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

Hiroki Izumi1, Zhiyong Wang1, Yusuke Goto1, Toshinori Ando1,2, Xingyu Wu1, Xuefeng Zhang1, Hua Li3, Daniel E. Johnson3, Jennifer R. Grandis3, and J. Silvio Gutkind1

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 survival 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.
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\(^{1068} \) (2236), pERK1/2\(^{202/204} \) (4370), ERK1/2 (4696), p53\(^{335/356} \) (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 and GAPDH (2118) were purchased from Cell Signaling Technology. Erlotinib was purchased from Selleck Chemical, and Antibody against EGFR was purchased from Sigma (MISSION siRNA human EGFR Knockout). siRNAs targeting sequences for PTEN, TSC2, STK11, and EIF4EBP1-knockout cells were engineered using the CRISPR/Cas9 system as described previously (26). Lentiviral constructs were purchased from Addgene (#52961). A single guide RNA (sgRNA) was designed according to Zhang Lab protocol (27). sgRNAs targeting sequences for PTEN, TSC2, STK11, and EIF4EBP1 were 5’-AGCGGAAATTTAATTGCAG-3’, 5’-GGTCGCCGATCTGTGGACGC-3’, 5’-CAGGTTGCTCGGCCGGGAA-3’, and 5’-GCCACCCGGCCAGTGCGC-3’, respectively. These oligos were cloned into lentiviral plasmid, and packaged into lentivirus in HEK 293T cells. HN12 and CAL27 cells were infected with lentivirus for 2 days. The infected cells were selected with puromycin (4 μg/mL) for 3 days. Single cell clones were obtained and knocked out of each gene was validated by Western blot analysis. siRNA for EGFR was purchased from Sigma (MISSION siRNA human EGFR SHH0659).

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 described previously (24, 25). All cells were cultured in DMEM (D-6429; Sigma-Aldrich) supplemented with 10% FBS at 37°C (24, 25). All cells were cultured in DMEM (D-6429; Sigma-Aldrich) with 5% CO2. All cell lines underwent DNA authentication by multiplex STR profiling (Genetica DNA Laboratories, Inc.) prior to the described experiments to ensure consistency in cell identity. Presence of mycoplasma was found according to Mycoplasma Detection Kit-Quick Test from Bioneer. PTEN-, TSC2-, STK11-, and EIF4EBP1-knockout cells were engineered using the CRISPR/Cas9 system as described previously (26). Lentiviral constructs were purchased from Addgene (#52961). A single guide RNA (sgRNA) was designed according to Zhang Lab protocol (27). sgRNAs targeting sequences for PTEN, TSC2, STK11, and EIF4EBP1 were 5’-AGCGGAAATTTAATTGCAG-3’, 5’-GGTCGCCGATCTGTGGACGC-3’, 5’-CAGGTTGCTCGGCCGGGAA-3’, and 5’-GCCACCCGGCCAGTGCGC-3’, respectively. These oligos were cloned into lentiviral plasmid, and packaged into lentivirus in HEK 293T cells. HN12 and CAL27 cells were infected with lentivirus for 2 days. The infected cells were selected with puromycin (4 μg/mL) for 3 days. Single cell clones were obtained and knocked out of each gene was validated by Western blot analysis. siRNA for EGFR was purchased from Sigma (MISSION siRNA human EGFR SHH0659).

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 [pH 7.5]), containing Halt Protease and Phosphatase Inhibitor Cocktail (98440; 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 5% BSA or 5% nonfat dry milk in TBS-T buffer [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 Biotech) 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), basic FGF (bFGF: 20 ng/mL, +13256029; 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 mm³, 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 ethanolss. The slides were extensively washed with distilled water and antigen retrieval was performed by high temperature treatment with 10 mM 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’-diaminobenzidine 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 (5.1, 2.2%) and less frequent mutations in the TSC2 (1.1, 0.5%), TSC2 (1.1, 1.8%), 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 knockouts using the CRISPR-Cas9 system. We employed CAL27 and HN12 cells, both of which are human HNSCC cell lines that harbor no genomic alterations and UM-SCC-17B cells. 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 IC\(_{50}\) 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 \( \mu \text{mol/L} \), respectively (Fig. 3A). The IC\(_{50}\) values in parental, PTEN, TSC2, STK11, and EIF4EBP1-knockout HN12 cells were 16.3, 101.1, 96.1, 30.9, and 32.4 \( \mu \text{mol/L} \), respectively (Fig. 3A). Remarkably, among all of these changes the IC\(_{50}\) 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 cetuximab between parental and TSGs knockout cells after treatment with cetuximab at 10 \( \mu \text{g/mL} \), 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
Genomic alterations in genes involved in PI3K/Akt/mTOR signaling in HNSCC. A, Frequency of genomic alterations in genes involved in PI3K/Akt/mTOR signaling in the HNSCC TCGA dataset (n = 504), including HPV-positive (n = 93) and negative (n = 409) lesions. B, Comparison of overall survival between patients with and without copy number loss of TSGs in either PTEN, TSC1, TSC2, STK11, or EIF4E-BPI. C, Isogenic cell panels of HNSCC cell lines, CAL27 and HN12, parental controls and with gene knockout in PTEN, TSC2, STK11, or EIF4E-BPI, as indicated.

(Fig. 4B), 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 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 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.

![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.](image-url)
to cetuximab treatment \textit{in vitro} and \textit{in vivo}. However, we have previously shown that genomic alterations in the \textit{PIK3CA} or \textit{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 \textit{PTEN} loss or HNSCC cells with endogenous \textit{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 \textit{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 \textit{PTEN} loss is sufficient to

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\caption{Resistance to cetuximab in HNSCC tumors with endogenous \textit{PTEN} loss, and PI3K/mTOR network-based analysis of refractoriness to cetuximab. \textbf{A}, UDSCC2 cells were transplanted into nude mice and treated with vehicle control diluent or cetuximab (40 mg/kg), three times per week. \textbf{B}, Representative tumors treated with vehicle control or cetuximab (HE staining). \textbf{C}, Representative immunohistochemical analysis of pEGFR (Y1068) and pS6, as indicated. \textbf{D}, Graphic depicting copy number variations in the PI3K/mTOR pathway in HNSCC. Resistance to cetuximab can be specifically conferred by \textit{PIK3CA} and \textit{RAS} mutations (from ref. 23), as well as from frequent \textit{PTEN} gene copy loss (red border).}
\end{figure}
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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 EGFR 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 WW1, and genetic deletion of the WW1 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 WW1 inhibitor indole-3-carbinol potently suppressed in vitro tumor growth and PTEN-mediated signaling. These findings suggest that targeted inhibition of WW1 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/BRCA 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 Medicines.

Authors’ Contributions

Conception and design: H. Izumi, Z. Wang, J.S. Gutkind

Development of methodology: H. Izumi, Z. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Izumi, Z. Wang, Y. Goto, T. Ando, X. Wu, X. Zhang, H. Li

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Izumi, X. Wu, X. Zhang, J.S. Gutkind

Writing, review, and/or revision of the manuscript: H. Izumi, Z. Wang, X. Wu, D.E. Johnson, J.R. Grandis, J.S. Gutkind

Study supervision: D.E. Johnson, J.S. Gutkind

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019;69: 7–34.
2. Lui WWY, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defined predictive biomarkers. Cancer Discov 2013;3:761–9.
3. Pickering CR, Zhang J, Yoo SY, Bengtsson L, Moorthy S, Neskey DM, et al. Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. Cancer Discov 2013;3:770–81.
4. Iglesia-Bartolome R, Martin D, Silvio Gutkind J. Exploiting the head and neck cancer oncogene: widespread PI3K-mTOR pathway alterations and novel molecular targets. Cancer Discov 2013;3:722–5.
5. The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015;517:1–7.
6. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor α and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53:3579–84.
7. Kalyanaraman S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 2006;24:2666–72.
8. Chung CH, Ely K, Mcgavan L, Varella-garcia M, Parker J, Parker N, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol 2006;24:4170–6.
9. Grandis JR, Melhem M, Gooding W. Levels of TGF-α/α and EGFR protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst 1999;90:824–32.
10. Vemorken JB, Trigo J, Hitt R, Koralewski P, Diaz-Rubio E, Rolland F, et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol 2007;25: 2171–7.
11. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006;354:567–78.
12. Vemorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2009;359:1116–27.
13. Mehanna H, Robinson M, Hartley A, Kong F, Foran B, Fulton-Lieuw T, et al. Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALATE HPV): an open-label randomised controlled trial of phase 3 trial. Lancet 2019;393:51–60.
14. Gillison ML, Trotti AM, Harris J, Eisbruch A, Harari PM, Adelstein DJ, et al. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. Lancet 2019;393:40–50.
15. Mitrodoni T, Morita S, Yatake Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11:121–8.
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16. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.

17. Cutsem EC, Kohno C-H, Hitre E, Zaluski J, Chien C-RC, Lim R, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009;360:1408–17.

18. Bokemeyer C, Bondarenko I, Makshon A, Hartmann JT, Aparicio J, de Braud F, et al. Fluorouracil, leucovorin, and oxaliplatin with or without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol 2009;27:663–71.

19. Brand TM, Iida M, Stein AP, Corrigan KL, Braverman CM, Luthar N, et al. AXL mediates resistance to cetuximab therapy. Cancer Res 2014;74:5152–64.

20. Leonard B, Brand TM, O'Keefe RA, Lee ED, Zeng Y, Kemmer JD, et al. BET inhibition overcomes receptor tyrosine kinase-mediated cetuximab resistance in HNSCC. Cancer Res 2018;78:4331–3.

21. Jiang N, Wang D, Hu Z, Shin HJC, Qian G, Rahman MA, et al. Combination of anti-HER3 antibody MM-121/SAR256212 and cetuximab inhibits tumor growth in preclinical models of head and neck squamous cell carcinoma. Mol Cancer Ther 2014;13:1826–36.

22. Madoz-Gurpide J, Zano S, Chamizo C, Casado V, Caramés C, Gavín E, et al. Activation of MET pathway predicts poor outcome to cetuximab in patients with recurrent or metastatic head and neck cancer. J Transl Med 2015;13:1–13.

23. Wang Z, Martin D, Molinolo AA, Patel V, Eglesias-Bartolome R, Degese MS, et al. mTOR co-targeting in cetuximab resistance in head and neck cancers harboring PIK3CA and RAS mutations. J Natl Cancer Inst 2014;106:439–51.

24. Amorosphimoltham P, Patel V, Sohdi A, Nikitakis NG, Saak JJ, Saussville EA, et al. Mammalian target of rapamycin, a molecular target in squamous cell carcinomas of the head and neck. Cancer Res 2005;65:9953–62.

25. Martin D, Abba MC, Molinolo AA, Vitale-Cross L, Wang Z, Zaida M, et al. The head and neck cancer oncogene: a platform for the development of precision molecular therapies. Oncotarget 2014;5:1–18.

26. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Middelsten TS, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 2014;343:84–7.

27. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 2014;11:783–4.

28. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4.

29. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1.
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