Response of Head and Neck Squamous Cell Carcinoma Cells Carrying PIK3CA Mutations to Selected Targeted Therapies

Eric D. Wirtz, MD; Daisuke Hoshino, PhD; Anthony T. Maldonado, BS; Darren R. Tyson, PhD; Alissa M. Weaver, MD, PhD

IMPORTANCE The PIK3CA mutation is one of the most common mutations in head and neck squamous cell carcinoma (HNSCC). Through this research we attempt to elicit the role of oncogene dependence and effects of targeted therapy on this PIK3CA mutation.

OBJECTIVES (1) To determine the role of oncogene dependence on PIK3CA—one of the more common and targetable oncogenes in HNSCC, and (2) to evaluate the consequence of this oncogene on the effectiveness of newly developed targeted therapies.

DESIGN, SETTING, AND PARTICIPANTS This was a cell culture–based, in vitro study performed at an academic research laboratory assessing the viability of PIK3CA-mutated head and neck cell lines when treated with targeted therapy.

EXPOSURES PIK3CA-mutated head and neck cell lines were treated with 17-AAG, GDC-0941, trametinib, and BEZ-235.

MAIN OUTCOMES AND MEASURES Assessment of cell viability of HNSCC cell lines characterized for PIK3CA mutations or SCC25 cells engineered to express the PIK3CA hotspot mutations E545K or H1047R.

RESULTS Surprisingly, in engineered cell lines, the hotspot E545K and H1047R mutations conferred increased, rather than reduced, IC50 assay measurements when treated with the respective HSP90, PI3K, and MEK inhibitors, 17-AAG, GDC-0941, and trametinib, compared with the SCC25 control cell lines. When treated with BEZ-235, H1047R-expressing cell lines showed increased sensitivity to inhibition compared with control, whereas those expressing E545K showed slightly increased sensitivity of unclear significance.

CONCLUSIONS AND RELEVANCE (1) The PIK3CA mutations within our engineered cell model did not lead to enhanced oncogene-dependent cell death when treated with direct inhibition of the PI3K enzyme yet did show increased sensitivity compared with control with dual PI3K/mTOR inhibition. (2) Oncogene addiction to PIK3CA hotspot mutations, if it occurs, is likely to evolve in vivo in the context of additional molecular changes that remain to be identified. Additional study is required to develop new model systems and approaches to determine the role of targeted therapy in the treatment of PI3K-overactive HNSCC tumors.

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ead and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer by incidence worldwide.1,2 Approximately two-thirds of patients present with advanced disease and undergo primary surgical treatment with adjuvant radiation and/or chemotherapy, or primary chemoradiation therapy with salvage surgical treatment. Although some advances in therapy have occurred, survival has not markedly improved in recent decades owing to locoregional recurrences, distant metastases, and second primary tumors.3 The only targeted therapy that is US Food and Drug Administration-approved for current use in HNSCC is cetuximab, a monoclonal antibody recognizing the epidermal growth factor receptor (EGFR), which gives a modest improvement in survival when combined with chemotherapy.4 Identification of novel targeted therapies for patients would make a large impact on disease that does not respond to traditional therapies.

To date, most targeted therapies are effective in patients who harbor a mutation or other specific genetic alteration in an oncogenic driver. Frequently occurring genetic mutations in HNSCC include a non–cancerogenic driver. Frequently occurring genetic mutations who harbor a mutation or other specific genetic alteration in this gene lead to PI3K overactivity. When evaluating multiple human malignant neoplasms,7 these mutations have been confirmed by visual inspection.

Oncogene addiction is the apparent dependence of some cancers on 1 or a few genes, promoting continued cell proliferation and maintenance of the malignant phenotype.11 Oncogene-addicted cancer cells are more sensitive to their inhibition than normal tissue. With the development of targeted therapy, oncogene-addicted cancers and the oncogenes they depend on are prime targets, given the favorable therapeutic index between cancer cell and normal cell susceptibility. However, to date, only a few oncogene-addicted cancers have been identified and successfully targeted. These cancers include chronic myelogenous leukemia, B-Raf-mutated melanoma, and EGFR-mutated non–small-cell lung cancers.12 Our goal in this study was to evaluate the role of one of the more common and targetable oncogenes in HNSCC, PIK3CA, in oncogene dependence and the effectiveness of newly developed targeted therapies.

Methods

Sanger and CCLE Data All available cell line data from the Sanger (Barretina et al13) and Cancer Cell Line Encyclopedia (CCLE) (Garnett et al14) databases were used to compare cell lines that contained PI3K or PTEN mutations with those cell lines that contained neither of these mutations. Published IC50 assay data for GDC-0941 and 17-AAG were plotted.15,14

Cell Lines and Engineering

We used the HNSCC cell lines: SCC25, SCC61, FaDu, HSC-2, CAL-3, and Detroit 562. PIK3CA genes wild type (WT), H1047R and E545K, were cloned using polymerase chain reaction into a pENTR vector and recombined into a pLent6/V5 vector as previously described into the SCC25 cell line.15 Lentiviral transduction was used to stably express the PIK3CA genes within the SCC25 cell line. We used commercially available antibodies for V5 (Invitrogen/Life Technologies) and actin (Sigma).

Cell Culture and Drug Treatment

Cells were grown in 10% or 20% fetal bovine serum in DMEM/F-12 medium. For IC50 assays, cells were plated in a 96-well plate with a density of 2000 cells per well in 200 μL of the same media. After 24 hours, the media was changed andserial dilutions of therapeutic agents 17-AAG, GDC-0941, trametinib, and BEZ-235 were added. The cells were next incubated for 96 hours.

Cell Viability

Calcein dye, Hoechst stain, and propidium iodide were applied to the cell culture at a concentration of 2 μM, 5 μg/mL, and 2 μM, respectively, followed by incubation for 30 minutes. The cells were imaged using an automated Cellavista system (Synentec). The cells that were fluorescent with both Calcein and Hoechst were determined to be viable, whereas those that fluoresced with propidium iodide were determined to be nonviable. Proper thresholding of cells by the software was confirmed by visual inspection.

Statistics and IC50 Determinations

The Sanger14 and CCLE15 IC50 assay data were plotted using box and whisker plots, including the 5th and 95th percentiles, using GraphPad. Statistical significance was then determined using the Mann-Whitney test with a value of P < .05. For analysis of experimental data, IC50 values were determined by plotting the log of the cell viability vs the log of the drug concentration. This linear graph was next solved for the point at which 50% of the cells were viable and 50% were nonviable. The inverse log of this drug concentration was calculated. Viability at each data point was calculated as the average number (from 3 wells on the same plate) of living cells treated with a certain concentration of drug divided by the average number (from 3 wells...
on the same plate) of living cells that received no treatment. GraphPad was used to display the data using the mean and standard error of the mean.

**Results**

The goal of our study was to investigate whether the commonly mutated PI3K pathway confers oncogene dependence or alters the response to targeted therapies in HNSCC. We began by analyzing publicly available drug response data from 2 recent studies\(^{13,14}\) that molecularly characterized a large number of cell lines across a diverse set of cancer types. To achieve an adequate sample size, it was necessary to include all cancer cell types. IC\(_{50}\) proliferation response data from the Sanger database\(^{14}\) (Figure 1A-C and G-I) were analyzed, comparing the responses of cell lines that contained a mutation in either the catalytic subunit of class Ia PI3K (PIK3CA) or in the opposing enzyme PTEN with those that did not contain these mutations. Only the data from Garnett et al.,\(^{14}\) from the Sanger center, included drug data from treatments with a direct PI3K inhibitor, the selective class I PI3K inhibitor GDC-0941. Using the Mann-Whitney test, we found no statistically significant difference in the IC\(_{50}\) response to GDC-0941 between those containing PI3K or PTEN mutations and nonmutated cells (Figure 1G-I), although there was a trend toward greater sensitivity. We also found a trend toward greater sensitivity when comparing solely the PI3K-mutated cells with the Mann-Whitney test.

**Figure 1. Analysis of Drug Response Data to 17-AAG or GDC-0941 From the Sanger\(^{14}\) and Cancer Cell Line Encyclopedia (CCLE)\(^{13}\) Data Sets**

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<th>Genetic Mutation Analyzed</th>
<th>CCLE 17-AAG PI3K</th>
<th>P = .48</th>
<th>n = 35</th>
<th>Non-PI3K/PTEN n = 401</th>
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<tr>
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<td>P = .75</td>
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<td>n = 50</td>
<td>Non-PI3K/PTEN n = 409</td>
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<td>Non-PI3K/PTEN n = 409</td>
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Box and whisker plots displaying published drug response data. The n number of CLs in each group indicated on graph. Statistical significance expressed in P values on graphs. The x-axes show the genetic mutation analyzed; the y-axes, IC50 value of cancer cell lines ln μm.
Whitney test that were treated with GDC-0941 with nonmutated cells, but statistical significance was not present (Figure 1). Of note, only 22 of the 526 cell lines tested were HNSCC cells. Of these, 3 had PI3K mutations and none had PTEN mutations.

Another molecule that might modify PI3K signaling is HSP90, a chaperone that controls the levels of many oncoproteins, including the downstream target of PI3K, the kinase Akt.13,14 The studies by Barretina et al13 and Garnett et al14 evaluated the response to cancer cell lines to the HSP90 inhibitor 17-AAG.13,14 Our analysis of the Garnett et al14 study indicates that the presence of PI3K or PTEN mutations is associated with greater sensitivity to 17-AAG (Figure 2A-B). Cell lines with PI3K or PTEN mutations analyzed together further increased the statistical significance of the difference between the response of nonmutated and mutated cell line I_{50} values (Figure 2C). However, our analysis of data from Barretina et al13 yielded no difference in response to 17-AAG (Figure ID-F). The reasons for these differences are unclear but could include cancer cell types tested or testing conditions.

The genomic studies by Barretina et al13 and Garnett et al14 included some HNSCC cell lines, but most of them were not tested for drug responsiveness. To investigate whether the presence of PI3K pathway-activating mutations might confer sensitivity to targeted therapies in HNSCC, we tested a panel of human papillomavirus–negative HNSCC cell lines with 17-AAG, of which FaDu and SCC25 parental were the only cell lines that did not carry PI3K mutations. These data yielded I_{50} results of 3 nM (FaDu), 10 nM (SCC25), 22 nM (Detroit 562), 13 nM (HSC-2), 50 nM (CAL-33), and 31 nM (SCC-61). In these cell lines, there was no clear difference to treatment with 17-AAG, although there seemed to be a trend toward increased resistance of those cells with PI3K mutations compared with those cells that did not contain a PI3K mutation (Figure 2A). These same cell lines were treated with GDC-0941, yielding I_{50} results of 86 nM (SCC25), 36 nM (Detroit 562), 75 nM (FaDu), 231 nM (SCC-61), 111 nM (HSC-2), and 158 nM (CAL-33). There was no clear difference in sensitivity to GDC-0941 between cell lines that contained a PI3K mutation compared with those that did not (Figure 2B).

The study by Lui et al6 evaluated HNSCC cell lines that contained PIK3CA hotspot mutations or WT expression and their sensitivity to BEZ-235, a combined PI3K/mTOR inhibitor, and found those cell lines with PIK3CA mutations were more sensitive to PI3K/mTOR inhibition compared with those with WT mutations. We evaluated the response of a panel of HPV-negative cell lines—SCC25, FaDu, Detroit 562, HSC-2, and CAL-33—of which SCC25 and FaDu were the only cell lines that did not carry PI3K mutations to BEZ-235. These data yielded I_{50} results of 31 nM (SCC25), 24 nM (FaDu), 5 nM (Detroit 562), 2 nM (HSC-2), and 34 nM (CAL-33). Since 2 of the 3 cell lines with hotspot PIK3CA mutations were more sensitive than control cells to BEZ-235, these data suggest a potential increased sensitivity to BEZ-235 in cell lines carrying PIK3CA mutations (Figure 2C).

Owing to the variability of drug response in PI3K-mutated and nonmutated cells in our panel, we sought to create a cleaner system in which the only difference between the cell lines was the presence of PI3K mutations. Therefore, we used lentiviral transduction to express WT PI3K or PI3K hotspot mutants E545K or H1047R in SCC25 HNSCC cells (Figure 3A). The resulting cell lines were then tested for response to candidate-targeted therapies by growing them for 96 hours in the presence of the selected drugs.

Similar to our results in the unmanipulated HNSCC cell lines (Figure 2A), E545K- and H1047R-expressing cells were found to have decreased sensitivity to 17-AAG compared with the SCC25 parental and WT cell lines (Figure 3B). Thus, E545K-
and H1047R-expressing SCC25 cells had IC\textsubscript{50} values of 83 nM and 53 nM, respectively, whereas the SCC25 WT PI3K-expressing and parental cell lines had IC\textsubscript{50} values of 9 nM and 12 nM, respectively. In contrast to our results with unmanipulated cell lines (Figure 2B), treatment with therapeutic agents (B) 17-AAG, (C) GDC-0941, (D) trametinib, and (E) BEZ-235. Mean cell viability plotted with error bars denoting standard error of the mean. B. Experiment was performed in triplicate measurements and with 4 independent experiments, except for PI3K WT, which was performed in 2 independent experiments for the concentrations of 5, 10, and 200 nM. C. Experiment was performed in triplicate measurements and with 3 independent experiments, except for SCC25 Parental, which was performed in triplicate measurements with 4 independent experiments for all concentrations. D. Experiment was performed in triplicate measurements with 3 independent experiments except for the 400-nM concentration, which was performed with triplicate measurements and 1 independent experiment for all CLs. E. Experiment was performed in triplicate measurements and with 3 independent experiments except for the SCC25 parental 200-nM concentration, which was performed only with triplicate measurements and 1 independent experiment.

We next tested the response of our engineered cell lines to combined PI3K/mTOR inhibition using BEZ-235. Similar to the trends we had seen in our unmanipulated cell line panel (Figure 2C), expression of the H1047R hotspot mutation led to increased sensitivity compared with the SCC25 parental cells with respective IC\textsubscript{50} values of 6 nM and 31 nM, respectively (Figure 3E). By contrast, expression of the E545K mutation led to a very small increase in sensitivity of unclear significance (27 nM compared with 31 nM for control). This may be due to the decreased activity of E545K compared with H1047R.\textsuperscript{18}

**Discussion**

In this study, we investigated the role of PI3K pathway mutations in mediating sensitivity to targeted therapies. Analysis of publically available data from large-scale cancer cell line studies\textsuperscript{13,14} yielded a mixed result, with sensitivity of PI3K and PTEN mutant cells to the HSP90 inhibitor 17-AAG in 1 study\textsuperscript{13} but not in another. Only 1 study\textsuperscript{14} tested a class I PI3K inhibi-
tor, and the results suggested sensitivity but were not statistically significant. In a small-scale panel of genetically unrelated HNSCC cell lines, the presence of PI3K mutations also yielded an unclear result, with some cell lines more sensitive than others to PI3K, HSP90, and combined PI3K/mTOR inhibitors. Finally, we created a set of isogenically related HNSCC cell lines and tested them for sensitivity to PI3K, HSP90, MEK inhibitors, and combination PI3K/mTOR inhibitors. Surprisingly, in the latter system, PI3K mutations conferred decreased sensitivity to the class I PI3K inhibitor GDC-0941, the HSP-90 inhibitor 17-AAG, and the MEK inhibitor trametinib. However, the H1047R-expressing cell line exhibited increased responsiveness to the dual PI3K/mTOR inhibitor BEZ235. These data suggest that PI3K mutations do not universally confer oncogene dependence, at least as measured by IC₅₀ assay. They also suggest that more sophisticated model systems are needed to understand the role of PI3K mutations in targeted therapy of HNSCC.

Our data do provide further support for the findings of Lui et al⁶ that cells expressing PI3K mutations may exhibit increased sensitivity to dual PI3K/mTOR inhibition. However, our data suggest that it is not universally applicable to all tumors and may not be applicable to all hotspot mutations. Nonetheless, our findings suggest that oncogene dependence in cells expressing PI3K mutations may be due to dependence on mTOR survival signaling and that proper targeting may depend on shutting down that pathway.

Another finding that was similar between the unmanipulated HNSCC cell lines and genetically engineered ones was the relative resistance of PI3K mutant-carrying cell lines to 17-AAG. This is in contrast to data from the analysis of data from Garnett et al¹⁴ in which PI3K-activated cell lines overexpressed AAG. This is in contrast to data from the analysis of data from Garnett et al¹⁴ in which PI3K-activated cell lines overexpressed AAG. One possible explanation for the discrepancy with the data from Garnett et al¹⁴ is that tumor type may influence the response to 17-AAG even in the context of oncogenic mutations. For example, in Figure 1A, only 3 of the 50 cell lines with PIK3CA mutations tested in that study were HNSCC cell lines.

Oncogene dependence is a complex phenomenon and may include diverse mechanisms. In hyperproliferative, early lesions, such as chronic myelogenous leukemia (CML), the oncogenic lesion may be an initiating factor that is required for ongoing imbalances in proliferation or differentiation state. In CML, approximately 95% of patients carry the BCR-ABL translocation and inhibition of that gene fusion product leads to remission in most patients. However, later-stage cancers may either be resistant to targeted therapy or have developed more complex molecular dependencies. For example, the presence of modifying molecular expression patterns and mutations may determine whether a patient with an “oncogene-addiction” mutation will respond to targeted therapy. In some cases, enhanced sensitivities to targeted therapies may develop over time in cancers expressing mutated oncogenes. PI3K mutations often develop in late-stage cancers.⁶ Thus, it is likely that cancers with PI3K mutations are not strictly dependent on this pathway for tumor growth. However, as the most commonly mutated pathway in cancer, it is an attractive target for therapy.⁶ One possibility that our data and the data from Lui et al⁶ suggest is that cancers with PI3K pathway mutations may develop dependence on certain pathways over time by a shift in their signaling state to adjust to the abnormally active PI3K/mTOR signaling. Another possibility is that PI3K is more important for controlling other hallmarks of cancer that our study and the studies by Garnett et al¹⁴ and Barretina et al¹³ did not test, such as invasion or serum-independent growth that are generally enhanced in late-stage cancers. A final possibility is that PI3K inhibition alone is not sufficient enough of a blockade to cause cell death owing to the ability of PI3K mutant-expressing cells to shift to other survival pathways, notably mTOR. Thus, only with dual PI3K/mTOR therapy are we able to see the increased sensitivity conferred by one of the PI3K hotspot mutations.

Our finding that exogenous expression of PI3K mutant molecules led to resistance to PI3K- and HSP90-targeted with sensitivity only to PI3K/mTOR dual inhibition therapy suggests several future directions. First, the advent of genome editing tools allows introduction of mutations into the endogenous gene. This might represent a better model system than the exogenous expression system we created because it should separate the effects of overexpressing proteins from the mutation status. Second, primary cancer cells or cell lines in which the mutation naturally occurred may be superior to engineered cell lines if the oncogene dependence requires evolution of the tumor in the presence of the oncogene. Third, it is possible that IC₅₀ analysis of cell proliferation is not sufficient to analyze the effect of PI3K mutations, despite its utility in other oncogene-addiction models. Finally, investigation of how the signaling state of PI3K mutant-expressing cells shifts on exposure to PI3K inhibitors may further identify optimal targeted therapy approaches.

Conclusions

This study demonstrates that PI3K plays a complex role in oncogene dependence. New model systems and approaches will likely need to be developed to determine the role of targeted therapy in the treatment of PI3K-overactive HNSCC tumors. 
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REFERENCES