximab (40 mg/kg, three times a week) or control diluent (10 tumors in 5 mice per each group). Tumor volume was calculated by using the formula length × width × width/2. The mice were euthanized at the indicated time points and tumors isolated for histologic and IHC evaluation.

Tissue analysis
All samples were fixed in zinc formalin (Z-Fix, Anatech) and embedded in paraffin; 5 μm sections were stained with hematoxylin–eosin for diagnostic purposes. For IHC studies, the sections were deparaffinized and hydrated through graded ethanol. The slides were extensively washed with distilled water and antigen retrieval was performed by high temperature treatment with 10 mmol/L citric acid in a microwave. After washing with water and PBS, the slides were successively incubated with the primary and secondary antibodies, and the ABC reagent (Vector Laboratories). The reaction was developed with 3-3’-diamobenzidine under microscopic control.

Genomic data analysis
Gene mutation and copy number variation analyses were performed using publicly available data generated by The Cancer Genome Atlas consortium, accessed through cBio portal (www.cbioportal.org; refs. 28, 29).

Data and statistical analysis
Data were analyzed using GraphPad Prism version 8.02 for Windows (GraphPad Software). The overall survival and progression-free survival time were assessed using the Kaplan–Meier method and compared using the log-rank test. Comparisons between experimental groups were made using unpaired t test. The correlation between
PTEN mRNA and protein expression was evaluated using Pearson test. P < 0.05 was considered to be statistically significant.

Results
The landscape of genomic alterations in PI3K/mTOR pathway in HNSCC
Pathway-specific analysis of the HNSCC oncogene suggests that most genomic alterations are involved in aberrant mitogenic signaling, with particular emphasis on the PI3K/mTOR pathway (2). In this study, we focused on the TSGs in the PI3K/mTOR signaling circuitry, including PTEN, TSC1/2, STK11, and EIF4EBP1, the loss of which are expected to result in persistent activation of mTOR signaling. We first analyzed the frequency of genomic alteration in the TCGA HNSCC dataset (n = 502). As shown in Fig. 1A, frequent mutations of PTEN (3.1, 2.2%) and less frequent mutations in the TSC2 (1.1, 0.5%), TSC2 (1.1, 1.5%), STK11 (2.2, 0.5%), and EIF4EBP1 (0, 0.2%) genes were observed in HPV-positive (n = 93) and HPV-negative (n = 409) patients with HNSCC, respectively. On the other hand, the loss of copy number in PTEN (35.8, 25.5%), TSC1 (9.5, 13.4%), TSC2 (11.6, 14.9%), STK11 (18.9, 36.0%), and EIF4EBP1 (16.8, 39.8%) were much more frequently observed in both patients with HPV-positive (n = 93) and HPV-negative HNSCC (n = 409), respectively. In addition, the copy number loss of either PTEN, TSC1/2, STK11, or EIF4EBP1 was associated with poorer survival of patients with HNSCC in TCGA HNSCC cohort (log-rank test P = 0.017; Fig. 1B), although mutations of these genes individually was not significantly associated with overall and progression-free survival (Supplementary Fig. S1). Thus, most of the alterations in TSGs in the PI3K/mTOR signaling pathway are not mutations, but losses of gene copy number, which are significantly associated with worse clinical outcome in patients with HNSCC. To elucidate the role of loss of PI3K/mTOR TSGs in resistance to cetuximab, we sought to generate HNSCC isogenic cell line panels with PTEN, TSC2, STK11, or EIF4EBP1 gene knockout using the CRISPR-Cas9 system. We employed CAL27 and HN12 cells, both of which are human HNSCC cell lines that harbor no genomic alterations in PI3K/mTOR and RAS pathway genes (25). sgRNAs targeting each gene were cloned into pLentiCRISPR-v2 vector, and packaged into lentiviruses using HEK 293T cells. The packaged lentiviruses were transduced into CAL27 and HN12 cells, and cells were selected with puromycin, (4 µg/mL) for 3 days. Single cell clones for each deleted gene were isolated and gene knockouts were validated by Western blot analysis of Cas9 expression and depletions of each protein expression (Fig. 1C).

Dependency of PI3K/mTOR signaling on EGFR activity in HNSCC cell lines: knockout of PI3K/mTOR TSGs confers EGFR-independent signaling
We next tested the impact of EGFR inhibition in a panel of different HNSCC cell lines, including cells that do not have obvious driver oncogene mutations (HN12, CAL27), using Detroit562 (PIK3CA mutant) and UM-SCC-17B (HRAS mutant) HNSCC cells (25), as controls. We performed knockdown of EGFR using siRNA, and analyzed Ser235/236 phosphorylation (p-ERK1/2) (p-ERK1/2, 235/236), referred herein to as p-ERK1/2, as markers of PI3K/mTOR and RAS/RAF/MAPK signaling activity, respectively. We found that p-S6 and p-Erk1/2 were suppressed by EGFR knockdown in both of HN12 and CAL27 cells under serum free conditions. As expected, Detroit562 (PIK3CA mutant) and UM-SCC-17B (HRAS mutant) cells were resistant to p-S6 and p-Erk1/2 reduction by EGFR knockdown (Fig. 2A). This finding was validated by EGFR inhibition using a clinically relevant small molecule EGFR tyrosine kinase inhibitor, erlotinib (Supplementary Fig. S2A). These results suggest that PI3K/Akt/mTOR and RAS/RAF/MAPK signaling are mainly regulated under basal conditions by EGFR in both CAL27 and HN12, but not in Detroit562 and UM-SCC-17B cells.

We next used these isogenic cell line panels to investigate which of the PI3K/mTOR pathway TSGs knockouts led to independence from EGFR activity with regard to downstream signaling. The isogenic CAL27 cells were treated with EGFR siRNA or erlotinib (3 µmol/L), followed by immunoblotting for total and phosphorylated forms of Erk1/2 or S6. Remarkably, both PTEN- and TSC2-knockout CAL27 cells were resistant to PI3K/mTOR signaling inhibition as shown by failure to reduce p-S6 in both EGFR siRNA and erlotinib treated cells. By contrast, STK11- and EIF4EBP1-knockout CAL27 cells remained sensitive to p-S6 reduction following EGFR knockdown or erlotinib treatment (Fig. 2B and C). Similar experiments were also performed in isogenic HN12 cell panels, revealing that PTEN-knockout cells were resistant to EGFR knockdown as well as to erlotinib treatment, whereas partial p-S6 reduction was observed in TSC2-knockout HN12 cells under these EGFR inhibiting conditions (Supplementary Figs. S2B and S2C).

Effects of PTEN knockout on the response to cetuximab in cell proliferation and orosphere formation
We next compared antiproliferative efficacy of erlotinib between parental and PI3K/mTOR pathway TSG knockout cells. Cells were treated with serial concentrations of erlotinib for 72 hours in 96-well plates. The IC50 values for erlotinib in parental, PTEN-, TSC2-, STK11-, and EIF4EBP1-knockout CAL27 cells were 0.9, 9.0, 4.4, 0.6, and 1.6 µmol/L, respectively (Fig. 3A). The IC50 values in parental, PTEN-, TSC2-, STK11-, and EIF4EBP1-knockout HN12 cells were 16.3, 101.1, 96.1, 30.9, and 32.4 µmol/L, respectively (Fig. 3A). Remarkably, among all of these changes the IC50 values for erlotinib in PTEN-KO cells were consistently 6- to 10-fold higher than that in parental cells. We next investigated whether the knockout of these PI3K/mTOR TSGs could interfere with the inhibition of the tumorigenic potential by cetuximab. For this, we compared the ability of sphere growth (orosphere) between parental and TSGs knockout cells after treatment with cetuximab at 10 µg/mL, which is equivalent to the serum trough concentration in patients treated with cetuximab (30). In parental HN12 cells, cetuximab significantly reduced the size of sphere formation (Fig. 3B). Notably, PTEN-knockout HN12 cells showed resistance to cetuximab-induced inhibition of sphere growth, whereas the other knockout cells were still partially or completely sensitive to cetuximab in terms of orosphere formation (Fig. 3B and C). Collectively, these results suggest that among all genes tested, knockout of PTEN showed the most robust phenotype, conferring independence from EGFR activity and resistance to EGFR inhibition in terms of downstream signaling, proliferation, and orosphere growth.

Effects of PTEN knockout on the response to cetuximab in HNSCC tumor xenografts
Given that PTEN knockout cells were the most robust in demonstrating resistance to EGFR inhibition, we first extended our genomics studies and analyzed the correlation between protein expression, mRNA expression, and PTEN copy number in the large TCGA HNSCC dataset. The protein expression of PTEN was significantly correlated with mRNA expression (Fig. 4A), and copy number

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suggesting that PTEN gene copy loss results in reduced transcripts and PTEN protein levels. Experimentally, we compared the efficacy of cetuximab between parental and PTEN-knockout tumors in vivo to confirm the role of PTEN knockout in conferring resistance to cetuximab. We transplanted parental or PTEN-KO CAL27 cells into the flanks of nude mice, and then treated with either cetuximab (40 mg/kg, three times a week) or control diluent (10 tumors per each group). The growth of PTEN-KO tumors treated with control diluent was almost identical compared with that of control-treated parental tumors. The volume of PTEN-KO tumors decreased after the cetuximab treatment, while parental CAL27 tumors responded to a greater extent. However, PTEN-KO tumors regrew rapidly, in contrast to wild-type CAL27 cells which exhibited prolonged tumor remission (Fig. 4C–E). These results suggest that cetuximab displayed greater efficacy in wild-type parental tumors compared with that achieved in PTEN-KO tumors. IHC analysis showed that cetuximab-treated PTEN-KO tumors exhibited sustained p-S6 staining (Fig. 4F), despite clear suppression of p-EGFR. In contrast, cetuximab treatment clearly suppressed p-S6 as well as p-EGFR in the wild-type parental tumors.

Efficacy of cetuximab on naturally occurring PTEN-deficient HNSCCs

To further confirm our findings that PTEN loss promotes cetuximab resistance in HNSCC, we employed UDSCC2 cells, which harbors an endogenous homozygous PTEN-loss (25). We injected the UDSCC2 cells into the flanks of nude mice, and treated with either cetuximab (40 mg/kg, three times a week) or control diluent (10 tumors per each group). The growth of UDSCC2 tumors treated with control diluent was almost identical compared with that of control-treated parental tumors. The volume of UDSCC2 tumors decreased after the cetuximab treatment, while parental UDSCC2 tumors responded to a greater extent. However, UDSCC2-KO tumors regrew rapidly, in contrast to wild-type UDSCC2 cells which exhibited prolonged tumor remission (Fig. 4G). These results suggest that cetuximab displayed greater efficacy in wild-type parental tumors compared with that achieved in UDSCC2-KO tumors. IHC analysis showed that cetuximab-treated UDSCC2-KO tumors exhibited sustained p-S6 staining (Fig. 4H), despite clear suppression of p-EGFR. In contrast, cetuximab treatment clearly suppressed p-S6 as well as p-EGFR in the wild-type parental tumors.
cells into the flank of nude mice, and treated UDSCC2-bearing mice with cetuximab (40 mg/kg, three times per week) or control solution (10 tumors per each treatment group) for 3 weeks. Consistent with our findings in isogenic genome-edited cells, UDSCC2 tumors were totally insensitive to cetuximab treatment (Fig. 5A and B). In the UDSCC2 tumors treated with cetuximab, persistent p-S6 staining was also observed, despite successful suppression of p-EGFR, strongly supporting the failure of cetuximab to perturb PI3K/mTOR signaling in the UDSCC2 xenograft model (Fig. 5C).

Discussion

Although cetuximab displays antitumoral activity in HNSCC and is approved by the FDA for treatment of this disease, only a small minority of patients benefit clinically from cetuximab treatment. Thus, precise stratification of patients that are sensitive or resistant to cetuximab is needed to harness the full clinical potential of this therapeutic agent. Here, we show that the HNSCC cells that are originally sensitive to cetuximab become resistant when the PTEN
Figure 3. Effects of PTEN knockout on the response to cetuximab in cell proliferation and orosphere formation assays. 

A, Parental and isogenic CAL27 and HN12 cells seeded in 96-well plates (2,000 per well) were treated with the indicated concentrations of erlotinib for 72 hours. Cell viabilities were normalized with that of the corresponding vehicle control (0.1% DMSO)-treated cells. 

B, Parental and isogenic HN12 cells were seeded in 96-well ultra-low attachment culture dishes at 100 cells per well (n = 10) and treated with vehicle control (0.9% NaCl) or cetuximab (10 μg/mL). Ten days after treatment, the size of spheres in each well were determined. 

C, Representative spheres obtained from parental and PTEN knockout cells treated with vehicle control or cetuximab (10 μg/mL).
gene is genetically disrupted. We also show that HNSCC cell lines that harbor endogenous PTEN loss are highly resistant to cetuximab treatment. These findings strongly indicate that PTEN loss, a frequent event in HNSCC, is sufficient to promote cetuximab resistance, and that this genomic alteration may be responsible for intrinsic cetuximab resistance.

Our analysis of TCGA data from 409 HPV-negative and 93 HPV-positive HNSCC samples revealed that a high percentage (69.3%) of HNSCC lesions exhibited the loss of at least one copy of candidate PI3K–mTOR pathway TSGs. Loss of these TSGs was observed to occur with much greater frequency than mutation of the genes. In addition, copy number loss of PI3K/mTOR TSGs seems to be clinically important, because they were found to be associated with poor survival in patients with HNSCC. Among the TSGs tested, copy number loss in PTEN gene was comparably observed in both HPV-negative (24.2%) and HPV-positive (31.2%) HNSCC cases, which is consistent with the frequency of reduced PTEN protein expression in HNSCC (31, 32).

Since EGFR is commonly overexpressed in HNSCC and HNSCC cells and tumors are often addicted to EGFR signaling for sustained survival and proliferation, EGFR targeting therapy including small molecule EGFR inhibitors (EGFRi), such as erlotinib, and targeting antibodies, for example, cetuximab, would be expected to be effective in a broader subset of patients with HNSCC. In fact, the HNSCC cell lines HN12 and CAL27, both of which do not have obvious driver oncogene mutations in PI3K/mTOR or RAS/RAF/MAPK signaling pathways, are dependent on EGFR signaling, and are highly sensitive to cetuximab treatment in vitro (33, 34).

Figure 4.
Effects of PTEN knockout on the response to cetuximab in HNSCC tumor xenografts. Correlation of PTEN protein expression with PTEN mRNA (A) and PTEN gene copy number (B) in the TCGA dataset. C, Parental and PTEN knockout CAL27 were transplanted into nude mice and treated with vehicle control diluent or cetuximab (40 mg/kg), three times per week. Cetuximab treatment was continued until 6 weeks (D). Representative tumors treated with or without cetuximab are shown. E, Tumor weight at the indicated day. Control diluent- and cetuximab-treated tumors were collected 18 and 74 days after treatment, respectively. F, Representative immunohistochemical analysis of pEGFR (Y1068) and pS6, as indicated.
to cetuximab treatment in vitro and in vivo. However, we have previously shown that genomic alterations in the PIK3CA or RAS genes that lead to aberrant signaling and EGFR-independent proliferation can confer cetuximab resistance in these HNSCC cell lines (23). Through the use of genome editing approaches we have now performed a systematic analysis of the contribution of PI3K/mTOR TSG copy loss to resistance to cetuximab. We found that HNSCC cells genetically engineered for PTEN loss or HNSCC cells with endogenous PTEN loss were resistant to cetuximab and erlotinib treatment, the latter as an example of EGFRi. Aligned with our observations, reduced expression, as judged by IHC or mutation of PTEN, correlates with poor response to cetuximab treatment in retrospective studies in metastatic HNSCC and colorectal cancer, respectively (17, 33). Collectively, these findings demonstrate that PTEN loss is sufficient to...
promote resistance to cetuximab and suggest that PTEN deficiency may play a role in the resistance to this agent in a large percentage of patients with HNSCC.

In addition to promoting resistance to EGF-targeting therapies, PTEN loss has previously been shown to confer resistance to clinically viable inhibitors of PI3K and CDK4/6 (34, 35). Hence, restoration of cellular PTEN activity, particularly in cells retaining only one copy of the PTEN gene, could prove highly valuable for restoring sensitivity to cetuximab, as well as other molecular targeting anti-cancer agents. Recent studies have discovered that the PTEN protein is negatively regulated by the upstream E3 ubiquitin ligase WWP1, and genetic deletion of the WWp1 gene led to upregulation of PTEN activity, and corresponding loss of PI3K/Akt signaling, in cells with only one copy of the PTEN gene (36). Moreover, treatment of cells characterized by heterozygous loss of PTEN with the WWP1 inhibitor indole-3-carbinol potently suppressed in vivo tumor growth and PI3K-mediated signaling. These findings suggest that targeted inhibition of WWP1 may be a promising strategy for reversing resistance to cetuximab, and other agents, resulting from genetic alteration of PTEN.

The use of EGFR targeting therapy for non–small cell lung cancer and metastatic colorectal cancer is restricted to selected patients based on molecular characteristics such as the presence of EGFR activating mutation and the absence of RAS/RAF mutations, respectively. However, in HNSCC cetuximab is currently prescribed regardless of the presence or absence of these, or other, genetic alterations. Our findings suggest that the PTEN loss, which can result in activation of the PI3K/mTOR signaling pathway independently of EGFR, can be used as a mechanistic biomarker for the selection of patients that could be considered for exclusion from cetuximab treatment.

Our study includes several limitations. First, the impact of PTEN loss on cetuximab resistance is based on in vitro and experimental mouse models rather than based on clinical studies. Second, HNSCC cells with PTEN-KO respond less to cetuximab compared with parental HNSCC cells, however they still show partial responses followed by tumor relapse. Thus, cetuximab may inhibit yet to be identified growth promoting signals in addition to PI3K/mTOR in PTEN-KO tumors, until PTEN deficiency and aberrant PI3K/mTOR signaling may drive tumor regrowth and treatment failure. Therefore, further clinical studies are warranted to investigate the clinical utility of PTEN loss as a negative predictive biomarker of cetuximab sensitivity and for patient stratification.

Disclosure of Potential Conflicts of Interest
D.E. Johnson has received speakers bureau honoraria from Kymera Therapeutics and has an unpaid consultant/advisory board relationship with STAT3 Therapeutics. J.S. Gutkind is a Scientific Advisory Board member at Domain Therapeutics, Vividion Therapeutics, and Oncoceutics and has received a commercial research grant from Revolution Medecines.

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Conception and design: H. Izumi, Z. Wang, J.S. Gutkind
Development of methodology: H. Izumi, Z. Wang
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References
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