PI3K Pathway Dependencies in Endometrioid Endometrial Cancer Cell Lines

Britta Weigelt1,4, Patricia H. Warne1, Maryou B. Lambros2, Jorge S. Reis-Filho4, and Julian Downward1,3

Abstract

Purpose: Endometrioid endometrial cancers (EEC) frequently harbor coexisting mutations in phosphoinositide 3-kinase (PI3K) pathway genes, including PTEN, PIK3CA, PIK3R1, and KRAS. We sought to define the genetic determinants of PI3K pathway inhibitor response in EEC cells, and whether PTEN-mutant EEC cell lines rely on p110β signaling for survival.

Experimental Design: Twenty-four human EEC cell lines were characterized for their mutation profile and activation state of PI3K and mitogen-activated protein kinase (MAPK) signaling pathway proteins. Cells were treated with pan-class I PI3K and mitogen-activated protein kinase (MAPK) signaling pathway proteins. Cells with concurrent PIK3CA and/or PTEN and KRAS mutations were sensitive to PI3K pathway inhibition, and only 2 of 6 KRAS-mutant cell lines showed response to MEK inhibition. KRAS RNAi silencing did not induce apoptosis in KRAS-mutant EEC cells. PTEN-mutant EEC cell lines resistant to the p110β inhibitors GSK2636771 and AZD6482, and only in combination with the p110α selective inhibitor A66 was a decrease in cell viability observed.

Conclusions: Targeted pan-PI3K and mTOR inhibition in EEC cells may be most effective in PIK3CA- and PTEN-mutant tumors, respectively, even in a subset of EECs concurrently harboring KRAS mutations. Inhibition of p110β alone may not be sufficient to sensitize PTEN-mutant EEC cells and combination with other targeted agents may be required.

Introduction

Endometrial cancer is the most common gynecologic malignancy in the western world with an estimated 49,560 new cases and 8,190 deaths in 2013 in the United States (1). Approximately 80% of endometrial carcinomas are of endometrioid histology and are associated with a hyperestrogenic state (2, 3). Although the outcome of women with early-stage endometrioid endometrial cancer (EEC) is favorable, it remains poor in patients with recurrent or metastatic disease (2). Thus, there is a need to improve our understanding of the disease at the molecular level and to refine current treatment strategies.

EECs have been shown to harbor, among other genetic aberrations (4–7), multiple cooccurring mutations in the phosphoinositide 3-kinase (PI3K) pathway, including PTEN, PIK3CA, PIK3R1, and KRAS (4–11). Given the role of the PI3K signaling pathway in cellular growth, survival, and endometrial cancer pathogenesis, inhibitors targeting different components of the pathway are currently being evaluated in preclinical and clinical studies (reviewed in refs. 12, 13). It is important to note, however, that there is a considerable intertumor genetic heterogeneity and that different combinations of coexisting PI3K pathway mutations can be found in EECs (4–6, 9–11). The functional effect of these distinct mutational patterns affecting different components of the same pathway on activation of the downstream effector PI3K and RAF/MEK/ERK pathways and response to targeted therapies has yet to be fully established.

Preclinical models of cancer have identified KRAS and BRAF mutations to confer resistance to PI3K pathway inhibition (reviewed in refs. 12, 13). Recent phase I/II clinical trials provided evidence to suggest that patients with colorectal cancer whose tumors harbored concomitant PIK3CA

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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and KRAS mutations are resistant to PI3K pathway inhibition (14, 15), whereas subsets of ovarian cancers with coexisting PIK3CA and KRAS/BRAF mutations may be sensitive (14, 16). These data imply that not only the mutational repertoires but also epistatic interactions between different components of the PI3K pathway may be distinct in different tumor types (12).

The most commonly altered gene in EECs is PTEN, and up to 60% of PTEN-mutant tumors also harbor a coexisting PIK3CA gain-of-function mutation (6–11). PTEN-deficient tumors, in particular breast and prostate cancer cells, have been reported to mainly depend on p110β signaling for tumorigenesis, proliferation, and survival (17–20), contrary to PIK3CA-mutant tumors which rely on p110α (21). A p110β isoform-specific inhibitor is currently being tested in patients with advanced PTEN-deficient solid tumors, including EECs, prostate, ovarian, breast, and colorectal cancer among others (NCT01458067).

Given that EECs frequently harbor coexistent mutations in PTEN, PIK3CA, PIK3R1, and KRAS, in this study we sought to determine the genetic predictors of response to small-molecule PI3K pathway inhibitors, and whether PTEN-mutant EEC cell lines are reliant on p110β for survival. To address these questions, we investigated the effects of different PI3K and RAF/MEK/ERK pathway inhibitors on cell viability in a panel of 24 EEC cell lines, and found that cells harboring PIK3CA and PTEN mutations were selectively sensitive to pan-PI3K and allosteric mTOR inhibition, respectively. In addition, we observed that subsets of EEC cell lines with concomitant PIK3CA and/or PTEN and KRAS mutations were responsive to PI3K pathway inhibition, and subsets of KRAS-mutant EEC cell lines to RAF/MEK/ERK pathway inhibition. We further found that EEC cell lines were not responsive to single-agent p110β inhibition irrespective of the PTEN status, and a reduction in cell viability was only observed upon combination with a p110α inhibitor.

**Materials and Methods**

**Cell lines**

The human EEC cell lines ECC-1, HEC-1-A, HEC-1-B, and RL95-2 were obtained from American Type Culture Collection (ATCC), AN3-CA, EFE-184, MFE-280, EN, and MFE-296 from the German Collection of Microorganisms and Cell Cultures (DSMZ), HJUERM-3 from RIKEN Cell Bank, and HEC-59, HEC-265, HEC-251, HEC-116, HEC-108, SNG-II, and SNG-M from the Japanese Health Science Research Resources Bank. Ishikawa were obtained from the Central Cell Services Facility at Cancer Research UK (CRUK). HEC-151, HEC-50B, HEC-6, HHU1A, and KLE were kindly provided by Dr. F. McCormick (University of California San Francisco, San Francisco, CA), and NOI-1 by Dr. R. Zeillinger (Medical University of Vienna, Vienna, Austria; Supplementary Table S1). Cell lines were authenticated by short-tandem repeat (STR) DNA profiling. As controls for KRAS-silencing experiments authenticated NCI-H460 and NCI-H727 lung cancer cell lines were obtained from the CRUK Central Cell Services Facility, for the p110β inhibitor experiments authenticated PC3 prostate cancer cells were obtained from the CRUK Facility and BT549 and HCC70 breast cancer cell lines from ATCC (22).

**Mutation analysis**

DNA from EEC cell lines was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and subjected to mutation screening to detect 238 mutations in 19 known cancer genes using the OncoCarta Panel v1.0 (SEQUENOM INC.) as previously described (23). Identified mutations were validated using Sanger sequencing. In addition, Sanger sequencing of the coding sequences of PTEN, PIK3CA, and PIK3R1 was conducted as previously described (ref. 22; see Supplementary Methods and Supplementary Table S2).

**Cell viability assays and small-molecule inhibitors**

Cells were plated in 96-well microtiter plates at densities ranging from 1,500 to 15,000 cells per well, optimized for untreated control cells to be 80% to 90% confluent at the endpoint of the experiment. After 24 hours, cells were treated with serial dilutions (100 pmol/L to 10 μmol/L) of PI3K and mitogen-activated protein kinase (MAPK) pathway inhibitors (Supplementary Methods and Supplementary Table S3). Cell viability was assessed after 72 hours of treatment by incubation with CellTiter Blue (Promega) for 1.5 hours. The drug concentration required for survival of 50% of cells relative to untreated cells [surviving fraction of 50%, SF50 (22, 24); for temsirolimus SF60, see Supplementary Fig. S1] was determined using GraphPad Prism version 5.0d. Cell lines that failed to achieve the SF50 to a given drug were nominally assigned as the highest concentration screened (i.e., 10 μmol/L; ref. 25). At least 3 independent
experiments in triplicate per cell line/targeted drug were carried out. Association between a mutation and response to a targeted agent was determined using a Fisher exact test and a two-tailed \( P \) value less than 0.05 was considered statistically significant.

**KRAS silencing**

A pool of 4 siRNA duplexes (siGENOME; "SMARTpool;" Thermo Scientific Dharmacon) was used to silence KRAS (26). Reverse transfection using 37.5 nmol/L siRNA was conducted using Lullaby (OZ Biosciences) or Lipofectamine RNAiMAX (Life Technologies) transfection reagents for EEC cell lines, selected from 25 transfection reagents tested for having the highest gene silencing efficiency but least toxicity, and Dharmafect 1 (Thermo Scientific Dharmacon) for lung cancer cell lines. Nontargeting siRNA pool #2 ("scrambled"). RISC-free, and SMARTpools targeting UBB and PLK1 were used as controls. Cell viability was determined 96 hours posttransfection using CellTiter Blue as described earlier, and apoptosis induction using the ApoONE Caspase-3/7 assay (Promega) by incubation of cells for 5 hours (22, 26, 27).

**Western blot analysis and protein quantification**

Standard Western blotting was conducted as previously described (22), using antibodies against PTEN, p110\(\alpha\), p110\(\beta\), PARP, \(\beta\)-Actin (Cell Signaling Technology; New England Biolabs), KRAS (Santa Cruz Biotechnology), and \(\alpha\)-tubulin (Sigma-Aldrich). For quantitative Western blotting, membranes were probed simultaneously with antibodies against (i) extracellular signal–regulated kinase 1/2 (ERK1/2) and phospho-ERK1/2 (Thr202/Tyr204), (ii) AKT and phospho-AKT (Ser473), (iii) AKT and phospho-AKT (Thr308), or (iv) S6 ribosomal protein (rpS6) and phospho-rpS6 (Ser235/236; all from Cell Signaling Technology). Conjugated secondary antibodies (IRDye 680LT; IRDye 800CW; LI-COR) were detected using the Odyssey Infrared Imaging System (LI-COR; ref. 22).

**Results**

**EEC cell lines harbor multiple mutations in the PI3K pathway**

To characterize the panel of EEC cell lines for their mutational patterns, and to assess whether the different combinations of coexisting PI3K pathway mutations observed in primary EECs can be found in EEC cell lines, we used the OncoCarta Panel v1.0 (Sequenom Inc.) and conducted Sanger sequencing of PTEN, PIK3CA, and PIK3R1 coding sequences. The OncoCarta Panel v1.0 screening of 238 common somatic mutations across 19 known cancer genes revealed that none of the EEC cell lines studied harbored mutations in ABL1, AKT1, AKT2, BRAF, CDK4, ERBB2, FGFR1, FGFR3, FLT3, IAK-2, KIT, MET, PDGFA, or RET (data not shown) but one EGFR (A389V; HEC-6), NRAS (G12D; HEC-151), and HRAS (Q61H; RL95-2) mutation were found (Table 1). Combined with Sanger sequencing, we observed that the prevalence of mutations in PTEN (17 of 24; 70.8%), PIK3CA (13 of 24; 54.2%), PIK3R1 (9 of 13; 73.8%), and KRAS (6 of 24; 25%; Table 1; Supplementary Fig. S2A) in the EEC cell lines was comparable with those reported in primary human EECs (7, 9, 10), as was the high frequency of PIK3CA mutations within exons 1 to 7 (9, 11). In line with previous observations (9), a subset (3 of 17; 17.6%) of PTEN-mутant EEC cell lines expressed detectable PTEN protein by Western blotting (Table 1 and Supplementary Fig. S2B). As observed in primary EECs (6, 9, 10), different components of the PI3K pathway were altered in the same cell line and included concomitant mutations in PTEN/PIK3CA (5 of 24; 20.8%), PTEN/PIK3R1 (6 of 24; 25%), PTEN/PIK3CA/PIK3R1 (2 of 24; 8%), PTEN/PIK3CA/KRAS (3 of 24; 12.5%), PIK3CA/KRAS (2 of 24; 8%), and PIK3R1/KRAS (1 of 24; 4.2%; Supplementary Fig. S2A). In EFE-184, JHUEM-3, and KLE cells, none of the PI3K pathway mutations assessed were identified (Table 1). For 9 and 19 cell lines, mutation profiles are available on the Catalogue of Somatic Mutations in Cancer (COSMIC; www.sanger.ac.uk/genetics/CGP/cosmic/) and the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE; www.broadinstitute.org/ccle/home; ref. 4) websites, respectively, and a high agreement between the results of our analysis and those available online was observed (data not shown). Taken together, the prevalence of the PI3K pathway mutations assessed in our EEC cell line panel mirrors that reported for primary EECs.

**AKT activation is associated with PTEN status**

As EEC cells harbor different combinations of concomitant PI3K pathway mutations, we sought to determine the associations between PTEN, PIK3CA, PIK3R1, and RAS mutations and effector pathway activation downstream of these genes. For this, baseline activation of AKT(Ser473), ribosomal protein (rp)-S6(Ser235/236), and ERK(Thr202/Tyr204) was determined by quantitative IR fluorescent Western blotting (LI-COR; Supplementary Fig. S3). In a way akin to breast cancer cells (28), we observed that in EEC cells PI3K pathway activation as determined by levels of AKT phosphorylation was significantly associated with PTEN mutation status [Mann–Whitney (MWU) test, two-tailed; \( P < 0.001; \) Fig. 1A], whereas no association between activating PIK3CA mutants and phospho-AKT(Ser473) levels was observed (MWU test; \( P = 0.772 \)). The 3 PTEN-mutant cell lines that retained PTEN protein did not differ in terms of their levels of phospho-AKT expression from those devoid of PTEN protein expression (MWU test; \( P = 0.344 \)). The key signaling node mTOR complex 1 (mTORC1) and one of its downstream targets rpS6 respond to numerous inputs including growth factors, amino acids, and energy, and is stimulated by the PI3K and RAF/MEK/ERK pathways (29). We observed that EEC cells with wild-type (wt) PTEN had lower levels of rpS6 activation than those with mutant PTEN (Fig. 1B), however, this association was no longer statistically significant (MWU test; \( P = 0.075 \)). Levels of ERK activation in EEC cell lines were not significantly associated with their PTEN, PIK3CA, PIK3R1, or RAS mutation status (Fig. 1C), and may in part be driven
by upstream aberrant growth factor receptor signaling not interrogated here. Of note, neither AKT and rpS6 activation nor ERK activation was associated with the RAS mutation status of the cells (MWU test; AKT, $P = 0.665$; rpS6, $P = 0.714$; ERK, $P = 0.973$). These data provide evidence to suggest that AKT activation is associated with the PTEN status and that PIK3CA gain-of-function and PTEN loss-of-function mutations may have distinct effects on AKT and PI3K pathway activation in EECs.

Genetic predictors of response are distinct between different types of PI3K pathway inhibitors

We next tested the response of the 24 EEC cell lines to targeted PI3K pathway inhibitors and determined the association with PTEN, PIK3CA, PIK3R1, and RAS mutations. Using a cell viability assay, we observed a range of responses to the pan-class I PI3K small-molecule inhibitor GDC-0941 in our EEC cell line panel after 72 hours of treatment, with 54% of cells having a $SF_{50}$ below 1 $\mu$mol/L (Fig. 2A). Responses were not associated with the cells’ doubling time (mean sensitive 38.84 ± 5.47 hours; mean resistant 36.96 ± 4.70 hours; MWU test; $P = 0.724$) but cell lines harboring a PIK3CA mutation were significantly more sensitive to GDC-0941 than PIK3CA wild-type cells (Fisher exact test; two-tailed; $P = 0.038$). We found no significant association between PTEN status and GDC-0941 response ($P = 0.386$). In addition, a subset of EEC cell lines tested here harboring coexisting PIK3CA/KRAS or PIK3CA/PTEN/KRAS mutations were GDC-0941 responsive (Fig. 2A).

Targeting the PI3K pathway more downstream revealed that PTEN mutations, rather than PIK3CA mutations, were significantly associated with response of EEC cells to the allosteric mTOR inhibitor temsirolimus ($P = 0.023$; Fig. 2B; doubling time mean temsirolimus-sensitive 32.98 ± 3.0 hours, mean resistant 43.88 ± 6.74 hours; MWU test; $P = 0.420$). Although EEC cell lines harboring RAS mutations were generally less responsive to temsirolimus, this association was not statistically significant (K/H/NRAS, $P = 0.391$; KRAS, $P = 0.357$). It should be noted, however, that only 3 cell lines in our EEC panel had coexisting PTEN and KRAS mutations, of which one was temsirolimus responsive. In addition, with exception of KLE cells, which are wild-type for PTEN, PIK3CA, PIK3R1, and RAS and show low levels of AKT and rpS6 activation (Fig. 1), all EEC cell lines were sensitive to the ATP-competitive mTOR kinase and dual

<table>
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NOTE: Mutations identified in 24 human EEC cell lines using Sequenom OncoCarta Panel v1.0 and Sanger sequencing for PTEN, PIK3CA, and PIK3R1 transcripts (amino acid changes shown). For PTEN protein, see Supplementary Fig. S2B.
PI3K/mTOR inhibitors AZD8055 and PF-04691502, respectively, irrespective of the RAS mutation status (Supplementary Fig. S4A and S4B).

To test whether EEC cells are responsive to RAF/MEK/ERK pathway inhibition, we treated the cell line panel with serial dilutions of the non-ATP-competitive mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitors PD0325901 and AZD6244 and the ATP-mimetic CRAF/BRAF inhibitor AZD628. In total, only 6 cell lines had a $SF_{50}$ below 1 $\mu$mol/L and in the vast majority of EEC cells treatment with 10 $\mu$mol/L of MEK/RAF inhibitors did not result in 50% inhibition of viability compared with the control (Fig. 2C and D; Supplementary Fig. S4C). Five cell lines (21%) were sensitive to the MEK inhibitor PD0325901, and response was significantly associated with the presence of KRAS, HRAS, or NRAS mutations ($P = 0.028$) but not with the doubling time of cells (mean sensitive 30.76 ± 0.90 hours; mean resistant 39.88 ± 4.45 hours; MWU test; $P = 0.743$). Only 2 of 6 KRAS-mutant EEC cells were sensitive to this small-molecule inhibitor ($P = 0.568$), which both harbored coexisting PIK3CA or PIK3R1 but not PTEN mutations. Three EEC cells showed $SF_{50}$ below 1 $\mu$mol/L when treated with the selective RAF inhibitor AZD628 (Fig. 2D), and the presence of KRAS, HRAS, or NRAS mutations was significantly associated with response to this agent ($P = 0.028$), whereas KRAS mutations alone failed to show a statistically significant association ($P = 0.143$). It should be noted, however, that although the associations between mutations in RAS and MEK/RAF inhibitor response was statistically significant and showed a high negative predictive value (i.e., the lack of RAS mutations predicted resistance to these agents), the

Figure 1. Associations between PTEN, PIK3CA, PIK3R1, and RAS mutations and AKT, rpS6, and ERK activation in EEC cells. A, AKT and phospho-AKT (Ser473); (B) rpS6 and phosphor-rpS6(Ser235/236); and (C) ERK and phospho-ERK(Thr202/Tyr204) were quantified using quantitative IR Western blotting (LI-COR; see Supplementary Fig. S3), and phospho-/total protein ratios ordered by increasing levels of activation. Mutational profiles of each cell line are shown below the graph where columns represent individual cell lines, rows represent genes, and colored boxes the presence of a mutation.

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positive predictive value of a KRAS, HRAS, or NRAS mutation to predict response to PD0325901 and AZD628 treatment was only 50% and 37.5%, respectively. As expected, given the absence of BRAFV600E gene mutations in the cell lines tested, none of the cell lines studied was sensitive to the BRAFV600E inhibitor PLX4032 (data not shown).

We next investigated whether the reduction in cell viability caused by the agents tested was the result of increased apoptosis. In the setting used, apoptosis induction as determined by PARP cleavage could not be observed (Supplementary Fig. S5A), suggesting that the single-agent small-molecule inhibitors tested in our cell line panel are likely to elicit preferentially cytostatic effects. The targeted agents used, however, inhibited their respective targets in EEC cells, as treatment with the MEK and RAF inhibitors PD0325901 and AZD628 resulted in decreased ERK phosphorylation, whereas the mTORC1 inhibitor temsirolimus led to a marked reduction in rpS6 phosphorylation, and the PI3K, mTOR kinase, and dual PI3K/mTOR inhibitors GDC-0941, AZD8055, and PF-04691502 to a decrease of both AKT and rpS6 phosphorylation compared with untreated control cells (Fig. 2E). In addition, as compared with single-agent treatment, combination treatment with PI3K and MAPK pathway inhibitors resulted in decreased cell viability and/or increased cell death of EEC cells (Supplementary Fig. S5B–S5D).

Taken together, our results show that the genetic predictors of response may be distinct between different PI3K pathway inhibitors in EEC cells. Our data further suggest that a subset of EEC cells harboring oncogenic RAS mutations and concomitant PIK3CA or PTEN mutations may still be sensitive to PI3K pathway perturbation. Finally, only a minority of EEC cells harboring KRAS mutations was shown to be sensitive to MEK or RAF inhibition.

**KRAS silencing does not induce apoptosis in EEC cells**

We used RNA interference (RNAi) to investigate whether EEC cells harboring KRAS mutations would be
dependent of the expression of this mutant oncogene for their survival. We determined the effect of KRAS silencing on cell viability in the 6 KRAS-mutant cell lines of our EEC cell line panel and 6 KRAS wild-type cell lines (Supplementary Fig. S6A). In addition, we used 2 KRAS-mutant lung cancer cell lines, NCI-H727 and NCI-H460, as controls (Supplementary Fig. S6B), in which we previously observed reduced cell viability upon KRAS silencing (26, 30). As expected, cell viability was markedly decreased upon KRAS depletion in the KRAS-mutant lung cancer cell lines as compared with scrambled control (Fig. 3A). On the other hand, only 1 of 6 KRAS-mutant EEC cells (HEC-1-B cells, a cell line that was sensitive to all PI3K and RAF/MEK/ERK pathway inhibitors tested earlier) showed a reduction in cell viability following KRAS silencing (26, 30). Expected, cell viability was markedly decreased upon KRAS depletion in the KRAS-mutant lung cancer cell lines as compared with scrambled control (Fig. 3A). On the other hand, only 1 of 6 KRAS-mutant EEC cells (HEC-1-B cells, a cell line that was sensitive to all PI3K and RAF/MEK/ERK pathway inhibitors tested earlier) showed a reduction in cell viability following KRAS silencing comparable with the levels seen in the lung cancer cells. The effect of KRAS silencing on cell viability in the other 5 KRAS-mutant EEC cell lines was significantly lower than that of KRAS-mutant lung cancer cell lines (t test; two-tailed; \( P = 0.0047 \)) and only marginally but not statistically significantly higher than that in KRAS wild-type EEC cells [KRAS mut: mean 72.13% ± 15.04% (95% confidence interval, CI, 53.45–90.81), KRAS wild-type: mean 89.52% ± 8.71% (95% CI, 80.38–98.65), t test, two-tailed; \( P = 0.06123 \)].

Previous work (31) has shown that the KRAS-mutant NCI-H727 lung cancer cells are "KRAS-dependent," given that caspase-3 cleavage was observed following KRAS ablation, whereas the KRAS-mutant NCI-H460 cells were classified as KRAS-independent, as KRAS silencing did not result in increased apoptosis. These observations were confirmed here using a fluorescent caspase-3/7 assay, and strong KRAS siRNA silencing-induced apoptosis was seen in NCI-H727 but not NCI-H460 cells (Fig. 3B). Irrespective of their KRAS status, some EEC cells showed a slight increase in caspase-3/7 activation compared with scrambled control, however, there was no statistically significant difference in apoptosis induction between KRAS-mutant and KRAS wild-type EEC cells (MWU test; \( P = 0.485 \)). Also the increase in caspase-3/7 activation observed in the KRAS-mutant HEC-1-B cells, which showed marked reduction in cell viability similar to that in lung cancer cells (Fig. 3A), was only modest (scrambled: 1.0, KRASsi: 1.51) as compared with the KRAS-dependent NCI-H727 cells (scrambled: 1.0, KRASsi: 5.92; Fig. 3B). These data provide additional evidence to suggest that a subset of EEC cell lines with activating KRAS mutations may not be necessarily dependent on KRAS signaling for their survival, and that KRAS silencing-induced apoptosis, a feature previously used to determine

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**Figure 3.** KRAS dependency in lung and EEC cell lines harboring activating KRAS mutations. A, cell viability was determined 96 hours post-siRNA-mediated KRAS silencing using a pool of 4 siRNA duplexes targeting KRAS (SMARTpool) in 2 KRAS-mutant lung, 6 KRAS-mutant, and 6 KRAS wild-type EEC cell lines. Cell viability is presented for each cell line relative to its nontargeting siRNA pool #2 ("scrambled") control, set to 100% (mean of at least 3 independent experiments in triplicate ± SD). B, apoptosis induction assessed 96 hours post-transfection, depicted as ratio of caspase-3/7 activation determined using the Apo-ONE assay over cell viability determined using CellTiter-Blue in KRAS-mutant lung cancer (striped bars), KRAS-mutant (open bars), and KRAS wild-type (checked bars) EEC cell lines (mean of at least 2 independent experiments in triplicate ± SEM). Apoptosis induction following siRNA-mediated silencing of KRAS ablation (red bars), and of the positive controls UBB (yellow bars) and PLK1 (blue bars) is presented for each cell line relative to its nontargeting siRNA pool #2 (i.e., scrambled) control (set to 1). mut, mutant; wt, wild-type.
"KRAS addiction" in lung and pancreas adenocarcinoma cell lines (31), was not observed in KRAS-mutant EEC cells.

p110β inhibition alone is not sufficient to sensitize EEC cells harboring PTEN mutations

Selective targeting of p110β is currently being assessed in patients with advanced PTEN-deficient cancers, including EECs. Given that (i) PTEN and PIK3CA mutations frequently coexist in EECs, (ii) that PIK3CA-mutant tumors have been shown to rely on p110α (21), and (iii) that here we showed that both p110α and p110β are expressed in EECs irrespective of the PTEN status (Fig. 4A), we sought to define whether inhibition of p110β alone would indeed be sufficient to sensitize PTEN-mutant EEC cells. Contrary to GDC-0941, which inhibits all class I PI3K isoforms (i.e., p110α, p110β, and p110γ), treatment with the p110β isoform-specific inhibitors GSK2636771, AZD6482, and TGX-221 or the p110α isoform-specific inhibitor A66 revealed that the EEC cell lines tested were resistant to these agents (SF50 above 1 μm/L; Supplementary Fig. S7A-S7D). In addition, upon 72 hours treatment with the p110β inhibitors GSK2636771 and AZD6482, cell viability was significantly more decreased in the control cells (i.e., previously described p110β-reliant PTEN-deficient PC3 prostate and BT549 and HCC70 breast cancer cell lines (17, 18)) than in PTEN-mutant and PTEN wild-type EEC cells (Fig. 4B). No significant difference in cell viability following p110β inhibition between PTEN-mutant and PTEN wild-type EEC cell lines was found (1 μm/L/10 μm/L GSK2636771: PTEN wt vs. PTEN mut EECs; MWU test, P = 0.128/P = 0.341; 1 μm/L/10 μm/L AZD6482: PTEN wt vs. PTEN mut EECs; MWU test, P = 0.849/P = 0.485; Fig. 4B).

Western blot analysis of activated AKT in PC3, BT549, and HCC70 controls and in EEC cell lines revealed a strong effect of pan-class I PI3K inhibitor GDC-0941 treatment on both AKT phosphorylation sites [i.e., AKT (Ser473) and AKT(Thr308)], in contrast to treatments with the p110α selective inhibitor A66 (Fig. 4C and D; Supplementary Fig. S8A and S8B). Inhibition of p110β by GSK2636771 or AZD6482 led to a marked decrease of AKT phosphorylation only in the control prostate and breast cancer cell lines, whereas only marginal effects on AKT activation were observed in EEC cells (Fig. 4C and D; Supplementary Fig. S8A-S8C). In fact, combination of the p110β inhibitors with the p110α inhibitor A66 at relatively high concentrations was required to decrease levels of AKT phosphorylation comparable with those seen following treatment with the pan-class I PI3K inhibitor GDC-0841 (Fig. 4D; Supplementary Fig. S8A and S8C), and to decrease cell viability in both PTEN-mutant and PTEN wild-type EEC cells to levels similar to those observed in the p110β-dependent PC3, BT549, and HCC70 cells (Fig. 4E; Supplementary Fig. S8D). Taken together, our data provide evidence to suggest that p110β inhibition alone is not sufficient to induce a marked reduction in viability of PTEN-mutant EEC cells, and that this is only obtained upon concurrent targeting of p110β and other p110 isoforms.

Discussion

The PI3K pathway is activated in more than 80% of EECs (9, 10), and understanding the biologic implications of mutations affecting genes in this pathway holds great promise for novel treatment strategies for the disease. Unlike in breast cancer (32), cooccurrence of mutations targeting different PI3K pathway components downstream of growth factor receptors in EECs is common. Using EEC cell lines as a model, we here show that PI3K pathway activation in EEC cells as assessed by AKT (Ser473) phosphorylation is associated with the presence of PTEN mutations, irrespective of the PIK3CA, PIK3R1, or RAS mutation status. These observations imply that the functional impact of mutations affecting different genes pertaining to the PI3K pathway (e.g., PTEN, PIK3CA, and PIK3R1) on AKT phosphorylation and PI3K pathway activation may be distinct (28). In addition, no associations between ERK activation and mutations in the PI3K pathway and RAS were found, suggesting that other aberrations, including those affecting growth factor receptors, may play a role in activation of the RAF/MEK/ERK pathway in EECs.

We further show that in EEC cell lines distinct genetic predictors are associated with response to different modalities of PI3K pathway inhibitors. In a way akin to breast cancer cell lines (25), PIK3CA mutations were predictive of response to the PI3K inhibitor GDC-0941. At variance with breast cancer cells, where PTEN-deficiency has been shown to be associated resistance to PI3K pathway inhibition (12, 22, 33, 34), response to the allosteric mTOR inhibitor temsirolimus was found to be associated with PTEN mutations affecting different genes in this pathway holds great promise for novel treatment strategies for the disease. Unlike in breast cancer (32), cooccurrence of mutations targeting different PI3K pathway components downstream of growth factor receptors in EECs is common. Using EEC cell lines as a model, we here show that PI3K pathway activation in EEC cells as assessed by AKT (Ser473) phosphorylation is associated with the presence of PTEN mutations, irrespective of the PIK3CA, PIK3R1, or RAS mutation status. These observations imply that the functional impact of mutations affecting different genes pertaining to the PI3K pathway (e.g., PTEN, PIK3CA, and PIK3R1) on AKT phosphorylation and PI3K pathway activation may be distinct (28). In addition, no associations between ERK activation and mutations in the PI3K pathway and RAS were found, suggesting that other aberrations, including those affecting growth factor receptors, may play a role in activation of the RAF/MEK/ERK pathway in EECs.
mutations in EEC cells. These observations provide evidence to suggest that mutations in different components of the PI3K pathway may have distinct functional effects depending on the tumor type, their overall genetic landscape and epistatic interactions, and that a predictive biomarker identified in one cancer type may not necessarily be a predictor in another type. In fact, the interactions between oncogenic RAS mutations and resistance to PI3K pathway inhibition (reviewed in refs. 12, 13) lend further credence to this notion, as their impact seem to be context- and tumor type-dependent. In recent early clinical trials, colorectal cancers harboring concomitant PIK3CA and KRAS mutations were found to be resistant to PI3K pathway inhibitor treatment (14, 15), whereas a subset of ovarian cancers with coexisting PIK3CA and KRAS/BRCA mutations were sensitive (14, 16). In line with previous in vitro studies (9, 35) and clinical trials (36), we did observe that EEC cells harboring KRAS mutations showed decreased sensitivity to allostERIC mTOR inhibition; however, we did not find significant associations between the presence of KRAS mutations and PI3K pathway inhibitor response. In particular, subsets of EEC cell lines with coexistent PIK3CA/KRAS and PTEN/ KRAS mutations were sensitive to GDC-0941 and temsirolimus treatment, respectively.

It should be noted, however, that contrary to our in vitro findings, 2 recent phase II clinical trials testing allostERIC mTOR inhibitors in patients with advanced or metastatic endometrial cancer did not identify PTEN status or any other marker assessed to be associated with response or clinical outcome (36, 37). Unlike in our cell line–based study, both clinical trials included patients not only with ECC but also with serous, adenosquamous, and clear cell cancers, which are known to harbor genetic aberrations distinct from those of EECs (38), which may in part be accountable for the disparate results. In addition, the clinical trials by Tredan and colleagues (36) and Oza and colleagues (37) were not sufficiently powered to conduct subgroup analyses of patients with ECC only, and results from ongoing clinical studies testing mTOR inhibitors in women with ECC are eagerly awaited.

One aspect that is germane for the translation of genetic information into clinical useful tests is the identification of driver mutations (39). Here, only a subset of KRAS-mutant ECC cells was responsive to MEK inhibition, and siRNA-mediated silencing of KRAS resulted in a decrease of cell viability to levels seen in KRAS-mutant lung cancer cells in only 1 of 6 KRAS-mutant ECC cell lines tested. In fact, apoptosis induction was only observed in the “KRAS-mutant” lung cancer cell line NCI-H727 (31) but not in KRAS-mutant ECC cells upon KRAS siRNA-mediated silencing. Although larger panels of KRAS-mutant ECC cell lines may be required to increase the statistical power for the study of KRAS dependency in ECCs and targeted drug response, our findings suggest that there may be subsets of KRAS-mutant ECC cells, where the activity of KRAS mutations proven to be oncogenic in other cancer types may not be required for their survival. This emphasizes the notion that driver mutations in one tumor type may not necessarily constitute drivers in another tumor type (39, 40). One could hypothesize that in these ECCs, epistatic interactions between the numerous mutations identified in this disease (4, 6) are such that loss of oncogenic KRAS signaling may be compensated for by activation of alternative signaling pathways. Further studies to determine the biologic and clinical importance of these epistatic interactions are warranted.

There are several lines of evidence from mouse models and other preclinical studies (17–20) to suggest that p110β activation may be required for tumorigenesis driven by PTEN loss-of-function in different cancer types. Analysis of hundreds of cell lines of different tumor types revealed that TGX-221 and AZD6482 p110β inhibitor response was not only selective for PTEN-mutant cells but also for Cdkn2a- and PIK3CA-mutant cells, respectively (Release 3, November 2012; www.cancerxgene.org/; refs. 5, 41). In particular, however, PTEN-deficient prostate cancer, breast cancer, and glioblastoma cell lines have been shown to be responsive to PIK3CB silencing and p110β inhibitor treatment in vitro and in vitro (17, 18). Our results fail to corroborate these observations in EEC cell lines, as single-agent treatment with the 3 different p110β selective inhibitors tested (i.e., GSK2636771, AZD6482, and TGX-221) and the p110α inhibitor A66 did not sensitize ECC cells. This is not entirely unexpected and may be the result of the epistatic interactions between PTEN loss-of-function and PIK3CA gain-of-function mutations, which are frequently observed concurrently in ECCs, and that the ECC cell lines studied here expressed both p110α and p110β at protein levels. Our results are consistent with the findings that sustained inhibition of selected p110 isoforms allows for functional redundancy of class IA PI3K isoforms (42), that inactivation of either p110α or p110β may counteract the impact of PTEN loss (43), and that inhibition of p110α/ p110β (PIK3G-A/D) isoforms do exert effects on cell viability in subsets of PTEN-deficient cell lines and xenografts (non-ECC; ref. 44). Given that activating genetic alterations targeting the PI3K pathway frequently cooccur with PTEN loss-of-function in ECCs, and that here we show that p110β inhibition alone may not be sufficient to sensitize PTEN-mutant ECC cells, our results suggest that combination of p110β inhibitors with other targeted agents may be required to increase efficacy. On the other hand, it is plausible that p110α inhibition may increase sensitivity of PIK3CA-mutant ECC cells to other agents targeting different components of the PI3K pathway or parallel pathways.

The limitations of this study include the analysis of cancer cell lines in vitro only, and the focus on the most commonly mutated PI3K pathway genes for their association with targeted therapy response in the ECC cell lines. With the ongoing next-generation sequencing efforts unraveling the entire mutational repertoire of primary endometrial cancers, additional genetic alterations may be identified in subgroups of the disease that may play a role determining response to PI3K pathway inhibitors.

In summary, we observed that targeted pan-PI3K and mTOR inhibition in ECC cell lines may be most effective in PIK3CA- and PTEN-mutant cells, respectively, and that
these PI3K inhibitors may even be effective in a subset of EEC cells concurrently harboring KRAS mutations. In addition, our data suggest that benefit from p110β inhibitors in PTEN-deficient EEC may be limited given the frequent coexistence of activating PIK3CA mutations. As other p110 isoform-specific inhibitors, in particular p110δ inhibitors, showed unexpected clinical efficacy due to effects on the tumor microenvironment (45), results from clinical trials are eagerly awaited.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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