Companion Diagnostics and Cancer Biomarkers

A Comprehensive Evaluation of Biomarkers Predictive of Response to PI3K Inhibitors and of Resistance Mechanisms in Head and Neck Squamous Cell Carcinoma

Tuhina Mazumdar1, Lauren A. Byers1,2, Patrick Kwok Shing Ng3, Gordon B. Mills2,3, Shaohua Peng1, Lixia Diao4, You-Hong Fan1, Katherine Stemke-Hale5, John V. Heymach1,2, Jeffrey N. Myers2,6, Bonnie S. Glisson1, and Faye M. Johnson1,2

Abstract

The PI3K/AKT/mTOR pathway is frequently activated in head and neck squamous cell carcinoma (HNSCC), but pathway inhibition has variable efficacy. Identification of predictive biomarkers and mechanisms of resistance would allow selection of patients most likely to respond and novel therapeutic combinations. The purpose of this study was to extend recent discoveries regarding the PI3K/AKT/mTOR pathway in HNSCC by more broadly examining potential biomarkers of response, by examining pathway inhibitors with a diverse range of targets, and by defining mechanisms of resistance and potential combination therapies. We used reverse-phase protein arrays (RPPA) to simultaneously evaluate expression of 195 proteins; SNP array to estimate gene copy number; and mass array to identify mutations. We examined altered signaling at baseline and after pathway inhibition. Likewise, we examined the activation of the PI3K/AKT/mTOR pathway in HNSCC tumors by RPPA. Cell lines with PIK3CA mutations were sensitive to pathway inhibitors, whereas amplification status did not predict sensitivity. While we identified a set of individual candidate biomarkers of response to pathway inhibitors, proteomic pathway scores did not correlate with amplification or mutation and did not predict response. Several receptor tyrosine kinases, including EGFR and ERK, were activated following PI3K inhibition in resistant cells; dual pathway inhibition of PI3K and EGFR or MEK demonstrated synergy. Combined MEK and PI3K inhibition was marked synergistic in HRAS-mutant cell lines. Our findings indicate that clinical trials of single-agent PI3K/AKT/mTOR pathway inhibitors in selected populations and of PI3K/EGFR or PI3K/MEK inhibitor combinations are warranted; we plan to conduct such trials. Mol Cancer Ther; 13(11); 2738–50. ©2014 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common and deadly disease in the United States and worldwide; in patients with this disease, the rate of treatment-related morbidity is high and recurrence is common. Although the unbiased genomic characterization of multiple cancers has fundamentally changed our approach to cancer therapy and translational research, targeted therapy based on biomarkers does not yet exist for HNSCC. Genomic characterization of HNSCC has demonstrated 4 common driver signaling pathways in the HNSCC oncogene: mitogenic signaling, NOTCH/differentiation, cell cycle, and p53/apoptosis (1–4). Of the mitogenic pathways affected, PI3K/AKT is by far the pathway most often altered in HNSCC, with about 80% of HNSCC tumors containing molecular alterations in one or more components of the pathway (2, 5). The frequent activation of the PI3K/AKT/mTOR pathway in HNSCC, the availability of pharmacologic inhibitors, and the pathway’s importance in cancer cell signaling make this pathway one of the most promising targets for desperately needed improvement in systemic therapy. Furthermore, the pathway’s activation by identifiable molecular alterations suggests the possibility of using suitable biomarkers for predicting response to pathway-inhibiting therapy.

The PI3K/AKT/mTOR signaling pathway is critical for the regulation of multiple cellular processes, including
proliferation, growth, differentiation, migration, and survival, in normal and cancer cells. Pathway components are frequently altered in cancer cells, leading to pathway activation and poor prognosis (6, 7). In HNSCC specifically, there are gain-of-function mutations in PIK3CA (6%–13%), LOH of PTEN (8%), reduced PTEN protein expression (30%), PIK3CA gene overexpression (52%), PIK3CA amplification (20%), PIK3CG mutations (4%), and inactivating mutations in PTEN (4%; refs. 2–6, 8–10). AKT1, PIK3R1, and mTOR mutations occur rarely (5). At the protein level, nearly all HNSCC tissues show phosphorylation of AKT and the downstream target S6, indicating pathway activation (11, 12).

Despite the frequent activation of the PI3K/AKT/mTOR pathway in HNSCC, inhibition of this pathway with a variety of inhibitors has had variable efficacy in vitro and in vivo (12–18). Identification of molecular markers able to predict benefit through identification of either sensitivity or resistance mechanisms could markedly improve the utility of PI3K/AKT/mTOR pathway inhibitors. Several candidate biomarkers have been identified through our knowledge of the pathway and findings in HNSCC and other cancers. Of these candidates, PIK3CA mutations are the ones most consistently related to sensitivity to pathway inhibitors as demonstrated in 2 recent studies with HNSCC patient–derived xenografts (PDX; refs. 5, 19): a recent series of phase I studies (20)and cell line and PDX models from multiple cancer histologies (19, 21, 22). Additional markers of sensitivity include HER2 amplification, 4EBP1 expression, PTEN loss (23–25). In contrast, in vitro inhibition of both signaling and proliferation by a dual PI3K/mTOR inhibitor was observed in breast cancer cells independently of PIK3CA mutation and basal pathway activation (26). PTEN loss, rather than PIK3CA mutation, was closely linked to breast cancer cell sensitivity to a PI3K inhibitor (25, 27). Non-responding HNSCC tissue had higher levels of multiple signaling components, including pSTAT3, EGFR, and c-Kit, than responding tumors tested ex vivo (18). Thus, while there are a number of potential markers of benefit, there is no consensus as to their utility and their applicability to HNSCC based on its underlying gene expression pattern and the patterns of co-mutations that occur.

Here, we build upon recent discoveries regarding the PI3K/AKT/mTOR pathway in HNSCC by significantly expanding the examination of potential biomarkers to include PI3KCA amplification, PTEN loss, and the expression and activation of 195 proteins; by examining pathway inhibitors with a diverse range of targets; and by identifying mechanisms of resistance that were previously unknown in HNSCC, leading to combination therapies with a strong potential for high clinical efficacy. We tested a panel of 18 HNSCC cell lines with and without detected PI3K/AKT/mTOR pathway alterations for sensitivity to PI3K, PI3K/mTOR, AKT, and mTOR catalytic inhibitors. In addition to studying the expected markers of sensitivity, we used reverse-phase protein and phosphoprotein arrays (RPPA) as unbiased approaches in a panel of 60 HNSCC cell lines. We inhibited activated pathways to identify several candidate drug targets for PI3K/AKT/mTOR pathway inhibitor combinations.

Materials and Methods

Materials

All PI3K pathway inhibitors, MEK162, erlotinib, OSI906, cabozantinib, and dovitinib were purchased from Selleck Chemicals and prepared as 10 mmol/L stock solutions in dimethyl sulfoxide. Antibodies against total and phosphorylated AKT, ERK, pS6 and 4EBP1, c-Myc, cyclin D1, phosphorylated SGK3, and the PathScan RTK signaling antibody array kit were purchased from Cell Signaling Technology; antibody against β-actin was purchased from Sigma-Aldrich.

Cell culture

The HOSC1 cell line was derived from an HNSCC tumor implanted into a mouse as previously described (28). A panel of 64 HNSCC cell lines used in this study was validated and obtained as previously described (29).

Cytotoxicity assay

The cytotoxicity of PI3K pathway inhibitors in HNSCC cell lines was assessed using an MTT assay as described elsewhere (28). Under each experimental condition, 4 independent wells were treated. We calculated IC50 and combination index (CI) values with methods previously described by Chou and using CalcuSyn software (Biosoft; ref. 30). CI values less than one were considered synergistic.

Western blotting

Western blot analysis was performed as described previously (28). In brief, cells were subjected to lysis on ice and the lysates to centrifugation at 20,000 × g for 10 minutes at 4°C. Equal amounts of protein samples were resolved by SDS-PAGE and then transferred to nitrocellulose membranes, immunoblotted with different primary antibodies, and detected with a horseradish peroxidase–conjugated secondary antibody (Bio-Rad Laboratories) and the ECL reagent (Amersham Biosciences).

PhosphoScan analysis

PhosphoScan analysis was done according to the protocol provided by the manufacturer (Cell Signaling Technology). In brief, HNSCC cells were subjected to lysis, and 100 μg of lysate was added to a slide coated with target-specific capture antibodies. The slide was then incubated with a biotinylated antibody cocktail. Streptavidin-conjugated HRP and LumiGLO reagent were used to visualize the bound detection antibody by chemiluminescence.

PCR sequencing

To confirm mutations found by Sequenom (see Results), the regions of interest were amplified using custom PCR primers. Sanger sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems) using BigDye Terminator v3 chemistry (Applied Biosystems). Mutation
analysis was performed using SeqScape Software v2.5 (Applied Biosystems).

**Hotspot mutational analysis**
Custom Sequenom assays for somatic variants across 45 different genes were designed by using Sequenom’s Assay Design program, with in silico analysis to avoid known SNP site interference (see Supplementary Table S1 for a list of the mutations). Point mutations were assayed using the Sequenom MassArray as described previously (27), with additional analysis comparing relative allelic frequencies of duplicate samples.

**RPPA analysis**
RPPA analysis and antibody validation were performed as previously described (31). Briefly, protein lysate was collected from subconfluent HNSCC cell cultures after 24 hours in full serum medium (10% FBS). Protein lysates were adjusted to a 1 μg/μL concentration, and a serial dilution of 5 concentrations was printed, with 10% of the samples replicated for quality control (2470 Arrayer; Aushon Biosystems) on nitrocellulose-coated slides (Grace Bio-Labs). Immunostaining was performed using a DakoCytomation-catalyzed system and diaminobenzidine colorimetric reaction. Spot intensities were analyzed and quantified using MicroVigene software (VigeneTech Inc.) to generate spot signal intensities.

**Statistical and RPPA analysis**
The software SuperCurveGUI (http://bioinformatics.mdanderson.org/Software/supercurve/) was used to estimate the IC₅₀ values of the proteins in each dilution series (on a log₂ scale) using the nonparametric, monotone increasing B-spline model; a correction for spatial bias before model fitting; a quality control metric for each slide; and loading correction using median centering across antibodies as previously described (31). As a measure of pathway activation, we calculated PI3K/Akt and TSC/mTOR scores; the loading-corrected data were first log₂ transformed, then median-centered and normalized by SD for each protein across samples. The score was obtained by calculating the sum of protein level of positive components minus the sum of the negative components of the pathway. The components of the PI3K/Akt and TSC/mTOR scores are listed in Supplementary Table S2.

**SNP array**
Whole-genome SNP array profiling was performed in cell lines using the Illumina Human1M-Duo DNA Analysis BeadChip (Illumina, Inc.). Before analysis, SNP data were normalized to the regional baseline copy number to account for aneuploidy, with the regional baseline copy number being assigned a value of 2.

**Results**

**PI3K pathway alterations are common in head and neck cancer cells and tumors**
We designed an HNSCC Sequenom panel that incorporates 191 mutations in 45 genes found in squamous cell cancers, including many of those identified in recently published whole-exome sequencing studies of HNSCC tumors and 24 PIK3CA mutations (refs. 3, 4; Supplementary Table S1). We isolated gDNA from 64 validated HNSCC cell lines (29) as well as control cell lines with known mutations in several of the tested genes. We identified several mutations, including HRAS (2 of 61, 3%), PIK3CA (5 of 61, 8%), NOTCH1 (1 of 61, 2%), EPHA2 (2 of 61, 3%), EPHA3 (2 of 61, 3%), and MET (1 of 61, 2%). PIK3CA mutations were validated by PCR and included the known missense activating mutations H1047R, E542K, and E545K (Table 1). Two of these 5 HNSCC lines did not have PIK3CA amplification (TR146, UMSCC19) and the other 3 did not have SNP array data. An analysis of 279 HNSCC tumors from The Cancer Genome Atlas (TCGA) yielded results consistent with both our cell line data and results of other recently published studies (2, 5), demonstrating that PI3K/AKT/mTOR pathway alterations are common in these cancers: 36% of the tumors had PIK3CA gene alterations (44 tumors with mutations, 43 with amplifications, and 14 with both), 2% had PTEN alterations (5 mutations and 1 deletion), 4% had AKT1 alterations (n = 10), and 1% had mTOR alterations (n = 4; Supplementary Fig. S1).

We identified 2 cell lines with PTEN loss and 7 with PIK3CA amplification (≥5 copies) using the SNP array. Both cell lines with PTEN loss demonstrated a complete lack of PTEN protein expression by Western blotting, but the cell lines with PIK3CA amplification did not consistently demonstrate higher PI3K p110α protein expression than HNSCC cells without amplification (Fig. 1). PIK3CA protein levels are regulated by multiple processes, including expression of PIK3R1, which is required for p110 stability. To confirm the results of the SNP arrays, FISH was performed on 8 HNSCC cell lines (Supplementary Methods). The copy numbers determined by FISH were consistent with those determined by the SNP array (Supplementary Table S3 and Supplementary Fig. S2).

**HNSCC cells with PIK3CA mutations, but not amplification, are sensitive to pathway inhibitors**
We tested 18 of the 64 HNSCC cell lines with PIK3CA mutations, PTEN loss, low PTEN protein expression, PIK3CA gene amplification, or no known pathway aberrations for sensitivity to 1 PI3K inhibitor (GDC0941), 2 dual PI3K/mTOR inhibitors (GDC0980 and GSK1059615), 1 AKT inhibitor (GSK690693), and 1 mTOR inhibitor (AZD8055;Table 1). The cutoff for sensitivity was the maximum concentration in humans or animals when available or the concentration at which pathway inhibition was observed by Western blotting (Supplementary Table S4; refs. 32–36). The 1 PI3K inhibitor and the 2 dual PI3K/mTOR inhibitors show similar trends in sensitivity (P < 0.05). Sensitivity to the AKT and mTOR inhibitors showed distinct trends that did not correlate with any of the other agents tested (Supplementary Table S5).

To confirm that the pathway inhibitors function at the concentrations used, we tested their ability to inhibit the...
PI3K Pathway Inhibition and Resistance Mechanisms in HNSCC

pathway over a wide concentration range in HNSCC cell lines of various sensitivities. In Detroit562 cells (PIK3CA-mutant, sensitive), all inhibitors except AKT inhibitor GSK690693 decreased pAKT and pS6K levels at concentrations of 100 nmol/L and 4EBP1 level at 500 nmol/L (Fig. 2A). As previously demonstrated, GSK690693 did not result in a decrease in pAKT level despite AKT inhibition because of pathway feedback (32). We observed a decrease in the AKT substrate pGSK3β at 1 μmol/L, which is consistent with AKT inhibition. Similar results were demonstrated with intermediate and resistant HNSCC cell lines (Fig. 2A and B).

The 5 HNSCC cell lines with PIK3CA mutations were all sensitive to most of the inhibitors, whereas those with PIK3CA gene amplification were predominantly resistant (Table 1). Of the only 2 HNSCC cell lines with PTEN loss in existence, one (UMSCC4) was sensitive to 3 of the 5 inhibitors and the other (MDA886LN) was resistant to all 5 inhibitors. Likewise, the HNSCC cell lines with low PTEN protein expression were predominantly resistant. The control cell lines, which did not have any known PI3K/AKT/mTOR pathway aberrations, had mixed sensitivity.

To test these correlations in a larger set of HNSCC cells, we determined the IC50 of GDC0941 in all 64 HNSCC cell lines and analyzed them in relation to the mutational status and copy number of PI3K. As before, we observed that cell lines with a PIK3CA mutation were more likely to respond to PI3K inhibition than cell lines without PIK3CA mutation ($P = 6.35 \times 10^{-13}$; Fig. 3A) and those with amplification (copy number $\geq 4$) were less likely to respond ($P = 0.365$; Fig. 3B), although the latter difference failed to reach statistical significance even with a copy number cutoff of 3 or 5.

**PI3K pathway and TSC/mTOR scores do not correlate with response to PI3K inhibition**

We hypothesized that the basal state of the PI3K pathway would correlate with cell sensitivity to PI3K inhibition. To test this hypothesis, we examined the basal activation of the PI3K/AKT/mTOR pathway by RPPA using 2 proteomic scores—the PI3K/AKT score (37) and TSC/mTOR score—in 60 HNSCC cell lines (Fig. 3C; Supplementary Fig. S3). Both scores measure pathway activation, but there are no overlapping proteins; the PI3K/AKT score measures proximal pathway components and the TSC/mTOR score measures components distal to TSC1/2 (Supplementary Table S2). In addition, the TSC/mTOR score integrates information from the RAS/MAPK and LKB1/AMPK pathways. Nevertheless, neither score predicted sensitivity to GDC0941. Specifically, we observed no correlation between sensitivity and the PI3K/AKT score when a sensitivity cutoff of 650 or a copy number cutoff of 3 or 5.

### Table 1. IC50 values of PI3K pathway inhibitors for HNSCC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PI3K pathway alteration</th>
<th>GDC0941 (PI3K inhibitor)</th>
<th>GSK1059615 (PI3K/mTOR inhibitor)</th>
<th>GDC0980 (PI3K/mTOR inhibitor)</th>
<th>AZD8055 (mTOR inhibitor)</th>
<th>GSK690693 (AKT inhibitor)</th>
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<tbody>
<tr>
<td>Detroit 562</td>
<td>PI3K mutation (H1047R)</td>
<td>3.0</td>
<td>0.9</td>
<td>1.2</td>
<td>0.01</td>
<td>1.0</td>
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<tr>
<td>TR146</td>
<td>PI3K mutation (E545K)</td>
<td>2.0</td>
<td>1.3</td>
<td>2.3</td>
<td>0.85</td>
<td>11.2</td>
</tr>
<tr>
<td>HOSC1</td>
<td>PI3K mutation (E542K)</td>
<td>0.74</td>
<td>0.2</td>
<td>0.50</td>
<td>0.43</td>
<td>11.8</td>
</tr>
<tr>
<td>UM195</td>
<td>None known</td>
<td>0.8</td>
<td>0.8</td>
<td>0.50</td>
<td>0.51</td>
<td>1.94</td>
</tr>
<tr>
<td>Detroit 562</td>
<td>PI3K mutation (H1047R)</td>
<td>0.40</td>
<td>0.34</td>
<td>0.23</td>
<td>0.35</td>
<td>1.8</td>
</tr>
<tr>
<td>TR146</td>
<td>PI3K mutation (E545K)</td>
<td>0.46</td>
<td>0.01</td>
<td>0.01</td>
<td>0.27</td>
<td>4.0</td>
</tr>
<tr>
<td>HOSC1</td>
<td>PI3K mutation (E542K)</td>
<td>0.34</td>
<td>0.11</td>
<td>0.23</td>
<td>0.37</td>
<td>2.8</td>
</tr>
<tr>
<td>UM195</td>
<td>None known</td>
<td>0.55</td>
<td>0.18</td>
<td>0.63</td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>UM195</td>
<td>PI3K mutation (H1047R)</td>
<td>0.5</td>
<td>0.11</td>
<td>0.38</td>
<td>0.71</td>
<td>2.5</td>
</tr>
<tr>
<td>MD1866 LN</td>
<td>PTEN loss</td>
<td>12</td>
<td>3.7</td>
<td>1.8</td>
<td>5.3</td>
<td>8.8</td>
</tr>
<tr>
<td>UM195</td>
<td>PTEN loss</td>
<td>1.1</td>
<td>0.16</td>
<td>0.18</td>
<td>1.0</td>
<td>0.82</td>
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<tr>
<td>MDA1866 LN</td>
<td>Low PTEN expression</td>
<td>2.6</td>
<td>2.0</td>
<td>2.0</td>
<td>0.19</td>
<td>1.5</td>
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<tr>
<td>MDA1866 TU</td>
<td>Low PTEN expression</td>
<td>2.2</td>
<td>0.28</td>
<td>0.22</td>
<td>1.9</td>
<td>0.3</td>
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<tr>
<td>1483</td>
<td>PI3K amplification</td>
<td>7.2</td>
<td>2.0</td>
<td>8.0</td>
<td>8.0</td>
<td>&gt;20</td>
</tr>
<tr>
<td>FADU</td>
<td>PI3K amplification</td>
<td>1.0</td>
<td>0.33</td>
<td>0.19</td>
<td>2.7</td>
<td>6.4</td>
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<tr>
<td>UM195</td>
<td>PI3K amplification</td>
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<td>0.72</td>
<td>0.44</td>
<td>0.09</td>
<td>2.9</td>
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<tr>
<td>JHU022</td>
<td>PI3K amplification</td>
<td>15.2</td>
<td>3.0</td>
<td>12.0</td>
<td>0.89</td>
<td>3.55</td>
</tr>
<tr>
<td>UM195</td>
<td>PI3K amplification</td>
<td>2.0</td>
<td>0.7</td>
<td>1.7</td>
<td>4.0</td>
<td>3.25</td>
</tr>
</tbody>
</table>

NOTE: Values that fall below the cutoff for sensitivity (Supplementary Table S4) are highlighted in green, intermediate values are in grey, and values above the cutoff for resistance are in red.
mTOR score when a sensitivity cutoff of 650 nmol/L ($P = 0.89$) or 1,300 nmol/L ($P = 0.12$) was used or when the top third and bottom third were compared ($P = 0.37$).

We also analyzed the sensitivities of the 18 HNSCC cell lines to the 5 pathway inhibitors in relation to the expression of individual pathway components measured by...
We measured the expression levels of several pathway components, including pAKT, pS6, 4EBP1, and pERK, in these cells. Of these 4 markers, pS6 was the best predictor of response, with a consistent trend for all inhibitors, but the correlation reached significance ($P < 0.05$) only with GSK690693 (Supplementary Table S6). Likewise, analysis of the 60 HNSCC cell lines in the RPPA dataset did not show a correlation between basal expression of pS6 (S235/236) or pS6 (S240/242) and sensitivity to GDC0941 (Supplementary Table S7).
Figure 3. PI3K mutation—but not amplification, PI3K score, or mTOR score—correlates with sensitivity to GDC0941. Sixty HNSCC cell lines were tested for sensitivity to GDC0941 using an MTT assay. Basal expression levels of 195 proteins and phosphoproteins were measured using RPPA, and the expression levels of 16 proteins were used to define PI3K and mTOR scores (Supplementary Table S2). Copy number was defined using SNP arrays. A, dot plot comparing GDC0941 IC50 values in HNSCC cells with and without a PIK3CA mutation. B, dot plot comparing GDC0941 IC50 values in HNSCC cells with and without PIK3CA amplification. C, heatmap of expression, IC50, copy number, PI3K/AKT score, and TSC/mTOR score sorted by GDC0941 sensitivity. D, dot plots comparing PI3K/AKT and TSC/mTOR scores to PI3K alteration (PTEN loss and/or PIK3CA mutant and/or PIK3CA amplified), amplification, or mutation in HNSCC tumors from the TCGA.
An analysis of 160 proteins from the RPPA data revealed several candidate biomarkers whose expression correlated with sensitivity to GDC0941. These data were analyzed using the IC₅₀ values as continuous variables, by comparing the most sensitive third to the least sensitive third and by using cutoffs of 650 and 1,300 nmol/L. The proteins whose expression correlated with sensitivity with a P < 0.05 in at least 3 analyses were VEGFR2, pCRAF, and PCNA (Supplementary Table S7).

Genetic alterations in the PI3K pathway do not lead to pathway activation

We expected that HNSCC cell lines with PIK3CA mutations, PTEN loss, or PIK3CA amplification would have basal activation of the PI3K/AKT pathway. However, Western blotting demonstrated that there was no consistent increase in pAKT, p70S6, pE-BP1, or pPDK1 in those cell lines (Fig. 1). Neither PIK3CA amplification nor PIK3CA mutation correlated with the PI3K/AKT score (P = 0.21 and 0.31). Likewise, PIK3CA mutation or amplification did not correlate with the TSC/mTOR score (P = 0.49, P = 0.58). Notably, the 2 HNSCC cell lines with PTEN loss did have high levels of phosphorylated S6 and AKT (Fig. 1).

Similarly, PIK3CA amplification did not correlate with an increase in the scores in 200 HNSCC tumors from the TCGA (Fig. 3D). However, tumors with PIK3CA mutation or amplification did not correlate with the TSC/mTOR score (P = 0.039, but not TSC/mTOR score, than those without PIK3CA mutation. There was no difference in the PI3K/AKT or TSC/mTOR scores between 8 tumors on which we have RPPA data and that have both PIK3CA amplification and mutation and HNSCC tumors without these genetic aberrations (P = 0.226 and 0.556).

PI3K inhibitors induce cell-cycle arrest in HNSCC cell lines with PIK3CA mutations

To further characterize the biologic effects of PI3K inhibition, HNSCC cells were treated with GDC0941 for 72 hours. In the sensitive Detroit562 and TR146 cells, but not in the resistant 1483 cells, GDC0941 caused cell-cycle arrest in G₁ with a reduction of the number of cells in S-phase (Supplementary Fig. S4). No significant apoptosis, mitotic catastrophe, or senescence was observed in any of the cell lines (all <5%, Supplementary Methods).

Activation of the MEK/ERK pathway induces resistance to PI3K inhibitors

Following incubation with GDC0941, pERK levels increased in the resistant cell lines JHU022 and 1483, whereas remaining unchanged in the sensitive cell line Detroit562 and the intermediate cell line OSC19 (Fig. 4A). Likewise, in the 2 HNSCC cell lines with PTEN loss, pERK was inhibited in the cell line with intermediate sensitivity but not in the resistant cell line (Fig. 4B). Treatment of both sensitive and resistant HNSCC lines with a combination of MEK and PI3K inhibitors led to additive or synergistic effects in all cell lines except Detroit562 (Fig. 4C). The combination also led to enhanced cell-cycle arrest (Supplementary Fig. S5) in TR146 (sensitive) and 1483 (resistant) compared with untreated or GDC0941 alone. In Detroit562 (sensitive), GDC0941 alone and combination showed almost same degree of cell-cycle arrest. However, we did not see any significant cell-cycle arrest in MDA886LN. Because the combination of MEK and PI3K inhibitors was particularly effective in a murine model of Kras-mutant adenocarcinoma (38), we tested it in the 2 cell lines with HRAS mutations and found that the drug combination was synergistic, with CIs of 0.015 ± 0.27 and 0.096 ± 0.12 for UMSCC17A and HN31 cells, respectively (Fig. 4D). Target inhibition was confirmed by Western blot analysis for both drugs (Fig. 4E).

Activation of multiple receptor tyrosine kinases following PI3K inhibition

To investigate potential pathways of resistance in HNSCC following PI3K inhibition, we incubated 2 sensitive and 2 resistant HNSCC cell lines with GDC0941 and measured the levels of 39 phosphoproteins. We discovered that the phosphorylation of EGFR, RET, FGFR3, and M-CSFR was increased in resistant lines but not in sensitive ones following incubation with GDC0941 (Fig. 5A). Phosphorylation of IGFR1 was increased in both sensitive and resistant cells although to a lesser extent in the sensitive lines.

Inhibition of activated receptor tyrosine kinase pathways partially reverses PI3K inhibitor resistance

To explore the functional significance of the EGFR, IGFR1, RET, and FGFR3 activation in resistant cell lines, we combined GDC0941 with the EGFR inhibitor erlotinib, the IGFR1 inhibitor OSI906, the RET inhibitor cabozantinib, or the FGFR inhibitor dovitinib (Fig. 5B). Only the combination of erlotinib and GDC0941 was synergistic in both resistant cell lines, with CIs of <0.01 and 0.096 ± 0.11.

Discussion

In an effort to study mutation-directed targeted therapy for HNSCC, we identified several missense mutations in HNSCC cell lines that mirror those found in HNSCC patient samples; the mutations included HRAS, PIK3CA, NOTCH1, EPHA2, EPHA3, and MET (3, 4). Like patient tumors, our cell lines had other alterations in the PI3K/AKT/mTOR pathway, including PIK3CA amplification and PTEN loss. HNSCC cell lines with PIK3CA mutations were universally sensitive to inhibitors of PI3K/AKT/mTOR, or both. Surprisingly, PIK3CA amplification, PTEN loss, and basal pathway activity did not predict response. There was an unexpected, statistically nonsignificant correlation between PIK3CA amplification and resistance. All the PI3K, mTOR, and dual inhibitors tested were equally efficacious in the 18 HNSCC cell lines tested; sensitivity to the PI3K and dual inhibitors correlated with each other.
Figure 4. The MEK/ERK pathway is engaged and increases cell survival after cells are treated with GDC0941. A, Detroit562, JHU022, 1483, and OSC19 cells were treated with GDC0941 for 3 hours, and Western blot analysis was performed using antibodies against various signaling molecules as indicated. B, Western blot analysis of 2 HNSCC cell lines with PTEN loss (UMSCC4 and MDA886LN) following incubation with GDC0941 for 3 hours. C and D, HNSCC cells without (C) or with (D) HRAS mutations were incubated with GDC0941, MEK 162, or both (GDC0941:MEK162 = 1.5:1) for 72 hours. Cell viability was estimated with an MTT assay. CIs are indicated. E, Western blot analysis confirmed target inhibition in the 1483 cell line following treatment for 3 hours with the indicated drugs.
Figure 5. Treatment with GDC0941 activates EGFR, IGF1R, FGFR, RET, and M-CSFR in resistant cell lines. A, 2 sensitive and 2 resistant HNSCC cell lines were treated with 500 nmol/L GDC0941 or vehicle alone for 6 hours and then processed for PhosphoScan analysis as described in Materials and Methods. Densitometric analysis was done to measure change in protein expression using ImageJ software. B, resistant cells were incubated with GDC0941 alone or in combination (1:1) with erlotinib, OSI906, cabozantinib, or dovitinib for 72 hours. i, inhibitor. Cell viability was estimated by an MTT assay. CIs are indicated.
but not with sensitivity to the mTOR or AKT inhibitor. More cell lines were resistant to the single AKT inhibitor tested than to the other inhibitors tested, but this may reflect the difficulty in choosing a cutoff for this drug in the absence of clinical pharmacokinetic data and feedback pathway reactivation of AKT. We found that the phosphorylation levels of ERK, EGFR, IGF1R, RET, FGFR3, and M-CSFR were increased by PI3K inhibition in the resistant lines. This reactivation had biologic significance as dual inhibition of MEK or EGFR with PI3K led to synergy. In particular, the 2 HNSCC cell lines with HRAS mutations had the most marked synergy of PI3K and MEK inhibition.

Ours is not the first study to demonstrate a correlation between PI3K mutation and response to pathway inhibitors. In HNSCC, this finding is particularly intriguing, as human papilloma virus–positive tumors have a higher rate of PIK3CA mutation (5). However, early clinical data demonstrate that even mutant tumors are neither profoundly nor universally sensitive to pathway inhibitors (20). Our in vitro data support this clinical finding in that the sensitive cell lines underwent cell-cycle arrest but not apoptosis, demonstrating that resistance pathways need to be further defined.

Several mechanisms of resistance to these pathway inhibitors have been elucidated. The best-studied interaction is between PI3K/AKT/mTOR and receptor tyrosine kinases (RTKs); this interaction can subsequently activate the PI3K/AKT/mTOR pathway. As expected with this model, AKT inhibition leads to the increased expression of multiple RTKs, and the combined administration of ERK and PI3K inhibitors leads to higher rates of cancer cell death (39). Likewise, we found that the addition of an MEK inhibitor to a PI3K inhibitor enhanced efficacy in both sensitive and resistant cell lines. That this was particularly the case in HRAS-mutant HNSCC cell lines is consistent with the findings in genetically engineered murine models of lung cancer with inducible expression of KRAS (G12D) or PIK3CA (H1047R; ref. 38). One potential mechanism leading to ERK activation is the loss of inhibition of the transcription factor FOXO3 following AKT inhibition, leading to increased expression of multiple RTKs (40). Enhanced signaling via HER2 following PI3K inhibition has been reported to lead to ERK activation (39). Similarly, we also found that PI3K inhibition in resistant HNSCC cells led to the activation of multiple RTKs and that EGFR and PI3K inhibitors were synergistic in HNSCC cells consistent with prior work in 2 HNSCC cell lines (41). This cross talk may be bidirectional, as MEK inhibition leads to PI3K/AKT activation via the loss of HER3 inhibition (42).

Additional mechanisms of resistance include mTOR inhibition leading to the induction of IRS-1 and subsequent AKT activation in cancer cells and patient tumors (45). IGF1R inhibition sensitizes cells to mTOR inhibition (45). In a transgenic breast cancer model, both PIK3CA-independent (Myc amplification) and -dependent (Met amplification) resistance pathways were identified (44). In addition, accumulation of nuclear β-catenin can subvert the proapoptotic signal of nuclear FOXO3a, induced by PI3K inhibition, into a prometastatic signal (45). In breast cancer cells, PI3K inhibition leads to DNA damage and BRCA1/2 downregulation; PI3K inhibition or knockdown sensitizes cells to PARP inhibition (46).

Although the drugs we used potently inhibited the PI3K/AKT/mTOR pathway in both sensitive and resistant cells, distal pathway inhibition (pS6 and p4EBP1) occurred at lower drug doses and was more complete in sensitive cell lines. Distal pathway components may be reactivated by ERK, RTKs, or AKT-independent pathways downstream of PI3P, such as JNK (47) and STAT3 (48). Our future studies will include RPPA as well as siRNA screens to identify targetable pathways that enhance the efficacy of PI3K/AKT/mTOR pathway inhibitors.

Multiple biomarkers for predicting drug sensitivity beyond PI3K mutations have been proposed, including PTEN mutations, PTEN loss, and AKT phosphorylation (21, 27); HER2 amplification (23, 24); 4EBP1 expression (25); NOTCH1 mutation (19); and pSTAT3, EGFR, and c-Kit expression (18). In a screen of the NCI-60 human tumor cell line collection, the expression of several genes, including Ogt and DDN, correlated with response to GDC0941; knockdown of either gene enhanced sensitivity to this agent (49). The presence of KRAS mutation, BRAF mutation, or EGFR amplification correlates with resistance to PI3K/AKT/mTOR pathway inhibition (20, 22). pAKT levels have been clinically validated as a biomarker of progression-free survival in 17 patients with pancreatic neuroendocrine tumors treated with everolimus (21). We identified 25 proteins whose expression correlates with response by at least one method of analysis and 3 that were consistently correlated by at least three methods. Our future studies will involve testing these biomarkers in an independent set and in clinical samples from patients with HNSCC treated with pathway inhibitors. Unlike breast cancer cell lines, HNSCC cell lines did not show a correlation between PTEN protein expression and sensitivity to PI3K inhibitors (27).

We did not find a correlation between PIK3CA mutation or amplification and protein markers of pathway activation. Likewise, pAKT, pS6, and p4EBP1 were not elevated in luminal/endoplasmic reticulum (ER)+ breast cancer with PIK3CA mutation (50). In an independent set of 77 breast tumors, there was no association between PIK3CA mutation and the phosphorylation of GSK3, AKT, S6, or AKT (27). In contrast, mRNA and protein signatures of PI3K pathway activations were enriched in basal-like breast cancer where PIK3CA mutations are uncommon. The activation of the PI3K/Akt/mTOR pathway in the basal subtype may be due to loss of PTEN and INPP4B and/or amplification of PIK3CA rather than mutation of PIK3CA (50). For a more direct comparison, we also used a protein signature score identical to that used in breast tumors in the TCGA (50), which includes components of both the PI3K and mTOR scores presented above, and did
not find a correlation with PI3KCA mutation or amplification in HNSCC tumors from the TCGA (data not shown).

Use of hotspot mutational analysis allows for screening of many samples at specific relevant bases across many patient samples; however, it can miss also relevant but rarer mutations. In a recent study that used whole-genome sequencing of 151 HNSCC tumors, additional PIK3CA mutations were detected. These mutations (R115L, G363A, C971R, R975S) were each found in less than 1% of patients with HNSCC and were not included in our study (5). Another limitation of our study was that we did not conduct animal experiments, but the efficacy of a dual mTOR/PI3K inhibitor in HNSCC PDX models has recently been demonstrated by 2 independent groups (5, 19).

Despite the frequent activation and importance of the PI3K/AKT/mTOR pathway, inhibition of this pathway has had variable efficacy in vitro, in vivo, and in clinical trials (12–18). Identification of biomarkers that predict sensitivity to these inhibitors could result in the first biomarker-driven targeted therapy for HNSCC. Furthermore, the development of rational drug combinations based on a comprehensive understanding of HNSCC cell resistance mechanisms could significantly improve outcomes for patients with HNSCC. In this study, we have identified potential biomarkers and combination therapies that will be tested in future clinical studies.

Disclosure of Potential Conflicts of Interest
G.B. Mills received a commercial research grant from AstraZeneca, Han AllBio, and GSK; has ownership interest (including patents) in Catena Pharmaceuticals, PTV Ventures, and Spindle Top Ventures; and is a consultant/advisory board member for AstraZeneca, Bind, Critical Outcome Technologies, Han AllBio Korea, Nuevolution, and Symphogen. K. Stemke-Hale has ownership interest (including patents) in GlaxoSmithKline and Exelixis. J.V. Heymach is a consultant/advisory board member for AstraZeneca, GlaxoSmithKline, Genentech, Boehringer Ingelheim, Exelixis, and Synta. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: T. Mazumdar, J.N. Myers, B.S. Glisson, F.M. Johnson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Mazumdar, L.A. Byers, Y.-H. Fan, K. Stemke-Hale, F.M. Johnson
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): T. Mazumdar, L.A. Byers, P.K.S. Ng, G.B. Mills, S. Peng, L. Diao, K. Stemke-Hale, B.S. Glisson, F.M. Johnson
Writing, review, and/or revision of the manuscript: T. Mazumdar, L.A. Byers, G.B. Mills, K. Stemke-Hale, J.V. Heymach, J.N. Myers, B.S. Glisson, F.M. Johnson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Mazumdar, G.B. Mills, S. Peng, F.M. Johnson
Study supervision: F.M. Johnson

Acknowledgments
The authors thank Arthur Gelmis, Kathryn Hale, and the Department of Scientific Publications for editorial assistance with the manuscript. They also thank Brenda Robinson for administrative assistance.

Grant Support
This work was supported by the National Cancer Institute MD Anderson Cancer Center Head & Neck SPORE P50CA097007 (to J.N. Myers, B.S. Glisson, and F.M. Johnson); RPPA, mutational analysis, FISH, and DNA sequencing were supported by the National Cancer Institute Cancer Center Support Grant P30CA16672 (to The University of Texas MD Anderson Cancer Center).

Received January 6, 2014; revised August 8, 2014; accepted August 22, 2014; published OnlineFirst September 5, 2014.

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Comprehensive predictive biomarker analysis for MEK inhibitor


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A Comprehensive Evaluation of Biomarkers Predictive of Response to PI3K Inhibitors and of Resistance Mechanisms in Head and Neck Squamous Cell Carcinoma

Tuhina Mazumdar, Lauren A. Byers, Patrick Kwok Shing Ng, et al.


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