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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 utilized 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. Knock-in 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.

Keywords

PIK3CA; AKT; mutation; gene targeting; GDC-0941; MK-2206

Introduction

Signaling through the PI3K/AKT/mTOR pathway is critical to normal and malignant cellular processes, including proliferation, apoptosis, and metabolism^{1,2}. Recurrent somatic

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mutations in this pathway have been found in many human cancers and shown to result in pathway activation. Specifically, *PIK3CA*, the gene that encodes the p110 catalytic component of PI3K is commonly mutated in ~36% of breast cancers, and 80–90% of mutations in *PIK3CA* cluster into three hotspot regions in Exon 9 (E545K and E542K) and Exon 20 (H1047R)^{3,4}. *AKT1* pleckstrin homology domain mutations have been found in ~3% of breast cancers. A dominant hotspot mutation results in glutamate 17 to lysine (E17K) substitution, but other, rarer non-hotspot *AKT1* mutations are functionally activating as well^{5–7}. Given the commonality of PI3K/AKT/mTOR pathway dysregulation in human malignancies, genes involved in this pathway are appealing targets for drug development.

GDC-0941 and MK-2206 are two PI3K pathway inhibiting drugs that are in advanced stages of clinical development. GDC-0941 is an oral class I pan-PI3K inhibitor⁸ with activity in the nanomolar range against a wide range of cancer cell lines⁹. MK-2206 is an oral pan-AKT allosteric inhibitor that has been shown to have activity in a panel of breast cancer cell lines which naturally harbor differing *PIK3CA* and *PTEN* mutations¹⁰. Both drugs are currently being evaluated in a number of phase I and II clinical trials, many of which are breast cancer-specific. Many of these trials are limiting recruitment to patients whose tumors harbor *PIK3CA* mutations or *PTEN* loss¹¹.

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 knock-in models of *PIK3CA* E545K and H1047R mutations and *AKT1* E17K mutations in non-tumorigenic MCF-10A human breast epithelial cells. We showed that the knock-in 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 knock-in of the *AKT1* E17K mutation was surprising and raised the question whether this mutation truly activates the PI3K pathway in human breast cancers and if it can predict for 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}. In order 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 (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 mutation¹⁸, and is *TP53* wild type. This luminal context may be especially relevant, since 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 utilized 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 ATCC. Parental MCF-7 cells and their derivatives were maintained in 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 non-transformed human breast epithelial cell line MCF-10A and its *PIK3CA* and *AKT1* knock in 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 knock-in 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 diploid²⁰, two 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 3' homology arm (a wild type version of the E545K knock-in vector described in Gustin et al. 2009). The second round used a vector with the E545 codon in the 5' homology arm, in an effort to reduce vector re-targeting. The *AKT1* E17K knock-in vector was described in Lauring et al. 2010¹⁵.

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 performed 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 4–12% gels (Invitrogen), transferred to Invitrolon polyvinylidene difluoride membranes (Invitrogen), and probed with primary and horseradish peroxidase–conjugated secondary antibodies. The list of antibodies is provided in Supplementary Table 1. Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer). Lysates from cells of different genotypes were always run together on the same gel for all results shown.

Growth assays

MCF-7 cells and their derivative clones were plated in triplicate at 5,000–10,000 cells/ well in 12-well plates. For experiments with estrogen cells were grown in phenol red-free DMEM/F12 with 0.5% CD-FBS to which 1 nM 17- β -estradiol (Sigma) was added. For growth in reduced growth factor conditions, the above medium was used without estradiol supplementation. Media were replaced every 3 days until the time of harvest. Cell counts were obtained using a Vi-Cell automated counter (Beckman Coulter).

Soft agar colony formation assay

3×10^4 exponentially growing cells were cast in 3 mL of top layer medium composed of phenol red-free DMEM/F12 with 5% FBS and 0.4% UltraPure agarose (Invitrogen) and poured on top of a 2 mL bottom layer containing 0.6% agarose in six-well tissue culture plates. Medium was added to the wells once a week. Colonies were stained with crystal violet and counted using Image J software. Two independent experiments were done in triplicate.

Drug sensitivity assays

GDC-0941, MK-2206, NVP-BEZ235, and perifosine were obtained from Selleck Chemicals. Inhibitors were reconstituted in DMSO. Cells were seeded at 1000 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 0.2ng/ml EGF for the MCF-10A cell line panel. On day one media was changed and ten dilutions of drug were added to each cell line in triplicate. On day seven, ATP was measured using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions, using an Omega plate reader. All experiments were repeated at least three separate times in triplicate.

Xenografts

The NIH Guide for the Care and Use of Laboratory Animals was followed in all experiments. 6 week old female NCR nu/nu mice (Taconic) were implanted subcutaneously with slow release β -estradiol pellets. $1\text{--}10^6$ cells in Matrigel (BD Biosciences) were injected in the flank subcutaneously. Tumors were measured weekly and tumor volumes were calculated using the formula $V/6(L \times W \times H)$.

Results

In order to assess the specific contribution of mutant *PIK3CA* to the growth and tumorigenic properties of MCF-7, we utilized gene targeting to “correct” the *PIK3CA* E545K mutation back to wild type, essentially creating a knock-in of wild type sequence on the mutant allele. Because MCF-7 cells contain three copies of *PIK3CA*²⁰, one wild type and two E545K alleles, two sequential rounds of gene targeting were necessary to create a *PIK3CA* wild type derivative of MCF-7, hereafter referred to as MCF-7^{PIK3CAWT}. 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, in order to compare the effects of distinct PI3K pathway mutations in the same luminal breast cancer cell line context, we knocked in the *AKT1* E17K hotspot mutation on the *PIK3CA* wild type background, deriving three independent targeted clones. Knock-in was verified by sequencing genomic DNA and cDNA (Figure 1 and Supplementary Figure 1). All mutants were expressed at the expected ratios to wild type transcripts based on the allelic ratios in genomic DNA.

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, Figure 2). One notable difference between *PIK3CA* and *AKT1* mutant cells is that the *PIK3CA* mutations led to increased phosphorylation of AKT2 S474 in addition to AKT1 S473, whereas the *AKT1* E17K mutant cells only show increased phosphorylation of AKT1, as one would expect. The reduced AKT activation in MCF-7^{*PIK3CA*WT} 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 demonstrate 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 cells²¹. 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 GSK3^{22,23}.

We and others have observed cross-talk between the PI3K and mitogen activated protein kinase (MAPK) signaling pathways¹⁴. Whereas mutant *PIK3CA*, but not *AKT1* E17K caused increased phosphorylation of the MAPKs ERK1/2 in MCF-10A breast epithelial cells¹⁵, correction of mutant *PIK3CA* to wild type and knock-in 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 (Figure 2). Despite modulation of PRAS40, which regulates the mTOR containing TORC1 complex, mutational 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% charcoal/dextran (CD)-treated fetal bovine serum (FBS) conditions in our MCF-7 cells and their wild type derivatives (Supplementary Figure 2). Similarly, *PIK3CA* and *AKT1* mutations did not lead to changes in cyclin D1 levels, even under low serum conditions, in contrast to 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 (Figure 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 levels seen in mutant cells. In fact, AKT target protein phosphorylation (FOXO1/3 and PRAS40) remained very low in MCF-7^{*PIK3CA*WT} 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 Figure 2 and Supplementary Figure 2).

Growth

Parental MCF-7 cells and derivatives with one helical domain mutation of *PIK3CA* grew faster than MCF-7^{*PIK3CA*WT} cells in all media conditions tested, including full serum, low serum, and estrogen-stimulated growth (Figure 3, A–C). Parental MCF-7 cells grew faster than targeted derivatives with only one mutant copy of *PIK3CA* E545K, suggesting that mutant gene dosage may confer an additional growth advantage beyond that provided by mutation alone. The introduction of the *AKT1* E17K mutation on the *PIK3CA* wild type background led to an increased growth rate, which was intermediate between wild type and *PIK3CA* E545K mutant growth. Interestingly, *AKT1* E17K mutant cells grew relatively faster in 0.5% CD-FBS containing media, intermediate between parental MCF-7 cells and single-mutant *PIK3CA* E545K cells. All of the cell lines were growth-stimulated by estrogen, and *PIK3CA* or *AKT1* mutation did not enhance estrogen responsiveness (Supplemental Figure 3). 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 knock-in cells (Figure 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/*PIK3CA*WT 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 (Figure 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 demonstrate 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 employed panels of human cancer cell lines which have been characterized to only a limited degree by DNA sequencing, and which 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 to *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^{*PIK3CA*WT} or *AKT1* E17K mutant cells (Figure 4A and Supplemental Figure 4). *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 (IC₅₀ ~200 nM versus ~7 μM for wild type), whereas *AKT1* E17K cells were only modestly more sensitive than wild type cells (Figure 4B and Supplemental Figure 4). *AKT1* E17K mutant knock-in MCF-10A cells were also less sensitive to MK-2206 than *PIK3CA* mutant cells and showed no increased sensitivity compared to a control MCF-10A cell line with knock-in of a wild type *AKT1* allele (Supplementary Figure 5)¹⁵. In contrast, IC₅₀ values were similar for all MCF-7 derived cell lines, irrespective of genotype, to the dual PI3K/mTOR inhibitor NVP-BE2235

(Figure 4C) and the alkylphospholipid inhibitor perifosine (Supplementary Figure 6), 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 (Figure 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 (400 nM) dose of GDC-0941. GDC-0941 showed differences in its ability to suppress PI3K-dependent signaling across the genotypes. *AKT1* mutant cells demonstrated greater residual AKT1 S473 phosphorylation and greater phosphorylation of downstream proteins FOXO 1/3, PRAS40, GSK3 β , and p70S6K at equivalent doses of GDC-0941, compared to *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 (Figure 5). *AKT1* mutant cells showed equivalent suppression of FOXO1/3 phosphorylation and slightly greater suppression of PRAS40 phosphorylation compared to *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 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 nM, a concentration where *PIK3CA* E545K mutant, but not *AKT1* mutant cells were already strongly growth inhibited. MK-2206 treatment up to 1 μ M 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.

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, non-tumorigenic 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 mutation²³. Taking a different approach, we used gene targeted knock-in to “correct” the E545K alleles back to wild type in MCF-7, and then to knock in the *AKT1* E17K mutations on a wild type background. In excellent agreement with their work, correction of the E545K mutation in MCF-7 cells led to a selective loss of AKT phosphorylation and phosphorylation of AKT targets FOXO 1/3 and PRAS40 in both low and high serum conditions, but mTOR-dependent signaling to p70S6K and EIF4EBP1 was not significantly affected. In contrast, knock in of *PIK3CA* mutations in MCF-10A cells led to a reduced EGF threshold for mTOR activation as well as activation of MAPK signaling^{14,15}. Collectively these results argue that the cellular context determines the differential signaling output of identical mutations.

Again, similar to the findings of Vogelstein et al. in colon cancer cells, *PIK3CA* E545K cells showed increased proliferation rates in all growth conditions compared to wild type cells. For the first time, however, we have shown a proliferative advantage with physiologic knock-in of the *AKT1* E17K mutation on the MCF-7 background. The reason we did not observe a proliferation effect in MCF-10A cells is unclear, although others have recently shown differential effects of *AKT1* E17K mutation in distinct breast epithelial contexts¹⁶. In our MCF-7 system, *AKT1* E17K mutants generally grew in vitro at an intermediate rate compared to wild type cells and *PIK3CA* E545K mutant cells. We did not observe a special synergy between *AKT1* mutation and estrogenic growth. In fact, the only media condition where the *AKT1* mutant grew as well as *PIK3CA* mutants in vitro was growth factor reduced 0.5% CD-FBS. In vivo, however, *AKT1* mutant tumors grew with similar kinetics to the parental MCF-7 cells and derivatives with a single E545K mutation. Since the mice were supplemented with estrogen, it is possible that this parity with *PIK3CA* mutants supports a role for *AKT1* mutations in an estrogenic context. For the first time we have demonstrated oncogenic activity for the *AKT1* E17K mutation compared to wild type cells using physiologic expression of the mutation from its endogenous locus. The *AKT1* mutant cells show evidence of activated 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 EGFR tyrosine kinase inhibitors for lung cancer and imatinib for gastrointestinal stromal tumors. Using panels of non-isogenic 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 non-isogenic 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 knock-in model of the *AKT1* E17K mutation allows for

assessment of this mutation as a predictive biomarker, since none of the human breast cancer cell lines identified to date has an *AKT1* mutation.

In agreement with previous reports using non-isogenic 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 to 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 to *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 proliferation¹². 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, then 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 genotypes⁶. 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 AKT 1 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. Whereas 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 cells²⁴. 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 performed 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 isoform^{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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Establishment of MCF-7^{PIK3CAWT} cells and *PIK3CA* WT/*AKT1* E17K derivatives
 Sequencing of genomic DNA and cDNA is shown. Parental MCF-7 cells have two mutant *PIK3CA* E545K alleles and one wild type allele. The cell line designated "Het" has one allele with targeted correction to wild type and one remaining mutant allele. A second round of gene targeting was performed to obtain the *PIK3CA* WT cells. The *PIK3CA* WT cells were used to knock in the *AKT1* E17K mutation.

Figure 2. PI3-kinase pathway signaling in the MCF-7 cell line panel

Cells were grown in medium containing 5% FBS. Equal amounts of protein were loaded on SDS-PAGE gels and blotted with the antibodies shown. The full list of antibodies is included in Supplementary Table 1.

Figure 3. Growth characteristics of MCF-7 cells with different PI3K pathway genotypes
 A–C. Proliferation in vitro in 0.5% charcoal/dextran-treated FBS medium (CD-FBS) (A), 1 nM α -estradiol in 0.5% CD-FBS (B), and 5% FBS medium (C). Cells were seeded at 5–10,000 cells/well in 12-well plates in triplicate and counted every 2–3 days. D. Colony formation in soft agar. Cells were seeded at 30,000 cells/well in triplicate in 0.4% agar atop a layer of 0.6% agar, and colonies were stained and counted after 21 days. E. In vivo growth of xenografts in estrogen-supplemented nude mice. 10 mice per group were inoculated with one million cells of each genotype in Matrigel. Tumors were measured weekly. In all cases error bars represent standard deviations.

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 performed in triplicate and repeated three times. Averages and standard errors of all three experiments are shown.

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 nM, 50 nM, 100 nM, and 400 nM) or MK-2206 (0 nM, 100 nM, 250 nM, 1000 nM). 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.