PDK1 Mediates NOTCH1-Mutated Head and Neck Squamous Carcinoma Vulnerability to Therapeutic PI3K/mTOR Inhibition

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Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) is driven largely by the loss of tumor suppressor genes, including NOTCH1, but lacks a biomarker-driven targeted therapy. Although the PI3K/mTOR pathway is frequently altered in HNSCC, the disease has modest clinical response rates to PI3K/mTOR inhibitors and lacks validated biomarkers of response. We tested the hypothesis that an unbiased pharmacogenomics approach to PI3K/mTOR pathway inhibitors would identify novel, clinically relevant molecular vulnerabilities in HNSCC with loss of tumor suppressor function.

Experimental Design: We assessed the degree to which responses to PI3K/mTOR inhibitors are associated with gene mutations in 59 HNSCC cell lines. Apoptosis in drug-sensitive cell lines was confirmed in vitro and in vivo. NOTCH1 pathway components and PDK1 were manipulated with drugs, gene editing, knockdown, and overexpression.

Results: PI3K/mTOR inhibition caused apoptosis and decreased colony numbers in HNSCC cell lines harboring NOTCH1 loss-of-function mutations (NOTCH1MUT) and reduced tumor size in subcutaneous and orthotopic xenograft models. In all cell lines, NOTCH1MUT was strongly associated with sensitivity to six PI3K/mTOR inhibitors. NOTCH1 inhibition or knockout increased NOTCH1MUT HNSCC sensitivity to PI3K/mTOR inhibition. PDK1 levels dropped following PI3K/mTOR inhibition in NOTCH1MUT but not NOTCH1WT HNSCC, and PDK1 overexpression rescued apoptosis in NOTCH1MUT cells. PI3K and AKT inhibitors together caused apoptosis in NOTCH1MUT HNSCC but had little effect as single agents.

Conclusions: Our findings suggest that NOTCH1MUT predicts response to PI3K/mTOR inhibitors, which may lead to the first biomarker-driven targeted therapy for HNSCC, and that targeting PDK1 sensitizes NOTCH1WT HNSCC to PI3K/mTOR pathway inhibitors.

Introduction

Although head and neck squamous cell carcinoma (HNSCC) has a high mutation rate, genomic sequencing has not detected druggable driver mutations in the disease. Rather, HNSCC is dominated by mutations in nontargetable tumor suppressor genes such as TP53 (72%), NOTCH1 (18%), RNF2 (16%), and AIP (6%; refs. 1, 2). The most frequently altered mitogenic signaling pathway in HNSCC is the PI3K/mTOR pathway, with 54% of patients having mutations or copy number alterations in PIK3CA (35%), PTEN (6%), RICTOR (7%), AKT1 (3%), PIK3R1 (2%), and MTOR (3%; ref. 3). PIK3CA, the third most frequently mutated gene in HNSCC (18%), has frequent hotspots in the helical (E542K or E545K) and kinase (H1047R) domains (3, 4). PI3K signaling and its effectors protein kinase B (AKT), mTOR, and phosphoinositide-dependent kinase 1 (PDK1), which play critical roles in cell proliferation and survival, are validated therapeutic targets in other cancers (5). With the exception of PI3K inhibitors that are approved for the treatment of hematologic malignancies and target of rapamycin complex 1 (TORC1) inhibitors that are approved for renal cell carcinoma, PI3K/AKT/mTOR inhibitors have elicited only modest response rates in solid tumors (5). Strategies guiding the selection of patients for these agents also remain elusive. Whether PIK3CA mutant (PIK3CA MUT) tumors have increased sensitivity to PI3K/AKT/mTOR inhibitors is unclear (4, 6, 7). PI3K/AKT/mTOR inhibitors have a low clinical response rate in PIK3CA MUT tumors (~30%; ref. 6) compared with other targeted therapies such as the epidermal growth factor receptor inhibitor osimertinib, which has a response rate of about 80% in EGFR MUT lung cancers (8). Consistent with these clinical findings, PIK3CA MUT HNSCC cell lines and patient-derived xenografts (PDX) are more sensitive to PI3K/mTOR pathway inhibitors than PIK3CA wild-type (PIK3CA WT) HNSCC cells; however, the drugs cause only cell-cycle arrest with no apoptosis in the mutant cell lines (4, 9–12).
Translational Relevance

The genomic landscape of head and neck squamous cell carcinoma (HNSCC) is dominated by nontargetable tumor suppressor genes such as NOTCH1, which is mutated in 18% of HNSCC. Molecurally targeted therapies directed towards activated onco genes are common for cancers generally but rare for can cers driven by the loss of tumor suppressor function. Our in vitro and in vivo studies revealed that PI3K inhibition causes apoptosis in only HNSCC with NOTCH1 loss-of-function mutations, independent of PIK3CA mutation status, via differential regulation of PDK1. This study, the first to establish a therapeutic vulnerability of HNSCC with NOTCH1 mutations to any class of drugs, may inform the development of the first biomarker-driven targeted therapy for HNSCC. Because NOTCH1 loss-of-function mutations are common in other squamous cell carcinomas, including lung (8%) and esophageal (21%) carcinomas, our findings likely have implications beyond HNSCC.

Molecular therapies targeting activated oncogenes are common, but only one such therapy has been approved for cancers driven by the loss of tumor suppressor function: PARP inhibitors for BRCA1/BRCA2 mutated breast cancer (13). Perhaps because of feedback pathways, targeting pathways activated downstream of tumor suppressors has not been effective for cancer therapy. To date, the new genomic information available for this disease has not been translated into clinical care largely because the genomic landscape is dominated by tumor suppressors. In this study, we hypothesized that an unbiased pharmacogenomics approach would identify novel, translationally applicable molecular vulnerabilities to HNSCC with the loss of tumor suppressor function. To address the lack of effective molecularly targeted therapies for HNSCC, we used an unbiased pharmacogenomics approach to integrate drug sensitivity data for seven diverse PI3K/mTOR pathway inhibitors in a panel of 59 molecularly characterized HNSCC cell lines (14, 15). We identified a striking correlation between NOTCH1 loss-of-function (LOF) mutations and sensitivity to PI3K/mTOR pathway inhibitors in HNSCC that we confirmed with both in vitro and in vivo studies.

To the best of our knowledge, this is the first study to establish a therapeutic vulnerability of NOTCH1 mutated HNSCC to any class of drugs. Thus, our findings have the potential to advance the approval of a biomarker-driven targeted therapy for HNSCC. Further, based on the mechanism of sensitivity of NOTCH1 mutated HNSCC, we have identified a combination therapy that may be effective against NOTCH1 mutated HNSCC.

Materials and Methods

Cells and reagents

We obtained and subjected 50 human papilloma virus (HPV)-negative and 9 HPV-positive HNSCC cell lines to whole-exome sequencing, reverse-phase protein array analysis, and gene expression profiling as described previously (14, 16, 17). All the cell lines were genotyped by short tandem repeat analysis, and all cell lines were mycoplasma-free at the time of testing with a Mycoplasma Detection Kit (Lonza). UMSCC49 parental and NOTCH1 knockout (KO) cells were purchased from Dr. Chad Brenner at the University of Michigan. An erythromycin ribosomal methylase (ERM) plasmid expressing PDK1-GFP was a gift from Dr. Gordon Mills. Cells were transfected with the PDK1-expressing plasmid with Lipoctectamine 3000 (Life Technologies) for 6 hours and selected with 1 to 2 mg/ml G418 (Sigma). To create the PJ34, FaDu, and MDAa66iLN NOTCH1 KO lines, we transfected the parental cell lines with a NOTCH1 CRISPR/Cas9 KO plasmid (sc-421930; Santa Cruz) using GenJet DNA transfection reagent (Signagen). The transfected cells were sorted based on green fluorescent protein expression, and individual clones were obtained. PQR309 was provided by PIQUR Therapeutics AG. All other drugs were purchased from Selleck Chemicals and prepared as 10 mmol/L stock solutions in DMSO.

Cell viability assays

HNSCC cell lines were treated with DMSO (vehicle) or PI3K/mTOR pathway inhibitors at seven different concentrations (0.018–9.613 μmol/L) for 72 hours. A CellTiter-Glo luminescent cell viability assay (Promega) was performed as described previously (30, 31). Inhibitory concentration (IC) and AUC values were calculated using the drexplore R package with a best-fit dose–response model (32). The combination indices were calculated using the Chou–Talalay method (33) in CalcuSyn (Biosoft). We tested the reproducibility and robustness of the data generated using three quality control parameters as described previously (28). These parameters were the concordance correlation coefficient, location shift, and maximum SD between two biological replicates for three technical and two biological replicates. Based on heuristics from our previous screening studies in lung cancer (28), the cut-offs for reproducibility were a concordance correlation coefficient greater than 0.8, a location shift less than 0.9, and a SD less than 0.23 based on the normal mixture fit model. Experiments not satisfying these criteria were repeated. The replicate with the smallest experimental variation measured by the median of SD was chosen as a representative of the replicates, and its IC values served as the final values for subsequent analysis.

Western blot analysis

Western blot analysis was performed as described previously (27). In brief, cells were lysed with ice-cold lysis buffer, and the lysates were centrifuged at 20,000 × g for 10 minutes at 4°C. Cell samples containing equal amounts of protein were resolved using SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with different primary antibodies. Protein expression was detected using a horseradish peroxidase–conjugated secondary antibody (Bio-Rad) and electrochemiluminescence reagent (Amersham Biosciences). The antibodies are listed in Supplementary Table S1.

Apoptosis and cell cycle assays

To measure apoptosis, we performed TUNEL staining with an APO-BRDU Kit (BD Biosciences) and Annexin V/propidium iodide staining with an FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) as described previously (35). For the cell-cycle analysis, cells were harvested, fixed, incorporated with bromodeoxyuridine (BrdUrd), and stained with 7-aminoactinomycin D using a BrdU Flow Kit (BD Biosciences). Data were acquired with a three-laser, 10-color Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza software (Beckman Coulter). All
apoptosis assays were performed in triplicate, and each test was completed twice on different days.

**Colonies formation assays**

HNSCC cells were seeded in 60-mm plates. One day later, the cells were treated with DMSO or the indicated drugs for 48 hours. The medium was changed, and the cells were incubated in drug-free medium for 14 to 21 days. The cell colonies were then washed, fixed in 10% formaldehyde, and stained with crystal violet (0.5%, w/v). Colony images were taken with a GelCount Tumour Colony Counter (Oxford Optronix Ltd.). The total colony number and area were counted and analyzed using the ImageJ software program (NIH, Bethesda, MD). Assays were performed in triplicate, and each test was completed twice on different days.

**Mouse models**

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by MD Anderson's Institutional Animal Care and Use Committee. Subcutaneous xenograft and orthotopic nude mouse tongue models were created as described previously (18). GSK2126458 was administered by oral gavage 5 days per week at 1 mg/kg (orthotopic model) or 3 mg/kg (subcutaneous xenograft model). PQR309 was administered by oral gavage 5 days per week at 30 mg/kg (orthotopic model).

**TUNEL tissue staining**

Harvested tumor tissues were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stored until further use. Deparaffinized and rehydrated tissue sections were processed for DNA labeling by terminal deoxynucleotidyl transferase, and then streptavidin-conjugated horseradish peroxidase/diaminobenzidine was detected using a TUNEL Apoptosis Detection Kit (Treven). Images were taken at 40× magnification, and Image J software was used to calculate the percentage of TUNEL-positive cells.

**Statistical analysis**

We used the β-uniform mixture model to control the FDR (16). To identify differentially expressed features between groups, we applied modified two-sample t-tests using the limma package in R. We used the Fisher exact test and Wilcoxon rank-sum test to evaluate associations between molecular characteristics and drug sensitivities. In vitro experiment results were compared using a two-sample t test and two-way analysis of variance corrected for multiple comparisons with the Tukey method in GraphPad Prism 7. In vivo experiment results were analyzed using a linear mixed model in R.

**Results**

HNSCC cell lines with LOF NOTCH1 mutations are sensitive to PI3K/mTOR pathway inhibitors in vitro

To identify novel predictive therapeutic vulnerabilities linked to PI3K/mTOR pathway inhibition, we treated 59 HNSCC cell lines with seven different PI3K/mTOR pathway inhibitors that were in clinical development at the start of this study (19–26). Of the drug–cell line combinations, 95% met our predefined quality control cutoff. The HNSCC cell lines exhibited diverse sensitivity to PI3K/mTOR pathway inhibitors (Supplementary Fig. S1A and S1B; Supplementary Table S2). Because the dose–response curves for PI3K/mTOR pathway inhibitors often plateau near the IC50 values (9), we used the more robust IC30 and AUC values as parameters for drug potency as described previously (27). Cell lines were classified as sensitive based on IC30 values less than the peak plasma concentration for each drug. To determine if cross-comparison of the PI3K/mTOR inhibitors as a class was feasible, we compared their drug sensitivity patterns with those of three cell-cycle kinase inhibitors we tested previously (17). The PI3K/mTOR pathway inhibitors clustered separately from the cell-cycle inhibitors (Supplementary Fig. S1C).

We then compared drug sensitivity to common driver genes identified in HNSCC tumors from The Cancer Genome Atlas (TCGA; ref. 17). Our cell lines did not have any mutations in 11 of the top 50 mutated genes, and six genes were mutated in only one cell line, precluding comparison. Of the 515 sequenced TCGA HNSCC tumors, 92% had a mutation in at least one of the remaining 33 genes, demonstrating that our cell lines were genomically representative of patients with HNSCC. Of the 33 genes, only mutated NOTCH1 and KRTAP5-5 were significantly correlated with sensitivity to six of the seven drugs (Fig. 1A). We did not study KRTAP5-5 further because it is mutated in only two cell lines and 6% of patients with HNSCC (1, 2).

Because PIK3CA mutations predict pathway inhibitor response in HNSCC (9–11), we specifically examined their relationship with drug sensitivity. We found no consistent correlation between PIK3CA mutations and drug sensitivity when we included all 12 PIK3CA WT cell lines (Fig. 1A). Compared with PIK3CA WT cell lines, PIK3CA WT HNSCC cell lines (excluding three cell lines with uncharcterized mutations) were significantly more sensitive to four drugs and substantially more sensitive to two drugs (Fig. 1B). The median IC50 values of six drugs for the NOTCH1 WT cell lines were significantly lower than those of the drugs for the NOTCH1 WT lines; the seventh drug, BKM120, was almost universally effective in HNSCC cell lines, likely owing to its multiple off-target effects (28). Two cell lines harbored both NOTCH1 and PIK3CA mutations and were sensitive to all the inhibitors tested. There was no mutual exclusivity of these two mutations in the 515 sequenced TCGA HNSCC patient samples, but there was a tendency towards co-occurrence (www.cbioportal.org; accessed on September 15, 2018).

HNSCC patient samples have frequent truncating NOTCH1 mutations (2). We found the distribution of these mutations to be very different from that of the activating mutations found in T-cell acute lymphoblastic leukemia (T-ALL); mutations in HNSCC cell lines were similar to the mutation pattern in HNSCC patient samples (Fig. 1C and D; ref. 29). Of the 17 HNSCC lines, we found to harbor NOTCH1 mutations, six had truncating mutations (nonsense mutations and deletions) and 11 had missense mutations. Of those 11, three were excluded from analysis because their NOTCH1 alterations occurred in the nuclear regulatory domain and more likely represented SNPs. Of the 14 HNSCC lines with NOTCH1 LOF mutations (Fig. 1E), two had both bona fide NOTCH1 and PIK3CA mutations. Both NOTCH1 and cleaved NOTCH1 [NOTCH intracellular domain (NICD)] protein levels were lower in NOTCH1 WT lines than in NOTCH1 WT lines, although the difference was not significant, likely owing to diverse protein expression levels in NOTCH1 WT lines. However, we found a significant positive correlation between NICD and total NOTCH1 protein levels in the cell lines (Fig. 1F). TCGA HNSCC patient samples also showed diverse NOTCH1 protein expression levels regardless of NOTCH1 mutation status (Supplementary
NOTCH1-Mutant Squamous Cancer Sensitivity to PI3K Inhibition

Basal activation of the PI3K/mTOR pathway and HPV status do not predict sensitivity to PI3K/mTOR pathway inhibitors

The diverse drug sensitivity suggested that subsets of cell lines have inherent molecular therapeutic vulnerabilities. Although there was a trend for PIK3CA**MUT** cell lines to be more sensitive to PI3K/mTOR pathway inhibitors, none of the 28 mutations in the PI3K/mTOR pathway described previously (4) consistently correlated with drug sensitivity (Supplementary Fig. S3A). Likewise, we found no significant correlation between the basal activation of the PI3K/mTOR pathway and drug sensitivity (Supplementary Fig. S3B and S3C, Supplementary Table S3). Of note, drug sensitivity did not correlate with **RICTOR** amplification or **PIK3CA** mutant gene expression (Supplementary Fig. S3D). Although HPV-positive HNSCC is molecularly distinct from HPV-negative HNSCC, drug sensitivity did not correlate with HPV status (Supplementary Fig. S4A and S4B).

We also compared basal gene and protein expression levels between resistant and sensitive cell lines (Supplementary Tables S4 and S5) and two additional proteomic scores (27). There was no robust correlation between DNA repair and epithelial to mesenchymal transition scores and sensitivity to drugs (r coefficient < 0.4; Supplementary Fig. S4C and S4D). We did not study these differentially expressed genes or proteins; these proteomic scores further owing to both inconsistency of the correlations and a lack of prior validation of the scores in HNSCC.

PI3K/mTOR pathway inhibition induces apoptosis and reduces clonogenic growth in NOTCH1**MUT** cell lines

We then validated the susceptibility of NOTCH1**MUT** HNSCC to PI3K/mTOR inhibition with GS2126458. GS2126458 induced significant apoptosis in all NOTCH1**MUT** lines; no apoptosis was detected in the two NOTCH1**WT**/PIK3CA**WT** lines or three NOTCH1**WT**/PIK3CA**MUT** lines (Fig. 2A and B). BAY806946 and PQR309 elicited similar results (Supplementary Fig. S5A and S5B). Consistent with these data, GS2126458, even at concentrations well below the peak plasma concentration value, significantly reduced the number of colonies formed and the total number of colonies formed after 3 days (Fig. 2C). GS2126458 induced G1 arrest in all cell lines but increased the sub-G1 fraction in only NOTCH1**MUT** lines (Supplementary Fig. S5C). We confirmed that GS2126458 had dose-dependent target inhibition in representative NOTCH1**WT** and NOTCH1**MUT** lines (Fig. 2D).

PI3K/mTOR pathway inhibition reduces tumor growth in both orthotopic tongue and subcutaneous xenograft models of NOTCH1**MUT** HNSCC

To assess the antitumor effect of PI3K/mTOR inhibition in NOTCH1**MUT** HNSCC, we first used two NOTCH1**MUT** lines to create subcutaneous xenograft models. The GS2126458-treated tumors regressed, whereas the vehicle-treated control tumors grew significantly. TUNEL staining showed that, consistent with the in vitro data, tumors treated with GS2126458 had significant apoptosis (Fig. 3A and B).

We also used an orthotopic xenograft model of HNSCC, whose local growth patterns and histology are similar to those of human HNSCC (18). In both NOTCH1**MUT** models, GS2126458 and PQR309 significantly reduced tumor size compared with vehicle alone. TUNEL staining showed that the NOTCH1**MUT** tumors underwent apoptosis at the end of treatment (Fig. 3C–F).

CRISPR-Cas9 NOTCH1 KO sensitizes NOTCH1**WT** HNSCC to PI3K/mTOR pathway inhibitor–mediated apoptosis

We used four NOTCH1**WT** lines with NOTCH1 to test the hypothesis that abrogating NOTCH1 signaling renders NOTCH1**WT** cells more sensitive to PI3K/mTOR pathway inhibition. Compared with their parental cell lines, the PJ34 and UMSCC49 lines with NOTCH1 KO showed decreased cell viability (Supplementary Fig. S7A and S7B), with combination indices of less than 1 for a range of fractions affected (Supplementary Table S6). Consistent with these data, tumors treated with GSK2126458 had significantly higher tumor cellularity (Fig. 3D). However, NOTCH1 KO did not significantly increase the sensitivity of the NOTCH1**WT** lines FaDu and MDA686LN to PI3K/mTOR pathway inhibition (Supplementary Fig. S6).

We also used the γ-secretase inhibitors N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyler and dibenzepine to inhibit the intramembrane proteolytic cleavage/activation of NOTCH1. The combination of PI3K/mTOR and NOTCH1 signaling inhibitors decreased cell viability in four NOTCH1**WT** lines (Supplementary Fig. S7A and S7B), with most combination indices of less than 1 for a range of fractions affected (Supplementary Table S6). Consistent with these data, tumors treated with GSK2126458 had significantly lower tumor volume compared with vehicle alone. TUNEL staining showed that the NOTCH1**WT** tumors underwent apoptosis at the end of treatment (Fig. 3C–F).

**Figure 1.**

NOTCH1 LOF mutations and NOTCH1 signaling in HNSCC are correlated with sensitivity to PI3K/mTOR pathway inhibitors. A, HNSCC cell lines (n = 59) were incubated with seven different PI3K/mTOR pathway inhibitors. The drugs’ IC50 and AUC values in wild-type cell lines were compared with those in cell lines with mutations in 33 common driver genes using a modified two-sample t test at an FDR of 0.05. Positive and negative correlations between the presence of a mutation and drug sensitivity are indicated by blue and red data points, respectively, with larger points representing significant differences. B, The IC50 values in cell lines with wild-type or mutant NOTCH1 and PIK3CA were compared using a Kruskal-Wallis test corrected for multiple comparisons by the Dunn test; P values are indicated in the figure. The dashed black lines indicate each drug’s peak plasma concentration. N.S., not significant. C, The frequency and location of mutations in NOTCH1 in HNSCC cell lines (upper gene map) are compared with those of T-ALL (lower map, below the gene) and HNSCC patients (upper map, above the gene) based on data from the Catalogue of Somatic Mutations in TCGA. Oncogenic T-ALL NOTCH1 mutations occur in a hotspot of mostly missense mutations within the negative regulatory helicoidization (NRD) domain or in a second hotspot of mostly truncating mutations near the C-terminus, deleting the proline-glutamic acid-serine-threonine (PEST) domain and increasing the stabilization of activated NOTCH1 in the nucleus. In contrast, the truncating mutations in HNSCC are scattered throughout the gene but not in the PEST domain, and the missense mutations cluster in the extracellular EGF ligand-binding domain. D, Most mutations in the NOTCH1 gene in TCGA HNSCC patient samples are missense mutations outside the C-terminal NRD and PEST domains. E, From our panel of 59 HNSCC cell lines, we identified 24 harboring NOTCH1 and/or PIK3CA mutations. Orange text corresponds to nonsense mutations outside the PEST domain; red text, frameshift mutations outside the PEST domain; blue text, missense mutations outside the NRD and PEST domains; and gray text, in-frame deletions. ¥, noncanonical mutations; †, suspected SNPs excluded from NOTCH1 LOF mutants. F, The basal expression levels of NOTCH1 and NOTCH1 intracellular domain (NICD) proteins in NOTCH1**WT** and NOTCH1**FRET** cell lines were compared using a two-sample t test. The correlation between the expression levels of NICD and NOTCH1 was assessed by Spearman correlation.

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Clin Cancer Res; 25(11) June 1, 2019

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Published OnlineFirst February 15, 2019; DOI: 10.1158/1078-0432.CCR-18-3276

Figure S2A). NOTCH1 and NICD levels did not consistently correlate with drug sensitivity in NOTCH1**MUT** or NOTCH1**WT** lines (Supplementary Fig. S2B–S2E).

Figure 1.
findings, the combination also increased apoptosis (Supplementary Fig. S7C–S7E). As with the NOTCH1-KO lines, combined γ-secretase and PI3K/mTOR inhibition was not synergistic in two NOTCH1WT lines, likely because of the diverse molecular mechanisms of resistance.

**PDK1 mediates resistance to PI3K/mTOR pathway inhibition in NOTCH1WT HNSCC**

To identify the mechanism of sensitivity in NOTCH1MUT HNSCC, we inhibited components of the PI3K/mTOR pathway in both NOTCH1MUT and NOTCH1WT cell lines. All cell lines were resistant to the mTOR inhibitors (rapamycin and ridofovir) and the AKT inhibitor (MK-2206), but both the dual PI3K/mTOR inhibitor (GSK2126458) and pan-PI3K inhibitor (BAY806946) had differential effects on NOTCH1MUT and NOTCH1WT cells (Supplementary Fig. S8A). These results suggest that an important signaling node that is downstream of PI3K but independent of AKT and mTOR is responsible for the differential sensitivity.

We then investigated PDK1’s role in the differential sensitivity, because PDK1 is activated by PI3K, mediates resistance to PI3K inhibition in breast cancer (30), and can regulate p70S6K, which was inhibited more robustly in NOTCH1MUT than in NOTCH1WT HNSCC (Supplementary Fig. S8B). PI3K inhibition using three different drugs reduced PDK1 expression in only NOTCH1MUT lines (Fig. 5A and B; Supplementary Fig. S9A). To assess PDK1’s role in PI3K/mTOR inhibition–induced apoptosis in NOTCH1MUT HNSCC, we induced PDK1’s overexpression in NOTCH1MUT HNSCC cells. Compared with parental lines, NOTCH1MUT cells overexpressing PDK1 had higher expression levels of p-AKT, p-S6, and p-4EBP1 (Fig. 5C), and they had decreased apoptosis after...
GSK2126458 treatment (Fig. 5D and E). As expected, PDK1 overexpression did not inhibit the effect of GSK2126458 on phosphatidylinositol (3,4,5)-trisphosphate (PIP3)-dependent molecules such as p-AKT (T308; Fig. 5E). In contrast, PDK1 overexpression did inhibit the effect of GSK2126458 on PIP3-independent molecules such as c-Myc and p-TSC2 (Supplementary Fig. S9B).

PDK1 inhibition with GSK2334470 alone had little effect on cell viability or apoptosis regardless of NOTCH1 status (Fig. 6). Therefore, we hypothesized that both AKT and PDK1 inhibition are necessary for apoptosis following PI3K inhibition. In all cell lines tested, AKT inhibition plus PDK1 inhibition decreased cell viability with combination indices consistent with a greater-than-additive effect (Fig. 6A; Supplementary Fig. S9C and S9D; Supplementary Table S7). The combination also led to apoptosis in all cell lines tested (Fig. 6B and C). In NOTCH1Wt lines, the effect of the combination was similar to that achieved with PI3K/mTOR inhibition alone (Fig. 6B).

Discussion

With this study, we are the first to establish NOTCH1 LOF mutations in HNSCC as a significant therapeutic vulnerability to PI3K/mTOR pathway inhibition. We found that, unlike PIK3CA Mut HNSCC cell lines, which underwent only cell-cycle arrest following PI3K/mTOR inhibition, NOTCH1Mut HNSCC cell lines also underwent apoptosis. Likewise, PI3K/mTOR inhibitors decreased NOTCH1 Mut HNSCC tumor size in vivo. Selective mTOR or AKT inhibitors did not have differential effects on NOTCH1 Wt or NOTCH1 Mut HNSCC. This led us to examine PDK1, whose expression decreased following PI3K/mTOR inhibition in only NOTCH1 Mut HNSCC, supporting the hypothesis that persistent PDK1 activation leads to PI3K/mTOR inhibition resistance in NOTCH1 Wt HNSCC despite robust AKT inhibition. Consistent with this hypothesis, a combination of drugs that inhibit both PDK1 and AKT led to apoptosis.

Two other lines of evidence support our findings. First, the PI3K inhibitor PX-886 significantly reduced tumor growth in two NOTCH1 Mut HNSCC PDX models (10). Second, NOTCH pathway activation confers resistance to PI3K/mTOR inhibitors via c-Myc activation in breast cancer (31). Although we found that basal c-Myc gene or protein expression did not predict response, and c-Myc expression did not differ according to NOTCH1 mutation status (data not shown), c-Myc may be differentially regulated downstream of PDK1 in a PIP3-independent manner. Although activating mutations in PIK3CA also occur in HNSCC and other solid tumors, PI3K/mTOR inhibitors have had limited...
clinical success. Compared with PIK3CA WT HNSCC, PIK3CA MUT HNSCC cell lines and PDX models are more sensitive to PI3K/mTOR pathway inhibitors (4, 9–12, 32). However, PI3K/mTOR inhibition does not cause significant apoptosis in PIK3CA MUT HNSCC cell lines (9). These published findings are consistent with those of this study and with clinical findings demonstrating that PIK3CA MUT tumors tend to be more sensitive than PIK3CA WT tumors to PI3K/mTOR pathway inhibitors but still have modest clinical responses (4, 6, 7, 33). Because HNSCC tumors with NOTCH1 mutations undergo cell death (rather than simple growth arrest) in response to PI3K/mTOR inhibitors, we hypothesize that drugs targeting the PI3K/mTOR pathway have greater clinical efficacy in this genomic subtype that in PIK3CA MUT or NOTCH1 WT HNSCC.

Resistance to PI3K/mTOR pathway inhibitors manifests as a lack of sustained pathway inhibition despite the use of potent agents. For example, mTORC1 inhibition blocks S6 kinase–dependent inhibition of insulin receptor substrate 1, leading to insulin-like growth factor 1 receptor and then PI3K/AKT activation (34). A second feedback loop occurs because AKT activation inhibits the nuclear localization of forkhead box class O, a transcriptional driver of receptor tyrosine kinase (RTK) expression. AKT inhibition relieves this feedback suppression, leading to RTK expression and subsequent ERK activation (35). Similarly, in HNSCC, the addition of MEK/ERK or RTK pathway inhibition enhances antitumor effects of PI3K/mTOR inhibition (9, 36–38). However, we did not find differential effects on p-ERK after PI3K/mTOR inhibition based on NOTCH1 status (data not shown). A third mechanism is the sustained PDK1 signaling that mediates residual mTORC1 activity despite potent PI3K inhibition in PIK3Ka inhibitor–resistant PIK3CA MUT breast cancers (30). Our findings suggest that sustained PDK1 activity is a mechanism of resistance to PI3K/mTOR inhibition in NOTCH1 WT HNSCC. Additionally, other factors may influence response of HNSCC to PI3K/mTOR pathway inhibitors such as TP53 and TGFβ (39).
The mechanism underlying this differential effect on PDK1 in HNSCC is unknown. Although crosstalk between the PI3K/mTOR and NOTCH1 pathways has been extensively studied in T-ALL, in which NOTCH1 acts as an oncogene (29), the way in which these pathways interact in solid tumors is unknown. In T-ALL, the activation of the hes family bHLH transcription factor 1 (HES1), a downstream target of NOTCH1, suppresses PTEN expression, leading to PI3K activation and resistance to NOTCH1 inhibition (29). In this study, NOTCH1 inhibition did not affect AKT activation in HNSCC. We speculate that in NOTCH1 WT HNSCC, PDK1 is activated independently of PI3K. In T-ALL (40) and thymocytes (41), PDK1 activation downstream of NOTCH1 signaling mediates survival and proliferation.

One candidate through which PDK1 becomes activated is collagen 9 type alpha 1 (COL11A1). In ovarian cancer cells, COL11A1 binds to PDK1 and attenuates its ubiquitination and degradation, leading to chemoresistance (42); and in mouse embryonic fibroblasts, COL11A1 expression is downregulated after NOTCH1 inhibition (43). A second candidate is peroxisome proliferator-activated receptors, which are transcription factors induced by NOTCH1 inhibition in fatty liver (44) and can activate PDK1, leading to increased keratinocyte survival (45). In addition, the NOTCH1 pathway can confer drug resistance through a variety of other mechanisms, including epithelial-to-mesenchymal transition (46), and by promoting the cancer stem cell phenotype (47), which leads to tumor proliferation despite PI3K pathway inhibition.

In our study, NOTCH1 inhibition did not sensitize some NOTCH1 WT HNSCC cell lines to PI3K/mTOR inhibitors. We speculate that other diverse mechanisms mediate resistance to...
PI3K/mTOR pathway inhibitors, including PTEN loss (48) and AXL activation (12). However, the consistent sensitivity of NOTCH1 MUT HNSCC to multiple PI3K/mTOR pathway inhibitors supports the vulnerability of this molecular subtype despite the varied effects of NOTCH1 manipulations in NOTCH1 WT HNSCC.

This is the first study to establish a therapeutic vulnerability of NOTCH1 MUT HNSCC to any class of drugs. Because NOTCH1 LOF mutations are common in other squamous cell carcinomas, including lung (8%) and esophageal (21%) carcinomas (49), our findings have wide-ranging implications. Our finding that NOTCH1 LOF mutations predict response to PI3K inhibitors may lead to the first biomarker-driven targeted therapy for HNSCC. In addition, targeting PDK1 may sensitize NOTCH1 WT HNSCC to PI3K/mTOR pathway inhibitors.

Acknowledgments

We thank Doriano Fabbro, PhD, of PIQUR Therapeutics AG for guidance regarding PQR309 use in experiments and Joe Munch in MD Anderson’s Department of Scientific Publications for editing the manuscript. This work was supported by philanthropic contributions to The University of Texas MD Anderson Cancer Center’s Oropharynx Discovery Program (to J.N. Myers, F.M. Johnson); by grants from the National Institutes of Health/National Institute of Dental and Craniofacial Research (U01DE025181, to J.N. Myers and M.J. Frederick; R01 DE021479-01A1, to M.J. Frederick); and by PIQUR Therapeutics AG. This work used the services of MD Anderson’s Flow Cytometry and Cellular Imaging Core and Bioinformatics Shared Resource, which are supported by the NIH through MD Anderson’s Cancer Center Support Grant (P30CA016672).

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Received October 16, 2018; revised December 20, 2018; accepted February 11, 2019; published first February 15, 2019.

Disclosure of Potential Conflicts of Interest

F.M. Johnson reports receiving commercial research support from PIQUR and Trovagene. No potential conflicts of interest were disclosed by the other authors.


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