Phosphoinositide 3-Kinase (PI3K) Pathway Alterations Are Associated with Histologic Subtypes and Are Predictive of Sensitivity to PI3K Inhibitors in Lung Cancer Preclinical Models

Jill M. Spoerke, Carol O'Brien, Ling Huw, Hartmut Koeppen, Jane Fridlyand, Rainer K. Brachmann, Peter M. Haverty, Ajay Pandita, Sankar Mohan, Deepak Sampath, Lori S. Friedman, Leanne Ross, Garret M. Hampton, Lukas C. Amler, David S. Shames, and Mark R. Lackner

Abstract

Purpose: Class 1 phosphatidylinositol 3-kinase (PI3K) plays a major role in cell proliferation and survival in a wide variety of human cancers. Here, we investigated biomarker strategies for PI3K pathway inhibitors in non–small-cell lung cancer (NSCLC).

Experimental Design: Molecular profiling for candidate PI3K predictive biomarkers was conducted on a collection of NSCLC tumor samples. Assays included comparative genomic hybridization, reverse transcription polymerase chain reaction gene expression, mutation detection for PIK3CA and other oncogenes, PTEN immunohistochemistry, and FISH for PIK3CA copy number. In addition, a panel of NSCLC cell lines characterized for alterations in the PI3K pathway was screened with PI3K and dual PI3K/mTOR inhibitors to assess the preclinical predictive value of candidate biomarkers.

Results: PIK3CA amplification was detected in 37% of squamous tumors and 5% of adenocarcinomas, whereas PIK3CA mutations were found in 9% of squamous and 0% of adenocarcinomas. Total loss of PTEN immunostaining was found in 21% of squamous tumors and 4% of adenocarcinomas. Cell lines harboring pathway alterations (receptor tyrosine kinase activation, PI3K mutation or amplification, and PTEN loss) were exquisitely sensitive to the PI3K inhibitor GDC-0941. A dual PI3K/mTOR inhibitor had broader activity across the cell line panel and in tumor xenografts. The combination of GDC-0941 with paclitaxel, erlotinib, or a mitogen-activated protein–extracellular signal-regulated kinase inhibitor had greater effects on cell viability than PI3K inhibition alone.

Conclusions: Candidate biomarkers for PI3K inhibitors have predictive value in preclinical models and show histology-specific alterations in primary tumors, suggesting that distinct biomarker strategies may be required in squamous compared with nonsquamous NSCLC patient populations.

Introduction

Aberrant activation of the phosphoinositide 3-kinase (PI3K) signaling pathway has been widely implicated in the pathogenesis of many cancers (1). Constitutive activation of this pathway leads to unregulated proliferation, evasion of apoptosis, and altered metabolism via the Warburg effect (2, 3). PI3K-dependent activity is frequently elevated because of amplification (4) or gain-of-function mutations of the PIK3CA gene (5), as well as loss of function of the PTEN tumor suppressor gene, a critical negative regulator of the pathway (6). In addition, increased pathway activity has been associated with resistance to anticancer therapies (7, 8).

The significance of PI3K signaling for tumor survival and proliferation has prompted investigation of this pathway as an anticancer drug target (2), leading to the development of candidate therapeutics designed to inhibit the activity of pathway components such as PI3K, mTOR, and AKT. GDC-0941 is a highly specific class 1 PI3K inhibitor with a favorable tolerability and pharmacokinetic profile (9), which is currently in clinical development. GDC-0941...

**Translational Relevance**

The class 1 phosphatidylinositol 3-kinase (PI3K) plays a major role in a wide variety of human cancers, and the PI3K signaling pathway is, therefore, an attractive target for therapeutic intervention. Predictive biomarkers help to identify likely responders (or nonresponders) to a particular therapy, improving the probability of successful drug development and maximizing benefit for patients. In this study, we evaluated candidate predictive biomarkers of sensitivity to selective PI3K/mTOR pathway inhibitors in lung cancer [non–small-cell lung cancer (NSCLC)]. These candidate biomarkers were associated with distinct histologic subtypes of NSCLC, suggesting that different predictive biomarker strategies may be necessary in squamous and nonsquamous patient populations. Sensitivity to a selective PI3K inhibitor was greatest in cell lines with RTK activation, PTEN loss, or PIK3CA mutation or amplification, whereas a dual PI3K/mTOR inhibitor had broader activity. These findings have implications for clinical predictive diagnostic development for this class of inhibitors.

NSCLCs comprise a highly heterogeneous group of diseases including multiple histologic subtypes. The most common forms of NSCLC in the USA and Europe are adenocarcinoma (50%) and squamous cell carcinoma (30%) with a variety of less common subtypes accounting for the remainder. Until recently, histologic subtype was not a factor in chemotherapy treatment decision making. However, histology is becoming an increasingly important factor both in terms of safety and efficacy. For example, bevacizumab (Avastin; Genentech) is contraindicated in patients with squamous cell carcinomas of the lung because of an increased risk of hemoptysis (17). Pemetrexed (Alimta; Eli Lilly) is also not prescribed for patients with the squamous subtype because of lack of efficacy, which may be related to higher expression of the drug target thymidylate synthase in squamous tumors making effective target blockade challenging (18). As a result, the standard of care regimens for front-line therapy are different for squamous and nonsquamous NSCLC. Thus, for the foreseeable future, it will be necessary to establish distinct clinical development plans for new molecular entities for these 2 subtypes of NSCLC.

Here, we present a detailed study of alterations relevant to PI3K and mTOR signaling in a collection of NSCLC tumor samples to assess the prevalence and overlap of these alterations and their relationship to histologic subtypes. The genes studied include core pathway components such as PIK3CA and PTEN, as well as FBXW7, which encodes a ubiquitin ligase that degrades mTOR (19), and GOLPH3, which is amplified in NSCLC and associated with mTOR inhibitor sensitivity (20). A detailed understanding of PI3K pathway alterations in NSCLC will enable a more precise delineation of candidate target populations within histologic subtypes, facilitating clinical trial design, and validation of predictive biomarkers.

**Materials and Methods**

**Tumor samples and cell lines**

A panel of NSCLC tumor samples was selected for this study from the Genentech tumor bank. Squamous or adenocarcinoma histology was confirmed by pathologic examination. This panel consisted of a total of 172 frozen and formalin-fixed paraffin-embedded (FFPE) lung tumor samples with a tumor content of at least 50%. It was not possible to run all assays on all samples, either because of samples being exhausted by one methodology, or incompatibility of assay format with sample fixation, as frozen samples are less amenable to the immunohistochemistry (IHC) and FISH assays. A complete list of the FFPE samples, along with associated clinicopathologic data and assay results, is shown in Supplementary Table S1.

Cell lines used in this study were obtained from the American Type Culture Collection, the Deutsche Sammlung von Mikroorganis men und Zellkulturen GmbH (DSMZ), and from A.F. Gazdar and J.D. Minna at the University of Texas Southwestern Medical Center (Dallas, TX). Cell lines were archived at early passage in the Genentech cell bank and authenticated using a multiplex short tandem repeat assay. All cell lines were maintained in RPMI 1640 medium...
supplemented with 10% fetal bovine serum, nonessential amino acids, and 2 mmol/L L-glutamine.

**Expression analysis in tumor tissue**

Gene expression analysis was carried out on RNA extracted from FFPE NSCLC tumor samples using the High Pure FFPE RNA Micro Kit (Roche) after deparaffinization with Envirome (xylene substitute), as described by Walter and colleagues (21). The 96-gene quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay consisted of epithelial–mesenchymal markers, HER family signaling factors, PI3K pathway signaling components, and 2 reference genes, and has been described previously (21). Cycle threshold (Ct) values were converted to relative expression as follows: relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta Ct$ is the mean of the target gene minus the mean of the reference genes. Hierarchical clustering of differentially expressed genes was carried out on median-centered data with the complete linkage method using Cluster v3.0 (22) and subsequently visualized using Freeview (23). RNA from frozen NSCLC tumors was run on the Human Genome U133 Plus 2.0 Array (Affymetrix) as previously described (24). PIK3CA expression was evaluated with probe set 204369_at, and compared with array comparative genomic hybridization (CGH) data (described below) from the same samples.

**Expression analysis in cell lines**

To identify genes that showed expression changes upon PI3K inhibition, cell lines H1975, H2170, H226, H441, LFXL529, and SW1573 were plated in T25 flasks in duplicate and treated with 1 $\mu$mol/L GDC-0941, 1 $\mu$mol/L GDC-0980, or dimethyl sulfoxide (DMSO; vehicle control) for 6 hours. RNA was extracted using the RNAeasy kit (Qiagen). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was preamplified with TaqMan PreAmplication Master Mix and 48 TaqMan gene expression assays (Applied Biosystems), and qRT-PCR was conducted on Fluidigm 48.48 Dynamic Arrays using the Biomark HD system (Fluidigm Corp.), as described in the Supplementary Materials and Methods.

**Mutation analysis**

Mutation analyses were carried out on genomic DNA extracted from FFPE tumors using the QIAaamp FFPE kit (Qiagen) after deparaffinization with Envirome, as well as genomic DNA extracted from cell lines using the QIAaamp kit (Qiagen). Mutations in PIK3CA, EGFR, KRAS, BRAF, and NRAS were detected using qPCR as described in the Supplementary Materials and Methods.

**Copy number and comparative genomic hybridization array analysis**

Array CGH studies were conducted as previously described (25) on frozen tumor samples. Raw log$_2$ (tumor/normal) values were corrected for GC bias according to the methods of Diskin and colleagues (26).

Corrected log$_2$ ratio values were segmented by circular binary segmentation (27) using an alpha of 0.001. CGH array data for specific genes of interest (FBXW7, INPP4B, LKB1, PTEN, EGFR, KRAS, TSC2, MET, PDK1, GOLPH3, PIK3CA, and AKT2) were analyzed using the Genomic Identification of Somatic Targets in Cancer (GISTIC) method (28). This assessed both frequency and magnitude of copy number variations for the chromosome region encompassing a gene of interest, and determined a significance threshold for any observed aberration. This enabled the statistical significance of copy number variation to be evaluated. NSCLC frozen tumor samples with both PIK3CA copy number data (array CGH) and mRNA expression data (Affymetrix array) were plotted together to confirm their correlation. Copy number data has been deposited in the GEO database under accession numbers GSE20393 and GSE39793.

Frozen tumor and cell line DNA was subjected to copy number analysis using TaqMan Copy Number Assays (Applied Biosystems) according to the manufacturer’s protocol, and described in more detail in the Supplementary Materials and Methods.

**FISH analysis**

FISH analysis was conducted on cell line and FFPE tumor samples as described previously (29, 30). A bacterial artificial chromosome contig comprising 2 overlapping clones, 245C23 and 355N16, covering the entire PIK3CA locus and adjoining areas (based on the University of California, Santa Cruz [UCSC] Genome Browser March 2006 assembly) was used as a probe for the FISH experiments. A commercially available probe for CEP3 (PathVysion; Vysis/Abbott Laboratories) was also used to enumerate the copies of chromosome 3 in the FISH experiments.

**PTEN immunohistochemistry**

PTEN IHC was conducted on FFPE tumor samples as previously described (31). The PTEN antibody was obtained from Cell Signaling Technologies (clone 138G6s), and the assay was run on the Discovery platform (Ventana). PTEN staining was scored in a semiquantitative fashion using an H-score method to account for heterogeneity of expression. The percentage of tumor cells present at each of 4 staining intensities was scored, and the H-score was calculated as follows:

$$H = (3 \times \% \text{ of strongly stained cells}) + (2 \times \% \text{ of moderately stained cells}) + (1 \times \% \text{ of weakly stained cells}) + (0 \times \% \text{ of cells without staining}).$$

This gave a score ranging from total absence of PTEN in the tumor compartment (H-score 0) to PTEN expression in tumor cells equivalent to surrounding normal and stromal cells (H-score 300).

**Western blot analysis**

Baseline and dose–response Western blot analyses were conducted as described previously (16). For PI3K inhibitor dose–response blots, cells were plated in 6-well plates and exposed 24 hours later to either GDC-0941 or GDC-0980.
Inhibitors and cell viability experiments

The PI3K inhibitors GDC-0941 (pan-PI3K) and GDC-0980 (dual PI3K/mTOR), and a MEK1/2 inhibitor used in this study were synthesized and provided by the Genentech Medicinal Chemistry group (9, 32, 33). Cell viability was assessed with the Cell Titer Glo ATP luminescence assay (Promega) using published protocols (16, 31). Cells were exposed to inhibitors for a total of 72 hours and cell viability was measured at the time of dosing and after 72 hours, to determine whether the inhibitory effect was because of growth inhibition or cell death. No growth (0%) on the dose–response curves represents the number of cells present before exposure to the inhibitors, and therefore viability values below that point are indicative of a cytotoxic effect. In vitro combination studies were conducted as described previously (25).

In vivo xenografts

NSCLC lines A549, H460, H520, HCC827, and H1975 were evaluated for in vivo efficacy as xenografts grown in immunocompromised mice. All lines were implanted in 6- to 8-week-old female athymic nude mice from Harlan Laboratories (Indianapolis, IN). Matrigel was used for implantation of A549 xenograft studies. After implantation, tumors were monitored until they reached a mean volume of 180 to 350 mm³ and mice were assigned to groups of 10 animals each before initiation of dosing. GDC-0941 and GDC-0980 were obtained as a solution dissolved in 0.5% methylcellulose/0.2% Tween-80 as the vehicle and dosed orally and daily at the concentrations indicated. Additional findings (39), we observed recurrent genomic alterations, including gains at 5p, 6p, 12p, and 14q and losses at 5q, 9p, and 10q (Supplementary Fig. S1). Supervised analysis of copy number alterations showed a high frequency of PI3K/mTOR pathway alterations, with distinct patterns between histologic subtypes (Fig. 2A). Specifically, loss of LKB1 and gain of EGFR were associated with adenocarcinoma samples, whereas AKT2 and PIK3CA gains and PTEN loss were associated with squamous histology.

To evaluate gene amplification across the chromosome 3 region encompassing the PIK3CA locus, GISTIC analysis (28) was conducted using data from the frozen NSCLC tumor samples. This analysis confirmed focal copy number gains at the PIK3CA locus in squamous tumors, but not in adenocarcinomas (Supplementary Fig. S2A). PIK3CA copy number in NSCLC tumors correlated with gene expression determined by microarray analysis (R = 0.90, Supplementary Fig. S2B). CGH plots from individual squamous NSCLC tumors showed that the amplicon is centered at the PIK3CA locus (Supplementary Fig. S3A). Copy number analysis of PIK3CA was also carried out by qPCR on an independent set of 93 frozen tumor samples and showed a similar pattern of increased copy number in squamous tumors (Supplementary Fig. S3B). Further investigation of PIK3CA copy number gains was carried out by FISH analysis. The assay was validated initially in the Calu3 squamous NSCLC cell line because this cell line is known from CGH array data to have increased PIK3CA copy number (Sanger COSMIC database). Amplification of PIK3CA relative to the centromeric probe CEP3 was observed in Calu3 cells (Supplementary Fig. S4). The assay was then applied to 41 adenocarcinoma and 43 squamous FFPE tumor samples; chromosomal amplification, defined by a PIK3CA/CEP3 ratio greater than or equal to 2.0, was observed in 37% of squamous but only 5% of adenocarcinoma samples, suggesting a significant association with squamous histology (P < 0.002, Fig. 2B and C). In 12 cases, matched primary and metastatic tumor samples were analyzed for PIK3CA copy

for 6 hours. Primary antibodies were used as previously described (16).

Results

PI3K pathway alterations in NSCLC tumor samples

To assess biomarker prevalence across NSCLC histologic subtypes, we conducted gene expression analysis on RNA from a set of 51 FFPE NSCLC tumor samples using Fluidigm Dynamic Arrays. Details of the gene expression assay are described in a separate manuscript (21). Tumors were defined as squamous cell carcinomas or adenocarcinomas by pathologic examination (Fig. 1, Supplementary Table S1). Mutation detection assays for common somatic alterations in EGFR, KRAS, BRAF, NRAS, and PIK3CA were carried out in parallel using a multiplex genotyping assay. Unsupervised analysis of the gene expression data revealed that the NSCLC tumors clustered into 2 separate groups. Cluster I showed high expression of markers associated with adenocarcinoma of the lung such as such as KRT-7 and NXX2-1 (TTF-1), as well as markers of an epithelial-like phenotype such as CLDN7 (21, 34, 35). Cluster 2 showed high expression of markers associated with a squamous cell carcinoma histology such as TP63 (36) and KRT6a, as well as markers of a mesenchymal-like phenotype such as TWIST1 and SNAI2 (Fig. 1). The majority of EGFR and KRAS mutations occurred in the epithelial-like adenocarcinomas, consistent with previous reports that these alterations are associated with adenocarcinoma (37). Several of the samples that were histologically squamous and harbored KRAS mutations clustered with the adenocarcinomas, which may reflect challenges inherent in pathologically distinguishing poorly differentiated adeno-squamous cancers; this is consistent with other reports that comprehensive profiling with multiple histology markers may be a more sensitive means of determining histologic subtype (38). Only 2 PIK3CA mutations were detected, and both were present in squamous cell carcinomas. As expected, mutations in NRAS and BRAF were not detected as they are rare in NSCLC.

To examine copy number alterations relevant to PI3K signaling, a panel of 79 frozen NSCLC tumor samples was analyzed by array CGH to determine genomic gains and losses for a series of candidate predictive biomarkers relevant to PI3K/mTOR pathway inhibitors. Of the tumor samples studied, 52 exhibited squamous histology and 27 had adenocarcinoma histology. Consistent with previous findings (39), we observed recurrent genomic alterations, including gains at 5p, 6p, 12p, and 14q and losses at 5q, 9p, and 10q (Supplementary Fig. S1). Supervised analysis of copy number alterations showed a high frequency of PI3K/mTOR pathway alterations, with distinct patterns between histologic subtypes (Fig. 2A). Specifically, loss of LKB1 and gain of EGFR were associated with adenocarcinoma samples, whereas AKT2 and PIK3CA gains and PTEN loss were associated with squamous histology.
number using the FISH assay (Fig. 2D). Amplification status differed between primary and metastatic samples in 5 of the cases: 2 patient samples were amplified (PIK3CA/CEP3 ratio ≥2.0) in the metastatic but not primary sample, whereas 3 were amplified in the primary but not the metastatic sample.

We also determined PTEN protein levels in 56 adenocarcinoma and 43 squamous cell NSCLC tumor samples by IHC, with intermediate levels of loss recorded as an H-score. Representative IHC staining is shown in Fig. 3A. The levels of PTEN protein in the panel of tumor samples were plotted in Fig. 3B, according to tumor histology. A continuum of PTEN loss was observed in NSCLC tumor samples with both squamous cell and adenocarcinoma histologies. The majority of tumors showed evidence of some loss of PTEN protein in neoplastic cells relative to stromal and normal tumor elements. The distribution of PTEN loss was significantly different between squamous cell and adenocarcinoma subtypes (P < 0.04, Mann–Whitney test), and total loss of PTEN was more common in NSCLC tumors with squamous histology (21%, 9/43 vs. 4%, 2/56).

PI3K pathway alterations in NSCLC cell lines

We aimed to determine whether similar pathway alterations were present in a panel of 29 NSCLC cell lines, and to use these cell lines to functionally validate the importance of PI3K pathway alterations in predicting sensitivity to selective inhibitors.

Baseline protein levels of selected pathway components were determined by Western blotting (Supplementary Fig. S5). Cell lines were also characterized for KRAS, NRAS, BRAF, PIK3CA, and EGFR mutational status, as well as copy number alterations in EGFR, HER2, MET, MYC, AKT2, and PIK3CA (Supplementary Table S2). Cell lines were classified into epithelial and mesenchymal subtypes based on published data (21). Sensitivity to GDC-0941 was assessed in different genetic backgrounds defined by the molecular analyses. We observed generally
greater sensitivity to the selective PI3K inhibitor GDC-0941 in epithelial compared with mesenchymal cell lines (P < 0.04, Fig. 4A); 4 of the 6 least sensitive epithelial cell lines harbored RAS mutations, whereas the 2 most sensitive mesenchymal lines harbored PIK3CA mutations (Fig. 4A and B). In addition, cell lines with receptor tyrosine kinase (RTK), PIK3CA alterations, or PTEN loss were significantly more sensitive to GDC-0941 as a class compared with those with RAS/RAF, LKB1, or no detected pathway alterations (P < 0.0001, Mann–Whitney test, Fig. 4B). Many of the cell lines with PI3K pathway activating events had more than 1 alteration in the pathway. One cell line, H1770, which showed evidence of PTEN loss but no other pathway alteration, was less sensitive than the models with at least 2 activating events. Expression of other candidate biomarkers such as FBXW7 and Golph3 was not associated with sensitivity to PI3K or dual PI3K/mTOR inhibition in this cell line panel (data not shown).

Comparison of half maximal inhibitory concentration (IC50) values from these experiments (Fig. 4B, bottom) showed that the PI3K/mTOR inhibitor GDC-0980 had the broadest activity across the panel, although it was also more potent in cell lines with PI3K and RTK alterations (P < 0.002, Mann–Whitney test). In contrast to GDC-0941, GDC-0980 was active in cell lines with LKB1 loss. An allosteric MEK inhibitor had minimal activity in PI3K-activated models and was more potent in cell lines with RAF and RAS alterations than in cell lines without these alterations (P < 0.03, Mann–Whitney test), consistent with previous reports (40). High-level MYC amplification was present in several cell lines, including the sensitive cell lines H1975 and H2170 (Supplementary Table S2), but was not associated with resistance to GDC-0941 or GDC-0980.

In vivo efficacy of GDC-0941 and GDC-0980 was determined in NSCLC xenograft models. Examples of dose-dependent inhibition in response to GDC-0941 in the H520 model, and GDC-0980 in the H460 model, are shown in Fig. 4C and Supplementary Fig. S6, respectively. Maximal tumor growth inhibition (TGI) observed in 5 NSCLC xenografts treated daily and orally with GDC-0941 at maximum tolerated doses of 100 or 150 mg/kg is plotted in Fig. 4D. Three of the models were also treated with GDC-0980 daily and orally at maximum tolerated doses of 2.5 or 5 mg/kg (Fig. 4D). Treatment of NSCLC models with a KRAS mutation (A549) or concomitant KRAS and PIK3CA mutations (H460) with GDC-0941 resulted in 33% or 20% TGI, respectively (Fig. 4D). GDC-0980 had greater antitumor activity in these models, with 62% TGI in A549 xenografts and 43% in H460 xenografts. In contrast, GDC-0941 and GDC-0980 showed comparable antitumor activity (67% and 68% TGI, respectively) in H520 tumors, which harbor...
both PIK3CA amplification and PTEN loss (Fig. 4D). In addition, in 2 RTK-driven models (HCC827 and H1975), GDC-0941 showed greater than 100% TGI, indicative of tumor regression (Fig. 4D).

Effects of GDC-0941 and GDC-0980 on signaling and feedback loops

We sought to determine whether sensitive and resistant cell lines showed distinct differences in pharmacodynamic effects on pathway signaling and pathway-regulated feedback loops, as such differences could have relevance to the clinical development of these inhibitors. The pharmacodynamic effects of the PI3K inhibitor GDC-0941 and the PI3K/mTOR inhibitor GDC-0980 were investigated by looking for changes in downstream components of the PI3K signaling pathway, in both sensitive (H1975) and resistant (H441) cell lines (Fig. 5A). In H1975 cells, GDC-0941 and GDC-0980 treatment resulted in increased levels of cleaved PARP and decreased levels of MCL-1 (both of which are hallmarks of apoptosis induction), consistent with the cytotoxic effect seen in the cell viability assays (Fig. 5A). The reduction in levels of Cyclin D1 was indicative of cell cycle arrest. Phospho-4EBP1 was decreased more dramatically with GDC-0980, which is consistent with 4EBP1 being directly downstream of mTOR. The effects on Cyclin D1, pAKT, and p4EBP1 were more modest in the resistant H441 cell line.

The effect of PI3K/mTOR inhibition on transcriptional output of the pathway was also assayed in a subset of NSCLC cell lines to assess whether there were pharmacodynamic differences in transcriptional readouts in sensitive compared with resistant cell lines. In this analysis, we focused on transcripts that have been previously identified as readouts of PI3K inhibition in breast cancer models (ref. 16; Fig. 5B). Treatment of sensitive cell lines with the PI3K inhibitors resulted in increased levels of mRNA for some RTKs and signaling pathway genes, including ERBB3, MET, and PIK3IP1. In addition, transcript levels of AA1370, CCNG2, and YPEL2 were also increased by treatment with GDC-0941 and GDC-0980, consistent with the previously described role of these genes as part of a negative feedback loop that is regulated by PI3K signaling (16). In contrast, the transcriptional effects of drug treatment on resistant cell lines were minimal.

In vitro combination studies with GDC-0941

Whether single-agent predictive diagnostic markers would be obscured in the setting of combination therapy is an important question with regard to clinical development. Clinical development of PI3K inhibitors in front-line treatment of NSCLC would require combination with a platinum-based chemotherapy regimen, such as carboplatin/paclitaxel, whereas development in later lines could also include combination with targeted agents, in particular, the epidermal growth factor receptor (EGFR) inhibitor erlotinib. In addition, MEK inhibitors have been suggested as rational combination partners to PI3K inhibitors (24, 41).

We also sought to determine whether predictive biomarker status would affect sensitivity to these combinations of agents by conducting in vitro combination screening. Combination effects were assessed in cell viability assays conducted across a range of concentrations of each compound. This approach allows visualization of percent inhibition at each combination of concentrations, as well as calculation of measures of synergy using the Bliss independence model, with positive Bliss scores indicating greater than additive effects (25). We found that the combination of GDC-0941 with paclitaxel was additive or modestly greater than additive, and that this effect was independent of candidate biomarkers (Fig. 5B). The GDC-0941/erlotinib combination showed slightly higher Bliss scores, with a trend toward greater combination effects in cell lines with RTK activation (Fig. 6B).
Figure 4. A, cellular sensitivity to GDC-0941 in NSCLC cell lines. Each data point represents the IC_{50} value for a particular cell line, grouped according to epithelial and mesenchymal classification as described in the text. The horizontal line represents the mean IC_{50} value ± SE. Cell lines with KRAS and/or PIK3CA mutations are indicated. B, in vitro sensitivity to PI3K pathway and MEK inhibitors in NSCLC cell lines. The bar chart at the top shows IC_{50} values for the pan PI3K inhibitor GDC-0941 from an ATP-based cell viability assay. Cell lines are grouped according to pathway alterations, as indicated in the middle of the figure. The heatmaps in the bottom show in vitro sensitivity to the selective PI3Ki GDC-0941, the dual PI3K/mTOR inhibitor GDC-0980, and the MEK1/2 inhibitor, with lower IC_{50} values shown in green and higher values in red. C, TGI with increasing doses of GDC-0941 in xenografted H520 NSCLC PTEN-null, PIK3CA-amplified tumors. Animals were dosed daily for 21 days with the indicated amount of GDC-0941, and the plot shows tumor volume over time. D, summary of NSCLC TGI in response to GDC-0941 and GDC-0980 in 5 xenograft models. The chart shows maximal TGI observed at either 100 mg/kg qd or 150 mg/kg qd for GDC-0941, or 2.5 mg/kg or 5 mg/kg for GDC-0980, as indicated. ND, GDC-0980 was not tested in HCC827 or H1975.
Finally, the GDC-0941/MEK inhibitor combination showed the strongest combination effects, and was particularly effective in cell lines with EGFR activation, PIK3CA mutations, or KRAS mutations (Fig. 6C).

**Discussion**

Lung cancer is a disease characterized by a high degree of molecular complexity. Whole-genome sequencing of a single NSCLC tumor sample revealed more than 50,000 high-confidence single-nucleotide variants in 1 cancer specimen (42). An important challenge is dissecting this complexity, and determining the association between alterations in specific signaling pathways and sensitivity to targeted therapeutics. Substantial progress has recently been made in the development of cancer therapeutics for targeted populations in NSCLC, as clinical studies have shown significant benefit in patients with EML4-ALK alterations (43), and have shown that the EGFR kinase inhibitors gefitinib and erlotinib have activity in patients with tumors harboring EGFR mutations (15%–20% of NSCLC cases; ref. 44). Despite this progress, nearly all of the patients with EGFR mutations or ALK fusions are nonsmokers, and thus represent an important but small fraction of the population with NSCLC. Of importance, there are still no validated targeted therapies for the majority of smoking-related NSCLC.
It has previously been suggested that the PI3K pathway may be a potential therapeutic target in NSCLC (45). Here, we extended earlier findings by conducting a detailed characterization of candidate biomarkers in the context of the major histologic subtypes of NSCLC, and using preclinical models to determine the association between these biomarkers and selective inhibitors of PI3K signaling. Our findings are consistent with those of previous studies showing that the incidence of PIK3CA mutations in NSCLC is relatively low (1) and they tend to occur in squamous tumors. Our data suggest that the pathway does undergo frequent pathologic activation through alternate mechanisms. In particular, whereas the PIK3CA and AKT2 loci were amplified specifically in squamous cell carcinomas, reduced PTEN expression was common in both histologic subtypes, although total PTEN loss was most frequent in squamous cell tumors. In addition to the known association of KRAS and EGFR alterations with adenocarcinoma (38), we also observed frequent loss of LKB1 in adenocarcinoma. The PI3K pathway thus seems to be important in both histologic subtypes of NSCLC, but appears to be activated by distinct molecular aberrations. Biomarker strategies may therefore need to be tailored to histologic disease subtypes to enable optimal patient selection. Our data have implications for diagnostic evaluation during the development of PI3K inhibitors in combination with taxane-containing regimens, as we found that this combination is additive across a range of genetic backgrounds. The implication of this is that the greatest activity may still occur in tumors harboring PI3K and RTK alterations, because these models have the highest intrinsic sensitivity to PI3K inhibition, thus showing the strongest absolute inhibition in combination with chemotherapy.

We initially aimed to analyze cell lines in which key pathway alterations occurred in isolation. However, most of the cell lines showed evidence of more than 1 molecular alteration in these signaling pathways, highlighting the molecular complexity of lung cancer. RTK activation, PTEN loss, or PIK3CA mutation or amplification were associated with selective PI3K inhibitor sensitivity in the cell line panel. Association of PIK3CA amplification with...
PI3K inhibitor sensitivity was limited by the relatively small number of cell lines that harbor PIK3CA copy number gains, and it should be noted that other studies have suggested that another gene colocализed under the amplicon, SOX2, is a lineage-specific amplicon and oncogenic driver (46). However, the observation that PIK3CA amplification is correlated with increased mRNA expression and associated with sensitivity to PI3K inhibition in vitro and in vivo suggests functional relevance that should be clinically tested. We also found that matched primary and metastatic NSCLC tumor samples were frequently discordant for PIK3CA amplification status, suggesting that archival tumor samples from primary lesions may not always reflect biomarker status in metastatic lesions, and that fresh biopsies from metastatic sites, or noninvasive methods such as circulating tumor cell biomarker analyses (47), may be helpful in accurately assessing the status of this biomarker in patients with metastatic disease. Consistent with previous reports (41), cell lines with KRAS and BRAF alterations were generally insensitive to selective PI3K pathway inhibition, although the dual PI3K/mTOR inhibitor did have some in vitro activity in these genetic backgrounds. In addition, while a class 1 selective PI3K inhibitor (GDC-0941) and dual PI3K/mTOR inhibitor (GDC-0980) showed comparable in vivo antitumor activity in the H520 xenograft model, which appears to be solely dependent on PI3K pathway activation, the dual PI3K/mTOR inhibitor GDC-0980 had greater antitumor activity than GDC-0941 in models harboring KRAS mutations or dual PIK3CA/KRAS mutations.

These findings may have clinical diagnostic implications, as they suggest that the activity of dual PI3K/mTOR inhibitors may extend to patients whose tumors are dependent on KRAS signaling. Similarly, GDC-0980 was more active than GDC-0941 in models with LKB1 loss, again suggesting that inhibitors targeting both PI3K and mTOR may have distinct diagnostic niches compared with selective PI3K inhibitors. The MYC oncogene has also been associated with resistance to PI3K inhibition in vivo (48), in breast cancer models, but did not appear to cause resistance in the NSCLC models studied here. The RTK-activated lines H1975 (16 copies of MYC) and H2170 (>20 copies of MYC) had low nanomolar in vitro IC_{50} values for GDC-0941, and strong in vivo antitumor activity was seen in H1975 cells, suggesting perhaps that the biology of PI3K resistance may differ between breast and NSCLC cancers.

Molecular analysis after treatment with GDC-0941 and GDC-0980 enabled the downstream effects of PI3K inhibition to be characterized in models with differential sensitivity to PI3K inhibition. The cell lines studied showed variation in the degree of inhibition of downstream signaling proteins related to apoptosis, cell cycle arrest, and the impairment of translation, with more sensitive cell lines showing more profound effects on these processes. Furthermore, treatment of inhibitor-sensitive cell lines with GDC-0941 and GDC-0980 resulted in increased expression of RTKs such as ERBB3 and MET, as well as other pathway-related genes such as PIK3P1. This suggests the possibility of feedback upregulation of RTKs and other upstream signaling molecules in response to PI3K inhibition, in agreement with recently published findings (49). Our data indicate that this phenomenon occurs to a greater extent in cell lines that are sensitive to PI3K inhibition, suggesting that assays for feedback-regulated genes may also serve as a pharmacodynamic measure of efficacy for inhibitors targeting this pathway. Feedback regulation of upstream receptors such as ERBB3 and Met may limit the activity and effectiveness of selective PI3K inhibitors, and it may be that combinations of agents targeting these signaling mechanisms will be required to fully overcome these feedback loops and maximize the potential of PI3K targeting. Indeed, the findings of our combination studies suggest that cotargeting EGFR or downstream at the level of MEK results in synergistic inhibition of cell growth, consistent with the hypothesis that such cotargeting may block PI3K inhibitor-induced pathway reactivation. Careful evaluation of predictive and pharmacodynamic biomarkers in the clinic will be essential to translate these findings into the most effective cancer therapy with PI3K inhibitors.

Disclosure of Potential Conflicts of Interest
A. Pandita is a scientist and has an ownership interest in Genentech. S. Mohan is a Senior Research Associate and has an ownership interest in Genentech. D. Sampath is an employee and has an ownership interest in Genentech, a member of the Roche Group. L.C. Amler is the Director of the Oncology Biomarker Development and has an ownership interest in Roche Holding. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: J.M. Spoerke, R. Brachmann, D. Sampath, M.R. Lackner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Spoerke, C. O’Brien, L. Huw, H. Koeppen, A. Pandita, D. Sampath, I.S. Friedman, L. Ross
Writing, review, and/or revision of the manuscript: J.M. Spoerke, H. Koeppen, R. Brachmann, A. Pandita, D. Sampath, I.S. Friedman, G.M. Hampton, L.C. Amler, D.S. Shames, M.R. Lackner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Spoerke, L. Huw, A. Pandita
Study supervision: D. Sampath, L. Ross, M.R. Lackner
Design of FISH experiments: A. Pandita

Acknowledgments
The authors thank the cell culture and technical support from the in vivo pharmacology group of Genentech, Zach Boyd for technical support with expression analysis, Helke Paul for administrative assistance, and Tim Wilson and Elizabeth Pannoose for comments on the manuscript. Support for third-party writing assistance for this manuscript was provided by F. Hoffmann-La Roche Ltd.

Grant Support
All studies were funded solely by Genentech, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 20, 2012; revised September 28, 2012; accepted October 21, 2012; published OnlineFirst November 7, 2012.
References


Phosphoinositide 3-Kinase (PI3K) Pathway Alterations Are Associated with Histologic Subtypes and Are Predictive of Sensitivity to PI3K Inhibitors in Lung Cancer Preclinical Models

Jill M. Spoerke, Carol O'Brien, Ling Huw, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-2347

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/11/06/1078-0432.CCR-12-2347.DC1

Cited articles
This article cites 44 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/24/6771.full.html#ref-list-1

Citing articles
This article has been cited by 18 HighWire-hosted articles. Access the articles at:
/content/18/24/6771.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.