PIK3CA/PTEN Mutations and Akt Activation As Markers of Sensitivity to Allosteric mTOR Inhibitors

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Abstract

Purpose: We sought to determine whether phosphoinositide 3-kinase (PI3K) pathway mutation or activation state and rapamycin-induced feedback loop activation of Akt is associated with rapamycin sensitivity or resistance.

Experimental Design: Cancer cell lines were tested for rapamycin sensitivity, Akt phosphorylation, and mTOR target inhibition. Mice injected with breast or neuroendocrine cancer cells and patients with neuroendocrine tumor (NET) were treated with rapalogs and Akt phosphorylation was assessed.

Results: Thirty-one cell lines were rapamycin sensitive (RS) and 12 were relatively rapamycin resistant (RR; IC50 > 100 nmol/L). Cells with PIK3CA and/or PTEN mutations were more likely to be RS (P = 0.0123). Akt phosphorylation (S473 and T308) was significantly higher in RS cells (P < 0.0001). Rapamycin led to a significantly greater pathway inhibition and greater increase in p-Akt T308 (P < 0.0001) and p-Akt S473 (P = 0.0009) in RS cells. Rapamycin and everolimus significantly increased Akt phosphorylation but inhibited growth in an in vitro NET model (BON). In patients with NETs treated with everolimus and octreotide, progression-free survival correlated with p-Akt T308 in pretreatment (R = 0.4762, P = 0.0533) and on-treatment tumor biopsies (R = 0.6041, P = 0.0102). Patients who had a documented partial response were more likely to have an increase in p-Akt T308 with treatment compared with nonresponders (P = 0.0146).

Conclusion: PIK3CA/PTEN genomic aberrations and high p-Akt levels are associated with rapamycin sensitivity in vitro. Rapamycin-mediated Akt activation is greater in RS cells, with a similar observation in patients with clinical responses on exploratory biomarker analysis; thus feedback loop activation of Akt is not a marker of resistance but rather may function as an indicator of rapamycin activity. Clin Cancer Res; 18(6); 1777–89. ©2012 AACR.

Introduction

mTOR signaling plays a key role in cell growth, protein translation, autophagy, and metabolism (1). Activation of phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling contributes to the pathogenesis of many tumor types. Rapamycin is an allosteric inhibitor of mTOR. Rapamycin analogs have been U.S. Food and Drug Administration (FDA) approved for the treatment of neuroendocrine tumors, renal cell carcinoma, and subependymal giant cell astrocytoma associated with tuberous sclerosis and have very promising clinical benefit in other tumor types such as breast and endometrial cancer. However, rapalogs have shown objective responses in only a subset of patients and unfortunately responses are frequently short lived. Therefore, there is a pressing need to identify predictors and pharmacodynamic (PD) markers of rapamycin response and mechanisms of therapy resistance.

Activation of Akt has been proposed to be a predictor of rapamycin response (2, 3). Rapamycin and its analogs have been shown to induce Akt activation (4–7). Insulin-like growth factor-I (IGF-I) and insulin-dependent induction of the PI3K/Akt pathway leads to feedback inhibition of signaling due to mTOR/S6K-mediated phosphorylation and degradation of IRS-1. Rapamycin-induced Akt activation has been primarily attributed to the loss of this negative feedback loop. This feedback loop activation of Akt was not only seen in vitro but was also observed in a phase I clinical trial of rapamycin analog everolimus (8). There is concern that Akt activation may limit the antitumor efficacy of
Translational Relevance

Rapamycin analogs are approved as antitumor agents for some tumor types, but they achieve objective responses in only a subset of patients and there is a pressing need to identify predictors of response and mechanisms of resistance. Rapamycin-induced feedback loop activation of Akt has been proposed as a mechanism of rapamycin resistance. Here we show that cell lines that are rapamycin sensitive (RS) are more likely to have PIK3CA and/or PTEN mutations or basal Akt phosphorylation. Unexpectedly, rapamycin leads to a greater increase in Akt phosphorylation in RS cells suggesting that feedback loop activation is an indicator of rapamycin response. Furthermore, patients with neuroendocrine tumors who had responded to rapalogs were more likely to have an increase in phospho-Akt with treatment. Thus PIK3CA/PTEN genomic aberrations and both basal and Akt activation should be pursued as response predictors. Further work is needed to identify markers of response and clinical benefit to rapalogs.

Materials and Methods

Cell growth analysis and IC\textsubscript{50}

Cell lines used are described in the Supplementary Methods. Cells were plated in triplicate at densities of 500 to 5,000 cells per well depending on growth characteristics of the cell lines. After adhering overnight, rapamycin response was determined by treating with 6 concentrations based on a 10-fold dilution series (range: 0–1,000 nmol/L). Cell growth was measured 5 days later using sulforhodamine B (SRB) assay as previously described (9). The IC\textsubscript{50} value of rapamycin was determined based on dose–response curve (10). Cell lines were categorized as rapamycin sensitive (RS) or rapamycin resistant (RR) using an IC\textsubscript{50} cut-off value of 100 nmol/L.

Reverse phase protein arrays

Reverse phase protein arrays (RPPA) were performed in the MD Anderson Cancer Center Functional Proteomics RPPA Core Facility as described previously (11–13). Cells were treated with different concentrations of rapamycin (0 (vehicle), 1, 10, and 100 nmol/L) and harvested at various time points (2, 24, and 72 hours) to capture dose and time effects. Two biologic replicates per condition were used. Samples were probed with monospecific, validated antibodies, enriched for components of PI3K/Akt/mTOR pathway. Protein levels were expressed as the mean expression values in log\textsubscript{2}.

Multiplex phosphoprotein assays

Xenograft lysates were prepared using RPPA buffer (Supplementary Methods). MSD assay (Meso Scale Discovery) was used to measure p-S6 S240/244, and total and p-Akt S473 in following vendor’s instructions. The signal was detected using an MSD Sector Imager 2400 in the MD Anderson Cancer Center Immune Monitoring Core Laboratory. Everolimus effect for individual samples was determined by calculating the ratio of p-Akt S473 to total Akt or p-S6 S240/244 to total Akt.

Immunohistochemistry

Immunohistochemistry (IHC) was done on 25 archival samples and pre- and on-treatment core biopsies. IHC was done at Cell Signaling Technology Inc. for PTEN, p-Akt S473, p-mTOR S2448, p-4E-BP1 T37/46, and p-S6 S235/236. The details of immunohistochemical technique have already been published (14). Briefly, antigen retrieval was carried out, and slides were washed and incubated in 3% hydrogen peroxide. Slides were stained overnight at 4°C, and this was followed by application of secondary antibodies (Vector) and avidin–biotin complex (Elite ABC; Vector). Immunostaining was scored dichotomously (staining intensity 0 vs. 1, 2, 3) by a dedicated gastrointestinal pathologist (AR).

In vivo studies

Xenograft studies were approved by the MD Anderson Animal Care and Use Committee. MCF7 xenografts were formed by inoculating 1.5 × 10\textsuperscript{7} cells in mammary fat pads of 8-week-old female nu/nu mice (Harlan Sprague Dawley Inc.). After tumors were formed, mice (10 mice per group) were given weekly intraperitoneal injections of either rapamycin (15 mg/kg) or dimethyl sulfoxide (DMSO) for 3 weeks. Mice were euthanized 24 hours after the first or fourth weekly injection (day 22). BON xenografts were formed by inoculating 2 × 10\textsuperscript{7} cells in the upper flank of 4-week-old male BALB/c mice (Department of Experimental Oncology at MD Anderson Cancer Center). In rapamycin treatment studies, after tumors were formed, mice (12 mice for treatment and 11 mice for control groups) were treated and euthanized as above. In the everolimus study, mice (8 mice per group) were given everolimus (10 mg/kg) or its control by oral gavage for 5 consecutive days each week throughout the study. Consistent with recommendations from Veterinary Medicine at MD Anderson Cancer Center with regard to ethical research of animals, treatment was ceased and animals were euthanized when average tumor burden in untreated control mice reached approximately 1,000 mm\textsuperscript{3} (day 24). In all 3 experiments, tumor growth was followed by caliper measurements and tumor volumes were calculated as previously described (15).

Everolimus clinical trial

Patients with neuroendocrine tumors received depot octreotide 30 mg every 28 days, and everolimus (RAD001, Afinitor; Novartis Pharmaceuticals) 5 or 10 mg orally daily on an open-label phase II trial (NCT00113360) and were
assessed for response by RECIST criteria (partial response (PR) stable disease (SD), and progressive disease (PD)) and progression-free survival (PFS; ref. 16). The primary objective of the trial was to assess the clinical activity of everolimus plus depot octreotide by PFS in treated and untreated patients with metastatic, unresectable low-grade neuroendocrine carcinoma. Secondary endpoints included correlative studies to determine the expression/phosphorylation status of components of the mTOR signaling pathway in the primary tumors, to determine whether these markers can be used as predictors of sensitivity, and to determine the effect of combination of everolimus and octreotide on the expression and phosphorylation mTOR targets in the accessible tumor tissue to identify PD markers of response.

Sixty patients (30 carcinoid and 30 islet cell) were enrolled on the trial. In the second half of the study, patients were approached to undergo pre- and on-therapy tumor biopsies as an optional procedure. Nineteen neuroendocrine cancer patients underwent pretreatment and on-treatment fine needle aspirates (FNA) and core needle biopsies for assessment of Akt/mTOR signaling by RPPA and IHC, respectively. Repeat biopsies were obtained 2 weeks (± 4 days) after initiation of therapy. Two patients did not have tumor in one of the 2 core biopsies and were eliminated from matched pair analysis. Sixteen patients who had paired evaluable biopsies received 10 mg everolimus orally per day, one patient with matched biopsies received 5 mg orally per day.

Statistical analysis
The association between PIK3CA/PTEN or KRAS mutation status and rapamycin sensitivity was tested with Fisher’s exact test. Bcl-2 expression in RS and RR cell lines was compared by Student t test. P-Akt levels in PTEN/PIK3CA, wild-type (wt) and mutants were compared with pairwise t test adjusting P values by false discovery rate (FDR). The cell line RPPA slide data consisted of 1,032 samples and 161 proteins (242 slides with replicates) and were collected from 43 cell lines, with 4 treatments (DMSO, Rap1, Rap10, and Rap100) per cell line, 3 time points (2, 24, or 72 hour) come with per treatment, and 2 biologic replicates (set A and B). To determine the differences in expression between RS and RR cell lines, we fitted a linear mixed model to each baseline protein expression level in the control vehicle (DMSO-treated cells). In this model, rapamycin sensitivity group and time were entered as fixed effects, and replicate was considered as a random effect (Supplementary Methods).

To determine differences in PD response to rapamycin treatment in RS versus RR cells, we also used a linear mixed model incorporating an interaction term. Explicit mathematical formulas for the models are presented in the Supplementary Methods. Means are reported for baseline measures and PD changes. We used the FDR to address the multiple comparison issue in our study. The FDR, defined as the expected proportion of false positives among all significant test, is a statistical method frequently used to correct for multiple comparisons. R package “fdrtool” was chosen to compute FDR. FDR less than 0.05 was considered statistically significant corresponding to P < 0.0366 for baseline and P < 0.433 for PD changes.

MSD data are presented as means ± SE vehicle and everolimus groups were compared using unpaired t test. Xenograft data are presented as means ± SE. Control and treatment groups were compared using unpaired t or Mann–Whitney U tests, where appropriate.

For the neuroendocrine trial, paired t test and 2-sample t test analysis were done as appropriate to compare the protein expression of pre- versus posttreatment for both cases. Pearson correlations were calculated between protein expression and PFS of all patients. ANOVA test were conducted to find the protein signature that manifests different expressions among response groups. FDR less than 0.2 was considered statistically significant. Analyses were done using R 2.10.1 (http://CRAN.R-project.org) or GraphPad Prism (GraphPad Software, Inc.).

Results
PIK3CA/PTEN mutations are associated with rapamycin sensitivity
To identify determinants of rapamycin sensitivity and mechanisms of resistance, we established a panel of 43 human cancer cell lines with differing genetic backgrounds, including different aberrations in the PI3K signaling pathway, including PIK3CA and PTEN mutations (Supplementary Table S1). This panel was specifically enriched for cell lines reported to be RR, on the basis of published literature. All 43 human cancer cell lines were treated with increasing doses of rapamycin for 120 hours and SRB assay was used to determine rapamycin IC_{50}. An IC_{50} of 100 nmol/L, a clinically achievable concentration (17, 18), was selected as a threshold for rapamycin sensitivity. Of 43 cell lines tested, 31 were RS and 12 were RR (Fig. 1A).

As PTEN and PIK3CA mutations are associated with activation of PI3K/Akt/mTOR signaling, we determined the association between mutation status and rapamycin sensitivity. PTEN/PIK3CA status was known in 40 cell lines (Supplementary Table S1). Ten (91%) of 11 PTEN mutant cell lines were RS; 18 (64%) of 28 cell lines that were PTEN wild type were RS (P = 0.1304; Fig. 1B). Ten (91%) of 11 cell lines with PIK3CA mutations were RS, 19 (66%) of the 29 PIK3CA wild-type cell lines were RS (P = 0.2326). Overall, 19 (90%) of 21 cell lines with either a PTEN or PIK3CA aberrations were RS, whereas only 10 of 19 cell lines that were known to be both PIK3CA and PTEN wild type were RS (P = 0.0123). KRAS alone or with other Ras–Raf pathway mutations (BRAF and NRAS) did not correlate with rapamycin resistance (KRAS alone, P = 0.3407; all Ras–Raf mutations, P = 0.668); however, we had a limited number of cell lines with KRAS (n = 6), BRAF (n = 1), and NRAS mutations (n = 1) in our panel.

Akt activation is associated with rapamycin sensitivity in vitro
To determine which proteins were differentially expressed between RS and RR cell lines, we measured the
functional proteomic profile in cells cultured in the presence of vehicle only and collected after 2, 24, and 72 hours of culture (Supplementary Fig. S1). When RS were compared with RR cells, 61 proteins or phosphoproteins were statistically significant at a FDR cut-off of 0.05, ($P < 0.0366$), and at a FDR cut-off of 0.01 ($P < 0.0046$), 36 proteins or phosphoproteins were highly significant (Supplementary Table S2).

$p$-Akt S473 (RS 0.742, RR 0.514) and $p$-Akt T308 (RS 0.345, RR 1.189) levels were significantly higher in RS cell lines ($P < 0.0001$; Fig. 2A). Other PI3K pathway members, $p$-FoxO1 T24/FoxO3a T32, $p$-S6K T389, $p$-GSK3α/β S21/9, $p$-PRAS40 T246, $p$-IRS-1 S1101, $p$-PKCα S657, $p$-ER S118, S6, Tuberin, PKCα, $p$-mTOR S2448, $p$-mTOR S2481, $p$-Tuberin T1462, and $p$-S6 S240/244 (in the order of decreasing significance) also showed differential expression (Fig. 2B, Table 1). As Bcl-2 overexpression has been associated with rapamycin resistance (19, 20), we also compared baseline Bcl-2 expression in RS and RR cell lines; there was no significant difference ($P = 0.3315$).

Next, we looked at rapamycin-induced Akt activation in cell lines of different genetic backgrounds. Baseline $p$-Akt
Figure 2. Rapamycin sensitivity and baseline Akt activation. All cell lines were cultured in the presence of vehicle only and collected after 2, 24, and 72 hours of culture. A, using functional proteomics analysis, log transformed p-Akt S473 and p-Akt T308 baseline expression and their change in 3 time points for RS and RR cell lines are shown. B, a protein signature of PI3K pathway activity and rapamycin sensitivity. Heat map showing preordered 43 cancer cell lines and 17 PI3K hierarchically clustered pathway proteins. Bar at the left of the heat map indicate whether a cell line was RS (green) or RR (red). Proteins’ data were derived from log transformation and median centering for each gene (red, high expression; green, low expression according to z scores). C, log transformed p-Akt S473 and p-Akt T308 baseline phosphorylation in PIK3CA/PTEN wild-type, PIK3CA, or PTEN mutant cell lines are displayed. The mid-line of each box is at the median for the data represented by that box. The upper and lower edges of each box mark the 75th and 25th percentiles of the data, respectively. The upper and lower bars mark the 95th and 5th percentiles of the data, respectively. D, log transformed p-Akt S473 and p-Akt T308 baseline phosphorylation in PIK3CA/PTEN wild-type, PIK3CA kinase domain mutant or other domain mutant cell lines are displayed. The mid-line of each box is at the median for the data represented by that box. The upper and lower edges of each box mark the 75th and 25th percentiles of the data, respectively. The upper and lower bars mark the 95th and 5th percentiles of the data, respectively.
S473 and T308 levels were significantly higher in cell lines with PIK3CA mutations as well as in those with PTEN mutations compared with PIK3CA and PTEN wild-type cell lines (Fig. 2C). PTEN mutant cell lines showed significantly higher levels of Akt phosphorylation compared with PIK3CA mutant cell lines (p-Akt S473, \( P < 0.0001 \); p-Akt T308, \( P = 0.0075 \)). Mutations in both PIK3CA kinase domain and other PIK3CA domains displayed significantly

<table>
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<th>Protein</th>
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<th>( P )</th>
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<th>Resistant</th>
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Figure 3. Feedback loop activation of Akt. A, cell lines were treated with DMSO or 100 nmol/L rapamycin for 24 hours. Western blotting was done with antibodies to p-Akt T308, p-Akt S473, and total Akt. B, cell lines were treated with DMSO or 1, 10, or 100 nmol/L rapamycin and harvested after 2, 24, or 72 hours. Changes in expression of p-Akt S473 and p-Akt T308 in response to rapamycin treatment were analyzed using RPPA. Expression levels at 3 time points are shown in log scale (RS, green; RR, red; median expression for all cell lines, black line).
higher levels of Akt phosphorylation compared with PIK3CA/PTEN wild-type cell lines; however, Akt phosphorylation was higher in PIK3CA kinase domain mutant cell lines (Fig. 2D).

Feedback loop Akt phosphorylation is greater in rapamycin-sensitive cell lines

To determine whether rapamycin-mediated Akt activation is associated with rapamycin sensitivity or resistance, we treated a panel of cancer cell lines with 100 nmol/L of rapamycin for 24 hours and assessed Akt phosphorylation by Western blotting. We observed Akt phosphorylation not only in cell lines that are relatively RR (MDA-MB-231 and MDA-MB-435) but also in cell lines that are RS (Fig. 3A).

We assessed the PD effects of rapamycin treatment compared with vehicle (DMSO) treatment in RS and RR cells. PD changes were defined as the difference between rapamycin treatment and DMSO. At a FDR cut-off of 0.05, levels of 73 proteins or phosphoproteins were significantly different \(P < 0.0433\), and at a FDR cut-off of 0.01, levels of 42 proteins or phosphoproteins were significantly different \(P < 0.0055\); Supplementary Table S3).

mTOR complex 1 (mTORC1), the target for rapamycin, phosphorylates 4E-BP1 and S6K, and S6K phosphorylates ribosomal protein S6; thus the phosphorylation of S6, S6K, and 4E-BP1 are commonly monitored as PD markers of mTOR inhibition (1). However, we and others have previously shown that rapamycin not only inhibits mTOR signaling in RS cell lines but also in RR cell lines (2). In this study, although both RS and RR cells showed inhibition of mTOR signaling, the quantitative RPPA approach showed that RS cells had a statistically greater inhibition of the pathway as shown by a more significant drop in p-S6K T389 \(P < 0.0001\), p-S6 S235/236 \(P < 0.0001\), and p-S6 S240/244 \(P < 0.0001\), and a greater increase in nonphosphorylated-4E-BP1 T46 \(P < 0.0001\); Supplementary Table S3). As expected based on the effects of rapalogs on cell-cycle progression (21), RS cells also had a statistically greater decrease in proliferation marker proliferating cell nuclear antigen compared with RR cell lines \(P < 0.0001\).

To determine the association of rapamycin-induced Akt activation with drug sensitivity, we compared p-Akt expression in DMSO- versus rapamycin-treated cells. Rapamycin led to a significantly greater increase in p-Akt T389 \(P < 0.0001\); Fig. 3B) and p-Akt S473 \(P = 0.0009\); Fig. 3B) in RS compared with RR cells (Supplementary Table S3). Rapamycin also led to a significantly greater increase in p-PRAS40 T246, an Akt target, indicating that the phosphorylation of Akt resulted in functional activation \(P < 0.0001\); Supplementary Table S3).

Eighteen cell lines displayed statistically significant increase in p-Akt S473 or p-Akt T380 upon rapamycin treatment on RPPA (Supplementary Table S4). To get mechanistic insight into differences between the cell lines that show significant Akt activation upon rapamycin treatment and those that do not, we compared their baseline proteomic profile. Forty-nine proteins were differentially expressed/phosphorylated \(FDR = 0.05, P = 0.0261\); Supplementary Table S4). Cell lines that had rapamycin-mediated Akt activation had higher levels of p-S6 and p-S6K, EF2K and p-EF2, p-MAPK (mitogen-activated protein kinase), as well as p-Akt, but lower p-AMPK. We next assessed differences in rapamycin treatment-induced changes between the cell lines that show significant Akt activation and those that do not. Fifty-eight proteins were differentially expressed/phosphorylated \(FDR < 0.05, P = 0.0300\); Supplementary Table S5). There was a significantly greater repression in p-S6 S235/236 and p-240/244 as well as in p-S6K T389 in the cell lines that had Akt activation than those that did not (all \(P < 0.0001\)).

Rapamycin treatment is associated with an increase in p-Akt in rapamycin-sensitive in vivo models

We have previously shown that rapamycin significantly decreases the \textit{in vivo} growth of the breast cancer cell line MCF7 and pancreatic carcinoid cell line BON; 2 cell lines harboring PIK3CA mutations (9, 22). We thus sought to determine the effect of rapamycin on Akt/mTOR signaling in in vivo models.

In MCF7 xenografts, rapamycin significantly inhibited mTOR signaling, as shown by a decline in p-S6 S235/236 and p-S6 S240/244 \(P < 0.001\) on RPPA. However, rapamycin treatment was associated with an increase in p-Akt T308 \(P = 0.089\); Fig. 4A, left panel). Rapamycin treatment was associated with a significant decrease \(P = 0.0044\) in tumor volume on day 21 in mice treated with 15 mg/kg rapamycin \(187 	ext{ mm}^3\) compared with vehicle \(388 	ext{ mm}^3\); Fig. 4A, right panel).

In BON xenografts, rapamycin significantly decreased p-S6 S235/236 \(P = 0.007\) and p-S6 S240/244 \(P = 0.002\) as assessed by RPPA (Fig. 4B, left panel). Similar to the MCF7 model, rapamycin treatment was associated with an increase in p-Akt T308 \(P = 0.02\). BON xenografts showed a significant decrease \(P = 0.0008\) in tumor volume on day 21 in mice treated with 15 mg/kg rapamycin \(200 	ext{ mm}^3\) compared with vehicle \(597 	ext{ mm}^3\); Fig. 4B, right panel).

In BON xenografts, everolimus significantly decreased p-S6 S240/244 \(P = 0.0003\) as shown by MSD multiplex phosphoprotein assay (Fig. 4C, left panel). Everolimus treatment also led to an increase in p-Akt S473 \(P = 0.0408\); Fig. 4C, middle panel). Everolimus treatment significantly decreased \(P = 0.0004\) tumor volume on day 30 in mice treated with 10 mg/kg everolimus \(367 	ext{ mm}^3\) compared with vehicle \(831 	ext{ mm}^3\); Fig. 4C, right panel). These studies, taken together, showed that rapamycin and its analogs increase Akt phosphorylation, even in RS \textit{in vivo} models.

Treatment associated increase in p-Akt is not associated with everolimus resistance in patients

Recently, everolimus has been shown to prolong PFS of pancreatic neuroendocrine tumors (23) and has received FDA approval. Therefore, we determined whether Akt activation correlated with PFS on everolimus-based therapy. Archival tumor blocks were available on 23 patients treated on the phase II trial of everolimus and octreotide. All tumors
expressed p-mTOR and almost all expressed PTEN. There were no significant differences in PFS based on expression of p-Akt S473, p-4E-BP1 T37/46, or p-S6 S235/236 on archival samples.

As biomarker analysis on the tumor being treated may be more clinically relevant than biomarkers on archival tissue, pretreatment and on-treatment FNAs were obtained in 17 patients on the trial after informed consent. Pretreatment

**Figure 4.** Rapamycin-mediated Akt activation in vivo. A, mice with established MCF7 tumor xenografts received 15 mg/kg rapamycin once a week by intraperitoneal injection. Mice were sacrificed 24 hours after last rapamycin injection. Protein was extracted from xenografts and p-S6 S235/236, p-S6 S240/244, and p-Akt T308 expression in DMSO and rapamycin treated xenografts was analyzed using RPPA. The mid-line of each box is at the median for the data represented by that box. The upper and lower edges of each box mark the 75th and 25th percentiles of the data, respectively. The upper and lower bars mark the 95th and 5th percentiles of the data, respectively (left and middle). The tumor volumes were measured using caliper and presented as the mean ± SE (right).

B, mice with established BON tumor xenografts received 15 mg/kg rapamycin once a week by intraperitoneal injection. p-S6 S235/236, p-S6 S240/244, and p-Akt T308 expression in DMSO and rapamycin treated xenografts was analyzed using RPPA. The mid-line of each box is at the median for the data represented by that box. The upper and lower edges of each box mark the 75th and 25th percentiles of the data, respectively. The upper and lower bars mark the 95th and 5th percentiles of the data, respectively (left and middle). The tumor volumes were measured using caliper and presented as the mean ± SE (right).

C, mice with established BON tumor xenografts received 10 mg/kg everolimus 5 days a week by oral gavage for 3 weeks. Mice were sacrificed 24 hours after last everolimus dose. Protein was extracted from xenografts and p-S6 S240/244, p-Akt-S473, and total Akt expression in vehicle and everolimus treated xenografts were analyzed using MSD assay. p-Akt S473 or p-S6 S240/244 to total Akt ratio is calculated and normalized to vehicle. Data were presented as mean ± SE (left and middle). The tumor volumes were measured using caliper and presented as the mean ± SE (right).
and on-treatment functional proteomics on FNAs samples were assessed by RPPA. We determined whether p-Akt levels on RPPA were associated with PFS. We found that high p-Akt T308 levels on baseline pretreatment FNAs as well as on-treatment FNAs correlated with longer PFS (pretreatment $R = 0.4762$, $P = 0.0533$; on-treatment $R = 0.6041$, $P = 0.0102$, respectively; Fig. 5A).

On RPPA, we showed that S6 phosphorylation was indeed significantly decreased on p-S6 S240/244 ($P = 0.0027$) and p-S6 S235/236 ($P = 0.0150$; Fig. 5B), showing inhibition of mTOR signaling. As RS cell lines were more likely to have feedback loop activation than RR cell lines, we assessed the effect of everolimus on p-Akt T308 levels. Patients who had a PR with everolimus treatment were significantly more likely to have an increase in p-Akt T308 than patients who had SD or progression ($P = 0.0146$; Fig. 5C).

Five patients had paired pretreatment and on-treatment core biopsies with IHC evaluable for p-Akt S473; one of these patients had activation of Akt signaling and had a PR.

**Discussion**

Rapamycin analogs have been FDA approved for the treatment of renal cell carcinoma, subependymal giant cell astrocytoma associated with tuberous sclerosis, and pancreatic neuroendocrine tumors and have shown promising antitumor efficacy in other cancer types. However, rapalogs have shown objective responses in only a subset of patients. Identification of predictors and PD markers of rapamycin response can help select patients most likely to benefit from rapalogs, and assess response early in the treatment course, and identify mechanisms of therapy resistance that can be targeted for combinatorial therapy. Our purpose was to determine whether PI3K pathway mutations/activation, that is, rapamycin-induced feedback loop activation of Akt is associated with rapamycin sensitivity or resistance. We showed that cell lines with $PIK3CA$ or $PTEN$ mutations were more likely to be RS. Baseline Akt phosphorylation was significantly higher in RS cells. Rapamycin also led to a significantly greater increase in Akt phosphorylation in RS cells. Furthermore, patients who had a PR were more likely to have an increase in p-Akt T308 with treatment compared with patients with SD or progression.

Rapamycin activates Akt in several models (6, 7). IGF-I and insulin-dependent induction of the PI3K/Akt pathway leads to feedback inhibition of signaling due to mTOR/S6K-mediated phosphorylation and degradation of IRS-1. Rapamycin-induced Akt activation has been attributed to the loss
of this negative feedback loop (6, 7). However, rictor-containing mTOR complex 2 (mTORC2) is involved with Akt phosphorylation on S473 (24). Rictor also regulates the ability of integrin-linked kinase (ILK) to promote Akt phosphorylation (25). Reducing rictor expression with rictor siRNA knockdown attenuates rapalog-induced Akt S473 phosphorylation, showing that increases in Akt S473 phosphorylation associated with mTORC1 inhibition are dependent on the presence of rictor (3). Although rictor was initially reported to lead to a rapamycin-insensitive companion of mTOR, we previously reported that rapamycin treatment leads to rictor dephosphorylation (26). It was subsequently shown that rictor T1135 is directly phosphorylated by mTORC1-dependent kinase (27). Although this phosphorylation does not affect mTORC2 complex formation or in vitro kinase activity, expression of a phosphomimetic kinase activity, expression of a phosphomimetic mutation of rictor (T1135A) increases Akt S473 phosphorylation. Thus, rapamycin-mediated rictor T1135 dephosphorylation may represent another mechanism by which mTORC1 inhibition leads to feedback activation of Akt signaling. Thus, there may be multiple regulatory links between mTORC1-dependent signaling and Akt, and multiple mechanisms of rapamycin-mediated activation of Akt. Furthermore, the effect of rapamycin on Akt phosphorylation varies with cell type (28). For example, rapamycin derivatives have been shown to inhibit Akt signaling by inhibiting mTORC2 formation in acute myeloid leukemia cells, both in vitro and in vivo (29). Further work to determine mechanism of differential regulation of Akt phosphorylation is ongoing.

We and others have observed Akt activation in many RS models (3, 7, 9). Breuleux and colleagues studied p-Akt levels at baseline and with treatment with everolimus in 13 cell lines and concluded that antiproliferative response to everolimus correlates with basal activation of the Akt pathway, but not with Akt phosphorylation response following everolimus treatment (3). Our results in terms of baseline pathway activation are similar, however in contrast, our data suggests that RS cells have a significantly greater Akt activation with rapamycin treatment potentially detected because of the quantitative RPPA approach. RS cells also had greater inhibition of mTORC1 phosphorylation compared with cell type (28). For example, rapamycin derivatives have been shown to inhibit Akt signaling by inhibiting mTORC2 formation in acute myeloid leukemia cells, both in vitro and in vivo (29). Further work to determine mechanism of differential regulation of Akt phosphorylation is ongoing.

Preclinically, combinations of rapamycin and IGFR inhibitors have been shown to decrease feedback loop activation and have additive antitumor effects (4). Indeed, this combination is being actively pursued in clinical trials (31). In addition, clinical trials are ongoing to test the safety and efficacy of targeting the pathway with mTOR kinase inhibitors that would inhibit mTORC1 as well as mTORC2 (thus inhibiting Akt S473 phosphorylation), or with dual PI3K/mTOR inhibitors. In addition, rapalog treatment has been linked to activation of MAPK signaling (32); thus dual targeting of PI3K/mTOR signaling and MAPK signaling is also being explored clinically (33, 34). Recently, inhibition of Akt with small molecule inhibitors have been shown to increase HER3 expression/signaling, and combined targeting of HER3 and Akt was shown to enhance efficacy (35). Thus feedback loop activation is clearly not a phenomenon restricted to allosteric mTOR inhibitors. Assessment of adaptive or survival responses to new targeted therapies should be pursued as an approach to design rational combinatorial therapies.

PI3K/mTOR signaling is a promising target in neuroendocrine tumors. In our phase II trial of everolimus and octreotide LAR in advanced low- and intermediate-grade neuroendocrine tumors, intent-to-treat response rate was 20% (16). Subsequently everolimus alone was shown to have antitumor efficacy in a phase II trial of daily oral everolimus in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy (36). Recently, a phase III trial (RADIANT 3) everolimus was shown to significantly improve PFS compared with placebo (23). These data recently led to the FDA approval of everolimus for pancreatic neuroendocrine tumors. However, even in this registration trial, objective PRs were observed in only 5% of patients receiving everolimus. Thus, the benefit from everolimus with respect to PFS was seen primarily in disease stabilization or minor tumor shrinkage. Thus it may be of great value to identify biomarkers that can upfront predict which patients with neuroendocrine tumors may derive the greatest clinical benefit. Recently, high-throughput characterization of pancreatic neuroendocrine tumors has identified variety of genomic aberrations including frequent aberrations DAXX, ATRX, TSC2, MEN1, PTEN, and PIK3CA (37).
Studies are ongoing to determine the role of these genomic aberrations in rapalog sensitivity. As expected, we showed that cell lines with PTEN mutations had increased Akt phosphorylation. There is no consensus on whether PIK3CA mutations activate PI3K signaling. PIK3CA mutations were reported to be associated with increased p-Akt levels in pancreatic cancer specimens (38) and in selected breast cancer cell lines (39), whereas others have found no clear association (40, 41). Our data supports an increase in Akt phosphorylation in PIK3CA mutant cell lines. However, the p-Akt elevation observed with PIK3CA mutations is not as robust as that seen with PTEN mutations. Furthermore, we did not analyze the differences in downstream signaling by genotype.

In vitro baseline high p-Akt levels are associated with rapamycin sensitivity. This is consistent with previous reports (42, 43). However, in spite of intense study of PI3K/mTOR signaling in cancer biology, currently there are no validated assays to assess Akt phosphorylation or pathway activation in the clinic. In our phase II study, p-Akt levels on archival tissue were not associated with outcome, whereas p-Akt levels on FNAs correlated with PFS. This could be a reflection of tumor evolution with time, or challenges with IHC with phospho-specific antibodies on archival samples. Consistent with this, we have previously shown that there is a significant discordance when IHC for p-Akt and p-4E-BP1 in primary breast tumors were compared with those in matched distant metastases (44). Thus more work is needed to determine whether p-Akt or another marker or markers of pathway activation can be brought into the clinic to test the value of PI3K activity as a predictive marker of response to rapalog or other PI3K pathway inhibitors.

Our in vitro data suggest that genomic aberrations such PIK3CA mutations and PTEN aberrations may also hold promise as potential predictors of response. Recently Weigelt and colleagues reported that breast cancer cells harboring PIK3CA mutations are selectively sensitive to mTOR kinase inhibitors as well as allosteric inhibitors (45), emphasizing that these pathway aberrations may also have predictive value for patient selection for new generation mTOR inhibitors. However, our recent studies show that there may also be discordance in PIK3CA mutation status between primary tumors and metastases (46). Thus to facilitate biomarker discovery and validation, pretreatment biopsies particularly in patients treated for recurrent or metastatic disease should be considered for assessment of pathway activation and mutation status in clinical trials.

Our study has several limitations. We have carried out the in vitro assays using a panel of 43 cell lines with different backgrounds, which we enriched for RR cell lines. However, there is also a selection bias with enrichment for breast cancer cell lines in this cell line set, which may have affected our results. Furthermore, we focused on in vitro cell growth inhibition, whereas in vivo cell signaling networks may differ, and in vitro approaches may not capture mechanism of growth inhibition in vivo. Finally, although our biomarker analysis in the NET trial is one of the largest series of pretreatment, and on-treatment biopsies of metastases reported to date, it was limited both because of overall study size and the number of responders seen in the study.

In conclusion, genomic aberrations of PIK3CA/PTEN are associated with rapamycin sensitivity. In addition, high p-Akt levels are associated with rapamycin sensitivity in vivo and may hold promise as a predictor in vivo. Feedback loop activation of Akt is greater in RS cells; thus treatment-associated increase in p-Akt is not a marker of resistance but rather of sensitivity. Further work is needed to better define the mechanism of differential regulation of Akt phosphorylation, and identify and validate markers of response and clinical benefit.

Disclosure of Potential Conflicts of Interest
G.B. Mills has received commercial research grant from AstraZeneca, Celgene, CeMines, Euxfixis, GlaxoSmithKline, IPath, Roche, SDI, and Wyeth/Pfizer; has ownership interest in Catena and PIV Ventures; and is a consultant/on the advisory board of Auranig, Aushon, Catena, Daichi, Foundation Medicine, and Komen. The other authors disclosed no potential conflicts of interest.

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