PIK3CA and AKT1 Mutations Have Distinct Effects on Sensitivity to Targeted Pathway Inhibitors in an Isogenic Luminal Breast Cancer Model System

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Abstract

Purpose: Activating mutations in the phosphoinositide-3-kinase (PI3K)/AKT/mTOR pathway are present in the majority of breast cancers and therefore are a major focus of drug development and clinical trials. Pathway mutations have been proposed as predictive biomarkers for efficacy of PI3K-targeted therapies. However, the precise contribution of distinct PI3K pathway mutations to drug sensitivity is unknown.

Experimental Design: We describe the creation of a physiologic human luminal breast cancer cell line model to study the phenotype of these mutations using the MCF-7 cell line. We used somatic cell gene targeting to "correct" PIK3CA E545K-mutant alleles in MCF-7 cells to wild-type sequence. The AKT1 E17K hotspot mutation was knocked in on this wild-type background.

Results: Loss of mutant PIK3CA dramatically reduced phosphorylation of AKT proteins and several known AKT targets, but other AKT target proteins and downstream effectors of mTOR were not affected. PIK3CA wild-type cells exhibited reduced proliferation in vitro and in vivo. Knockin of the AKT1 E17K hotspot mutation on this PIK3CA wild-type background restored pathway signaling, proliferation, and tumor growth in vivo. PIK3CA, but not AKT1 mutation, increased sensitivity to the PI3K inhibitor GDC-0941 and the allosteric AKT inhibitor MK-2206.

Conclusions: AKT1 E17K is a bona fide oncogene in a human luminal breast cancer context. Distinct PI3K pathway mutations confer differential sensitivity to drugs targeting the pathway at different points and by distinct mechanisms. These findings have implications for the use of tumor genome sequencing to assign patients to targeted therapies.
Translational Relevance

The phosphoinositide-3-kinase (PI3K) signaling pathway is of fundamental importance to both normal and malignant biology. Activation of the pathway by mutations in PIK3CA, PTEN, AKT1, and other genes is one of the most common events in cancer and is especially common in human breast cancer, making the PI3K pathway an excellent therapeutic target. As with other targeted therapies, there is great clinical interest in knowing whether mutations in the PI3K pathway can predict for efficacy of targeted therapies and guide selection of patients for clinical trials. We have used gene targeting to establish an isogenic panel of MCF-7 cell lines with differing mutational status of PIK3CA and AKT1. We show that AKT1 functions as a bona fide oncogene in luminal breast cancer, and that common mutations in PIK3CA and AKT1 confer distinct sensitivities to some, but not all, PI3K pathway inhibitors currently in clinical trials.

Given the clinical interest in understanding and targeting this pathway, there is intense interest in developing biomarkers that can predict drug activity. Several studies have tested in vitro sensitivity to these targeted agents in panels of human cancer cell lines and have attempted to identify genetic, transcriptional, or proteomic correlates of sensitivity and resistance. In such panels, PIK3CA-mutant and/or PTEN-deficient cancer cell lines have been shown to be more sensitive to GDC-0941 and MK-2206 (10, 12, 13). However, some apparently wild-type cell lines are also sensitive. As cancer cell lines are genetically complex and heterogeneous, even a handful of PIK3CA-mutant breast cancer cell lines in such studies will differ in TP53 status, ERBB2/Her-2, or other chromosomal amplifications, and many unknown genetic, genomic, and epigenetic aberrations.

As a complementary model system to isolate the phenotypic effects that can be ascribed to a single genotypic change, our laboratory creates human cell line models of recurrent breast cancer mutations using somatic cell gene targeting. Gene targeting to knock in an oncogene mutation allows physiologic expression and regulation of the oncogene from its endogenous regulatory elements. We have previously used this technology to create knockin models of PIK3CA E545K and H1047R mutations and AKT1 E17K mutations in nontumorigenic MCF-10A human breast epithelial cells. We showed that the knockin of PIK3CA but not AKT1 mutations into MCF-10A cells resulted in growth factor independence and increased sensitivity to the PI3K inhibitor LY294002 and the allosteric mTOR inhibitor rapamycin (14, 15). The lack of phenotype associated with knockin of the AKT1 E17K mutation was surprising and raised the question whether this mutation truly activates the PI3K pathway in human breast cancers and whether it can predict greater sensitivity to drugs targeting the PI3K pathway.

However, the distinct patterns of genetic alteration observed in breast cancer subtypes suggest that mutations occur and interact in a specific cellular context, and we and others have shown that oncogenes can have distinct phenotypes depending on expression level and regulation as well as cellular background (16, 17). To study the function of PIK3CA and AKT1 mutations in an isogenic context in a breast cancer cell line representative of the human breast cancers where these mutations commonly occur, we chose to use the MCF-7 cell line. MCF-7 has been used for decades to elucidate aspects of human breast cancer biology and as a preclinical model to test breast cancer treatments. MCF-7 expresses estrogen receptors (ER) and progesterone receptors, and in many respects, is a typical example of the luminal subtype of human breast cancer. MCF-7 carries mutations typically found in human luminal breast cancers, including PIK3CA E545K and a GATA3 frameshift mutation (18), and is TP53 wild-type. This luminal context may be especially relevant because the PIK3CA and AKT1 mutations are enriched in the luminal breast cancer subtypes. In addition, MCF-7 cells are capable of growing as tumor xenografts in nude mice, enabling the study of oncogene contributions to in vivo growth and preclinical modeling of drugs targeting these oncogenes.

Here, we report the creation of a panel of MCF-7–derived cell lines in which we used gene targeting to “correct” the naturally occurring PIK3CA E545K mutation back to wild-type and then to introduce the AKT1 E17K hotspot mutation. These isogenic cell lines, which differ by individual mutations in the same pathway, exhibit differences with respect to growth phenotypes and drug sensitivity. Our findings suggest that the relationship between pathway mutations and response to individual targeted drugs is not straightforward, but this cell line panel will be a useful tool to unravel these complexities and inform clinical practice in the age of personalized oncology.

Materials and Methods

Cell lines

MCF-7 cells were originally obtained from American Type Culture Collection. Parental MCF-7 cells and their derivatives were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 5% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Identity of MCF-7 cells was verified by sequencing for the described PIK3CA and GATA3 mutations, as well as by identification of described chromosomal amplifications by comparative genomic hybridization. The nontransformed human breast epithelial cell line MCF-10A and its PIK3CA and AKT1 knockin derivatives were maintained as described (14, 15). All cells were cultured at 37°C at 5% CO2.

Gene targeting of the PIK3CA and AKT1 loci

Targeted correction of the PIK3CA E545K mutation and knockin of the AKT1 E17K mutation were conducted with an adeno-associated viral vector as described (14, 15, 19). 5’- and 3’-homology arms were constructed by PCR using genomic DNA (gDNA) from MCF-10A as template for the
homology arms. Primer sequences for PCR are available on request. Because MCF-7 cells are not diploid (20), 2 rounds of gene targeting were necessary to “correct” both E545K alleles back to wild-type sequence. The first round of targeting used a vector with the E545 codon on the 5’-homology arm (a wild-type version of the E545K knockin vector described in Gustin and colleagues; ref. 15). The second round used a vector with the E545 codon in the 5’-homology arm, in an effort to reduce vector retargeting. The AKTI E17K knockin vector was described in Lauring and colleagues (15).

**gDNA and RNA extraction, cDNA synthesis, PCR, and DNA sequencing**

Genomic DNA and total RNA were prepared from cells using QIAamp DNA Blood kits and RNeasy kits (Qiagen), respectively. cDNA was synthesized with First-Strand cDNA Synthesis kits (GE Biosciences). PCR amplification was conducted with a GeneAmp 9700 (Applied Biosystems) and Phusion Hot Start II polymerase (NEB). For cDNA amplification, forward and reverse primers were located in distinct exons. DNA sequencing was carried out by the Johns Hopkins DNA Synthesis and Sequencing Facility. Primer sequences for PCR and sequencing are available on request.

**Immunoblotting**

Whole-cell protein extracts prepared in Laemmli sample buffer were resolved by SDS-PAGE using NuPage 4% to 12% gels (Invitrogen), transferred to Invitronol polyvinylidene difluoride membranes (Invitrogen), and probed with primary and horseradish peroxidase-conjugated secondary antibodies. The list of antibodies is provided in Supplementary Table S1. Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer).

**Growth assays**

MCF-7 cells and their derivative clones were plated in triplicate at 5,000 to 10,000 cells per well in 12-well plates. For experiments with estrogen, cells were grown in phenol red-free DMEM/F12 with 0.5% charcoal/dextran treated (CD)-FBS to which 1 nmol/L 17-estradiol (Sigma) was added. For growth in reduced growth factor conditions, the medium was changed and 10 dilutions of drug were added to each cell line in triplicate. On day 7, ATP was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions, using an Omega plate reader. All experiments were repeated at least 3 separate times in triplicate.

**Drug sensitivity assays**

GDC-0941, MK-2206, NVP-BEZ235, and perifosine were obtained from Selleck Chemicals. Inhibitors were reconstituted in dimethyl sulfoxide. Cells were seeded at 1,000 cells per well in triplicate in DMEM/5% FBS in 96-well plates on day zero for the MCF-7 cell line panel and supplemented DMEM/F12 (1:1) media with 0.2 ng/mL EGF for the MCF-10A cell line panel. On day 1, media were changed and 10 dilutions of drug were added to each cell line in triplicate. On day 7, ATP was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions, using an Omega plate reader. All experiments were repeated at least 3 separate times in triplicate.

**Xenografts**

The NIH Guide for the Care and Use of Laboratory Animals was followed in all experiments. Six-week-old female NCR nu/nu mice (Taconic) were implanted subcutaneously with slow release β-estradiol pellets. A total of 1 × 10⁶ cells in Matrigel (BD Biosciences) were injected into the flank subcutaneously. Tumors were measured weekly and tumor volumes were calculated using the formula π/6 (LxWxH).

**Results**

To assess the specific contribution of mutant PIK3CA to the growth and tumorigenic properties of MCF-7, we used gene targeting to “correct” the PIK3CA E545K mutation back to wild-type, essentially creating a knockin of wild-type sequence on the mutant allele. Because MCF-7 cells contain 3 copies of PIK3CA (20), one wild-type and 2 E545K alleles, 2 sequential rounds of gene targeting were necessary to create a PIK3CA wild-type derivative of MCF-7, hereafter referred to as MCF-7PIK3CAWT. Three clones with correction of one mutant PIK3CA E545K allele (leaving one remaining mutant PIK3CA allele) were derived as intermediates designated “Het” 1–3. Finally, to compare the effects of distinct PI3K pathway mutations in the same luminal breast cancer cell line context, we knocked in the AKTI E17K hotspot mutation on the PIK3CA wild-type background, deriving 3 independent targeted clones. Knockin was verified by sequencing gDNA and cDNA (Fig. 1 and Supplementary Fig. S1). All mutants were expressed at the expected ratios to wild-type transcripts based on the allelic ratios in gDNA.

**Signaling**

Correction of PIK3CA mutations in MCF-7 led to a marked reduction in activation of AKT proteins as measured by phosphorylation of threonine 308/309 and serines 473/474 (of AKT1 and AKT2, respectively; Fig. 2). One notable difference between PIK3CA- and AKTI-mutant cells is that the PIK3CA mutations led to increased phosphorylation of AKT2 S474 in addition to AKT1 S473, whereas the AKTI E17K-mutant cells only show increased phosphorylation of
AKT1, as one would expect. The reduced AKT activation in MCF-7\textsuperscript{PIK3CA\_WNT} cells in turn caused greatly reduced phosphorylation of known AKT target proteins, including PRAS40, FOXO 1/3, and AS160. The AKT1 E17K mutation fully restored phosphorylation of FOXO 1/3 and PRAS40 to levels equivalent to those in PIK3CA-mutant cells. Although AS160 has been proposed to be an AKT2-specific substrate, which regulates translocation of the glucose transporter GLUT4 to the plasma membrane in insulin-stimulated fibroblasts, our AKT1 E17K cells show comparable phosphorylation of AS160 serine 318 to PIK3CA-mutant cells, despite a lack of evident AKT2 activation. This supports the finding that AKT1 E17K can mediate gain of functions not normally carried out by the AKT1 isoform in untransformed cells (21). As others have noted, ablation of mutant PIK3CA did not have strong effects on phosphorylation of other proteins thought to be direct AKT targets, such as GSK3β (22, 23).

We and others have observed cross-talk between the PI3K and mitogen-activated protein kinase (MAPK) signaling pathways (14). Although mutant PIK3CA, but not AKT1 E17K, caused increased phosphorylation of the MAPK extracellular signal-regulated kinase (ERK)-1/2 in MCF-10A breast epithelial cells (15), correction of mutant PIK3CA to wild-type and knockin of AKT1 E17K did not affect ERK1/2 phosphorylation levels in MCF-7 cells, indicating that such cross-talk is likely cell type or context specific (Fig. 2). Despite modulation of PRAS40, which regulates the mTOR-containing TORC1 complex, mutual activation of PIK3CA or AKT1 did not consistently increase phosphorylation of mTOR at serine 2448 or phosphorylation of mTOR target proteins and their targets, including p70-ribosomal protein S6-kinase (p70S6K), eukaryotic elongation factor 4 binding protein 1 (eIF4EBP1), and ribosomal protein S6. These mTOR targets were significantly phosphorylated at baseline even in 0.5% CD-FBS conditions in our MCF-7 cells and their wild-type derivatives (Supplementary Fig. S2). Similarly, PIK3CA and AKT1 mutations did not lead to changes in cyclin D1 levels, even under low serum conditions, in contrast with what we observed previously in MCF-10A cells (14, 15). This is in concordance with the fact that MCF-7 cells continue to proliferate, albeit more slowly, under these growth factor-reduced conditions (Fig. 3A). When cells were stimulated with serum, increased signaling through the PI3K–AKT pathway was observed; however, the addition of serum did not restore phosphorylation levels of wild-type cells to the levels seen in mutant cells. In fact, AKT target protein phosphorylation (FOXO1/3 and PRAS40) remained very low in MCF-7\textsuperscript{PIK3CA\_WNT} cells even in the presence of full serum, suggesting that mutant PIK3CA and AKT1 lead to qualitative signaling changes, rather than merely quantitative changes that reduce the threshold for growth factor stimulation (compare Fig. 2 and Supplementary Fig. S2).

### Growth

Parental MCF-7 cells and derivatives with one helical domain mutation of PIK3CA grew faster than MCF-7\textsuperscript{PIK3CA\_WNT} cells in all media conditions tested, including full serum, low serum, and estrogen-stimulated growth (Fig. 3A–C).
Parental MCF-7 cells grew faster than targeted derivatives with only one mutant copy of \(\text{PIK3CA}^{E545K}\), suggesting that mutant gene dosage may confer an additional growth advantage beyond that provided by mutation alone. The introduction of the \(\text{AKT1}^{E17K}\) mutation on the \(\text{PIK3CA}\) wild-type background led to an increased growth rate, which was intermediate between wild-type and \(\text{PIK3CA}^{E545K}\)-mutant growth. Interestingly, \(\text{AKT1}^{E17K}\)-mutant cells grew relatively faster in 0.5% CD-FBS-containing media, intermediate between parental MCF-7 cells, and single-mutant \(\text{PIK3CA}^{E545K}\) cells. All of the cell lines were growth stimulated by estrogen, and \(\text{PIK3CA}\) or \(\text{AKT1}\)
mutation did not enhance estrogen responsiveness (Supplementary Fig. S3). In addition, AKT1-mutant cells increased soft agar colony formation above wild-type cells as well as parental MCF-7 and single copy PIK3CA-mutant knockin cells (Fig. 3D).

As our previous MCF-10A cell line models of PIK3CA and AKT1 mutations did not allow us to assess in vivo tumor growth properties, we tested the ability of the MCF-7 series of cell lines to grow as xenografts in nude mice implanted with estradiol pellets. Parental MCF-7 cells, derivatives with a single copy of PIK3CA E545K and AKT1 E17K/PIK3CAWT cells, all readily formed tumors, which grew with similar kinetics. In contrast, PIK3CA wild-type cells formed only very small tumors, even after 7 weeks of observation (Fig. 3E). Of note, parental MCF-7 cells remained absolutely dependent on estrogen supplementation for in vivo growth (data not shown). Thus, loss of mutant PIK3CA in MCF-7 cells impairs two-dimensional and three-dimensional growth in vitro and tumor growth in vivo. Collectively, these data also show for the first time that the AKT1 E17K mutation does confer an in vitro and in vivo growth advantage in a luminal breast cancer context.

Drug sensitivity

Other investigators have attempted to correlate the sensitivity of human cancer cell lines to drugs targeting the PI3K pathway with mutation of pathway genes. Several, but not all of these studies, have found a trend toward greater sensitivity of PIK3CA and PTEN-mutant/deficient cells to PI3K inhibitors (10, 12, 13, 24). Most of these studies have used panels of human cancer cell lines, which have been characterized to only a limited degree by DNA sequencing and necessarily differ from each other in many respects in terms of mutations in other genes, copy number changes, and epigenetic modifications. In addition, there have been no studies comparing AKT1-mutant cell lines with PIK3CA-mutant cell lines for sensitivity to PI3K pathway-targeted drugs. We therefore exposed our isogenic panels of MCF-7 cells to several PI3K pathway inhibitors currently being evaluated in clinical trials.

PIK3CA E545K-mutant cells were approximately 5-fold more sensitive to the class I PI3K inhibitor GDC-0941 than MCF-7/PIK3CAWT or AKT1 E17K-mutant cells (Fig. 4A and Supplementary Fig. S4). AKT1-mutant cells displayed equivalent sensitivity to PIK3CA wild-type cells. PIK3CA E545K-mutant MCF-7 cells also showed greatly increased sensitivity to the allosteric AKT inhibitor MK-2206 (IC_{50} ~200 nmol/L vs. ~7 μmol/L for wild-type), whereas AKT1 E17K cells were only modestly more sensitive than wild-type cells (Fig. 4B and Supplementary Fig. S4). AKT1 E17K-mutant knockin MCF-10A cells were also less sensitive to MK-2206 than PIK3CA-mutant cells and showed no increased sensitivity compared with a control MCF-10A cell line with knockin of a wild-type AKT1 allele (Supplementary Fig. S5; ref. 15). In contrast, IC_{50} values were similar for all MCF-7–derived cell lines, irrespective of genotype, to the dual PI3K/mTOR inhibitor NVP-BEZ235 (Fig. 4C) and the alkylphospholipid inhibitor perifosine (Supplementary Fig. S6), indicating that PIK3CA mutations sensitize to some, but not all, drugs targeting the PI3K pathway.

The differential sensitivity of PIK3CA and AKT1 E17K-mutant cells to GDC-0941 and MK-2206 led us to explore the impact of these inhibitors on pathway signaling (Fig. 5). At low doses, where parental MCF-7 cells were already significantly growth inhibited, GDC-0941 caused a surprising increase in phosphorylation of ERK proteins as well as AKT2 S474. We hypothesize that this increased phosphorylation may be mediated by relief of negative feedback mechanisms, as has been observed with other PI3K pathway inhibitors (25–27). This signaling was observed across PIK3CA and AKT1 genotypes and was suppressed at a higher

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**Figure 4.** Differential sensitivity of PIK3CA- and AKT1-mutant cells to targeted pathway inhibitors. MCF-7–derived cells of the indicated genotypes were treated with increasing concentrations of the class I–specific PI3K inhibitor GDC-0941 (A), the allosteric AKT inhibitor MK-2206 (B), and the dual PI3K-mTOR inhibitor NVP-BEZ235 (C). Viable cell number was determined with a luminescence assay. Results are depicted as viable cell number relative to untreated controls. Experiments were carried out in triplicate and repeated 3 times. Averages and SE of all 3 experiments are shown. WT, wild-type.

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A (400 nmol/L) dose of GDC-0941. GDC-0941 showed differences in its ability to suppress PI3K-dependent signaling across the genotypes. AKT1-mutant cells showed greater residual AKT1 S473 phosphorylation and greater phosphorylation of downstream proteins FOXO 1/3, PRAS40, GSK3β, and p70S6K at equivalent doses of GDC-0941, compared with PIK3CA-mutant cells. PIK3CA E545K-mutant cells showed a reduction in ribosomal protein S6 phosphorylation with increasing concentrations of GDC-0941, whereas the AKT1 E17K-mutant cells did not show suppression of S6 phosphorylation.

The differential sensitivity of PIK3CA- and AKT1 E17K-mutant cells to MK-2206 is not due to an inability of MK-2206 to inhibit signaling by the AKT1 E17K pleckstrin homology domain mutant (Fig. 5). AKT1-mutant cells showed equivalent suppression of FOXO 1/3 phosphorylation and slightly greater suppression of PRAS40 phosphorylation compared with PIK3CA-mutant cells. Both genotypes showed a reduction in the threonine 308 phosphorylation event that is a marker of AKT activation, and AKT1-mutant cells showed greater and more consistent suppression of T308 phosphorylation than PIK3CA-mutant cells. The effect of MK-2206 on the serine 473 phosphorylation mark of AKT1 was uneven and did not correlate with the inhibition of AKT activity, but the AKT1-mutant cells maintained higher levels of AKT1 serine 473 phosphorylation with increasing concentrations of MK-2206. Of note, MK-2206 completely inhibited AKT2 activation (as measured by serine 474 phosphorylation) at 100 nmol/L, a concentration where PIK3CA E545K-mutant, but not AKT1-mutant cells were already strongly growth inhibited. MK-2206 treatment up to 1 μmol/L did not have strong effects on other protein biomarkers such as ribosomal protein S6, even though growth was suppressed by approximately 80% in MCF-7 cells at this concentration.

Figure 5. PI3K pathway signaling in GDC-0941 and MK-2206 treated MCF-7 derivatives with PIK3CA or AKT1 mutations. Cells were grown in medium containing 5% FBS and treated with vehicle or increasing concentrations of GDC-0941 (0 nmol/L, 50 nmol/L, 100 nmol/L, and 400 nmol/L) or MK-2206 (0 nmol/L, 100 nmol/L, 250 nmol/L, and 1000 nmol/L). After 24 hours of drug treatment, lysates were prepared and equal amounts of protein were loaded onto SDS-PAGE gels and blotted with the indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Discussion

A decade of research has established widespread mutational activation of the PI3K pathway as a central event in many types of cancer, and breast cancer in particular. Drugs targeting this pathway at various points are approved for use or under evaluation in clinical trials. Despite this rapid progress in knowledge, there is much that we still do not understand about how mutational activation of this pathway differs from activation in response to stimuli and how mutations relate to sensitivity to PI3K pathway-targeted drugs. As an approach to these questions, we have established an isogenic human luminal breast cancer model in the MCF-7 cell line to study the biology of different PI3K pathway mutations in their native cellular context. We and others previously modeled these same mutations in the immortalized, nontumorigenic human breast epithelial cell line MCF-10A (14, 15, 28). Although MCF-10A has been a good model to study the effects of oncogenes and growth and morphogenesis, there may be situations where cellular context is important for revealing phenotypes induced by mutant genes. PIK3CA mutations occur at the highest frequency in luminal type breast cancers, and to date AKT1 mutations have only been observed in ER" breast cancers (3, 4, 29). Our previous study in MCF-10A cells failed to show major phenotypic differences between AKT1 E17K and AKT1 wild-type MCF-10A cells, raising the possibility that AKT1 mutations might be less oncogenic than PIK3CA mutations. For these reasons, we sought to evaluate these mutations in a distinct cellular context. MCF-7 cells have long served as a faithful model of luminal breast cancer biology.

Previously, the Vogelstein laboratory used gene targeting to knock out either the wild-type copy or the mutant copy of PIK3CA in human colon cancer cell lines with either E545K or H1047R mutation (23). Taking a different approach, we used gene-targeted knockin to "correct" the E545K allele back to wild-type in MCF-7, and then to knock in the AKT1 E17K mutation on a wild-type background. In excellent agreement with their work, correction of the E545K mutation to wild-type MCF-7 cells led to a reduced EGF threshold for AKT signaling (similar in intensity to mutant PIK3CA-induced signaling), increased in vitro proliferation and soft agar colony formation, and stimulation of tumor xenograft growth in vivo. Thus, we conclude that AKT1 E17K is a bona fide oncogene in luminal breast cancers, when expressed physiologically from its endogenous locus, and we have created a relevant human breast cancer model to study its biology.

There is great clinical interest in determining whether mutations in the PI3K pathway can serve as biomarkers to predict sensitivity to drugs targeting the pathway. Work in other cancers has shown that in some cases only mutational activation of a pathway confers sensitivity to targeted therapies, as in the case of EGF receptor tyrosine kinase inhibitors for lung cancer and imatinib for gastrointestinal stromal tumors. Using panels of nonisogenic cancer cell lines, previous investigators have shown a trend toward increased sensitivity of PIK3CA-mutant cell lines to PI3K inhibitors, the allosteric AKT inhibitor MK-2206, and the allosteric mTOR inhibitor everolimus (10, 12, 13, 24). Although suggestive, the use of nonisogenic lines necessarily means that in addition to the presence or absence of a PIK3CA mutation, each cell line may have other alterations that could impact sensitivity. Isogenic cell lines offer a complementary approach to determining the impact of genotype on drug sensitivity. In addition, our knockin model of the AKT1 E17K mutation allows for assessment of this mutation as a predictive biomarker because none of the human breast cancer cell lines identified to date has an AKT1 mutation.

In agreement with previous reports using nonisogenic cell lines, PIK3CA helical domain mutant MCF-7 cells had a 5-fold lower IC50 for the class I PI3K inhibitor GDC-0941 than PIK3CA wild-type cells. It seems logical that cells with greater activation of the pathway would be more sensitive to its inhibition. AKT1-mutant cells showed the same sensitivity as wild-type cells, however, despite having a nearly identical signaling profile as PIK3CA-mutant cells. Therefore, the AKT1 E17K mutation does not necessarily predict...
for resistance to GDC-0941: it simply does not predict for greater sensitivity, in contrast with the E545K PIK3CA mutation. Alternatively, one could hypothesize that the AKT1 mutant, being downstream from PI3K, might be resistant to inhibition of PI3K. Our Western blotting data do show that AKT1 phosphorylation and phosphorylation of AKT targets FOXO 1/3 and PRAS40 are less inhibited at equivalent drug exposure compared with PIK3CA E545K cells. However, it is important to note that AKT1-mutant cells were at least as sensitive as wild-type cells to GDC-0941. As others have shown, a reduction in S6 phosphorylation is a pharmacodynamic marker of GDC-0941 activity, which correlates with the effect on proliferation (12). However, mutational activation of the pathway per se does not predict whether the drug will lead to reduced S6 phosphorylation. A key difference between PIK3CA-mutant and AKT1-mutant cells is that PIK3CA mutation leads to activation of both AKT1 and AKT2. If activation of AKT2 is critical to the proliferation of PIK3CA-mutant cells, PI3K inhibition might have a greater impact on PIK3CA-mutant cells.

For MK-2206, equivalent suppression of AKT-dependent signaling was observed in both sensitive and resistant cell lines. Although AKT1-mutant cells showed greater residual AKT1 serine 473 phosphorylation than PIK3CA-mutant cells, AKT-dependent target protein phosphorylation was shut down at least as effectively in AKT1-mutant cells as in PIK3CA-mutant cells. Other investigators have shown that purified AKT1 E17K protein is more resistant than wild-type AKT1 to the allosteric AKT inhibitor VIII but not to the ATP-competitive AKT inhibitors, GNE-929 and GSK690693 (5, 6). Inhibitor VIII caused greater growth inhibition of AKT1 wild-type than AKT1 E17K-mutant–transfected cells, whereas GNE-929 inhibited proliferation of both genotypes (6). We did not observe a great difference in sensitivity to MK-2206 of wild-type versus AKT1 E17K-mutant cells in a viability assay. Western blotting showed that MK-2206 was able to effectively shut down AKT-dependent signaling at doses well below those where significant growth inhibition occurred. MK-2206 is structurally different from inhibitor VIII, which may allow it to inhibit the E17K mutant effectively. Again, the greater effectiveness of MK-2206 against PIK3CA-mutant cells may be due to a greater reliance of PIK3CA-mutant cells on AKT2 for viability. Another possibility is that MK-2206 inhibits a target other than AKT1 and AKT2, and this target is somehow activated or expressed selectively in PIK3CA helical domain mutant cells. Although SGK3 has been proposed as one such AKT-independent, PI3K-dependent target, we could find no evidence for significant or differential SGK3 phosphorylation in our cell lines (data not shown).

The differential sensitivity of mutant versus wild-type and PIK3CA-mutant versus AKT1-mutant cells was not a universal finding to all the pathway inhibitors we tested. Although GDC-0941 and MK-2206 exhibited differential sensitivities (and possibly for different reasons), the dual PI3K/mTOR inhibitor NVP-BEZ235 potently inhibited growth irrespective of genotype. This effect may be related to the ability of NVP-BEZ235 to more potently suppress mTOR-dependent signaling to ribosomal protein S6 and EIF4EBP1, which were not completely dependent on mutant PIK3CA status in MCF-7 cells (24). Lack of selectivity for mutations was also seen with perifosine.

The general mutual exclusivity of distinct PI3K pathway mutations in primary human breast cancers in large sequencing studies conducted to date has suggested that distinct mutations in the pathway are “sufficient” to cause tumor initiation or progression. This has led to the idea that targeted therapies can be selected by identifying patients whose tumors harbor any PI3K pathway mutations. Some early-phase clinical trials have suggested better response rates to PI3K inhibitors or everolimus in patients with PIK3CA mutations (30–34). Various investigators have shown that PTEN-deficient cancers depend on PIK3CB, rather than PIK3CA, signaling, and these cancers are more sensitive in preclinical models to inhibitors that target this PI3K isofrom (9, 35). There are currently no clinical data on the sensitivity of AKT1-mutant cancers to PI3K pathway-targeted drugs. Our data suggest that different agents targeting the pathway at different levels and using distinct biochemical mechanisms may have differential efficacy against cancers with PIK3CA mutations and AKT1 mutations. Correlative analyses of clinical trials using these agents will complement studies, such as this one, to determine the predictive value of specific PI3K pathway mutations for response to targeted therapies.
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