

An Integrative Genomic and Proteomic Analysis of PIK3CA, PTEN, and AKT Mutations in Breast Cancer

Katherine Stemke-Hale,^{1,7} Ana Maria Gonzalez-Angulo,^{1,2,7} Ana Lluch,⁸ Richard M. Neve,⁹ Wen-Lin Kuo,⁹ Michael Davies,^{1,3,7} Mark Carey,^{1,7} Zhi Hu,⁹ Yinghui Guan,⁹ Aysegul Sahin,⁴ W. Fraser Symmans,⁴ Lajos Pusztai,² Laura K. Nolden,¹ Hugo Horlings,¹⁰ Katrien Berns,¹¹ Mien-Chie Hung,⁵ Marc J. van de Vijver,¹⁰ Vicente Valero,² Joe W. Gray,⁹ René Bernards,¹¹ Gordon B. Mills,^{1,7} and Bryan T. Hennessy^{1,6,7}

Departments of ¹Systems Biology, ²Breast Medical Oncology, ³Melanoma Medical Oncology, ⁴Pathology, ⁵Molecular and Cellular Oncology, and ⁶Gynecologic Medical Oncology and ⁷Kleberg Center for Molecular Markers, The University of Texas M. D. Anderson Cancer, Houston, Texas; ⁸Universidad de Valencia Clinic Hospital, Valencia, Spain; ⁹Lawrence Berkeley National Laboratory, Berkeley, California; and ¹⁰Division of Experimental Therapy and ¹¹Division of Molecular Carcinogenesis and Center for Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, the Netherlands

Abstract

Phosphatidylinositol 3-kinase (PI3K)/AKT pathway aberrations are common in cancer. By applying mass spectroscopy-based sequencing and reverse-phase protein arrays to 547 human breast cancers and 41 cell lines, we determined the subtype specificity and signaling effects of PIK3CA, AKT, and PTEN mutations and the effects of PIK3CA mutations on responsiveness to PI3K inhibition *in vitro* and on outcome after adjuvant tamoxifen. PIK3CA mutations were more common in hormone receptor-positive (34.5%) and HER2-positive (22.7%) than in basal-like tumors (8.3%). AKT1 (1.4%) and PTEN (2.3%) mutations were restricted to hormone receptor-positive cancers. Unlike AKT1 mutations that were absent from cell lines, PIK3CA (39%) and PTEN (20%) mutations were more common in cell lines than tumors, suggesting a selection for these but not AKT1 mutations during adaptation to culture. PIK3CA mutations did not have a significant effect on outcome after adjuvant tamoxifen therapy in 157 hormone receptor-positive breast cancer patients. PIK3CA mutations, in comparison with PTEN loss and AKT1 mutations, were associated with significantly less and inconsistent activation of AKT and of downstream PI3K/AKT signaling in tumors and cell lines. PTEN loss and PIK3CA mutation were frequently concordant, suggesting different contributions to pathophysiology. PTEN loss rendered cells significantly more sensitive to growth inhibition by the PI3K inhibitor LY294002 than did PIK3CA mutations. Thus, PI3K pathway aberrations likely play a distinct role in the pathogenesis of different breast cancer subtypes. The specific aberration present may have implications for the selection of PI3K-targeted therapies in hormone receptor-positive breast cancer. [Cancer Res 2008;68(15):6084–91]

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

K. Stemke-Hale and A.M. Gonzalez-Angulo contributed equally to this work.

Requests for reprints: Bryan T. Hennessy, Departments of Gynecologic Medical Oncology and Systems Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-563-1792; Fax: 713-745-1541; E-mail: bhennessy@mdanderson.org.

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Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway mediates key cellular functions, including growth, proliferation, survival, angiogenesis, and motility (1). PI3K phosphorylates membrane phosphatidylinositols, thereby recruiting AKT and phosphoinositide-dependent kinase (PDK) 1 to the cell membrane. PDK1 and PDK2, likely the TORC2 complex, phosphorylate AKT and initiate a downstream signaling cascade. The tumor suppressor PTEN reverses the effects of PI3K by dephosphorylating the same site on membrane phosphatidylinositols that is phosphorylated by PI3K. The growth factor receptor signaling and energy sensing LKB1-AMPK pathways integrate at the tuberous sclerosis complex (TSC), resulting in information transfer to the TORC1 complex and protein synthesis. In normal cells, the PI3K pathway is under tight homeostatic control through feedback regulatory loops that maintain normal cellular function and regulate glucose homeostasis (2).

Activating mutations in *PIK3CA*, *PIK3R1*, and *AKT1* and inactivating mutations in *PTEN*, *LKB1*, and *TSC2* are present in a broad range of tumor types (1). Further, germ-line mutations in *PTEN*, *LKB1*, and *TSC* result in hamartomatous cancer predisposition syndromes (Cowden's, Peutz-Jeghers, and tuberous sclerosis, respectively). Additional pathway components, including *PIK3CA*, *PIK3CB*, *AKT1* and *AKT2*, *PDK1*, p70S6 kinase (p70S6K), and *IKBKE*, are frequently amplified in tumors. Because genomic aberrations can predict responsiveness to targeted therapies, and because multiple PI3K pathway members are frequently aberrant in breast tumors, targeting this pathway may provide a highly effective therapeutic approach (1, 3).

PIK3CA mutations, primarily at hotspots in exons 9 and 20 that encode portions of the helical and kinase domains of PI3K, have been reported to occur in approximately one third of breast cancers (4). These mutations have been reported to activate AKT and downstream signaling in model systems but their effects in patient tumors are unknown (5). *PTEN* mutations are relatively uncommon in breast cancer (<5%); however, PTEN protein loss (e.g., promoter methylation, loss of heterozygosity, and regulation at the RNA or protein level) is more common (~30%; ref. 1). A recent report identified a somatic mutation in the PH domain of *AKT1* (E17K) in 8% of breast cancers in a small sample set (6). This mutation activates AKT1 by recruiting it to the membrane through a PI3K-independent mechanism (6). However, two large-scale sequencing studies failed to detect mutations at this site in any AKT isoform across multiple tumor types, raising the possibility

Table 1. Frequency of mutations in the *PIK3CA*, *AKT1*, and *PTEN* genes in 547 human breast cancers and 41 breast cancer cell lines

Tumor subtype	Mutation				
	<i>PIK3CA</i> catalytic domain*	<i>PIK3CA</i> other [†]	<i>PIK3CA</i> total	<i>PTEN</i> [‡]	<i>AKT1</i> E17K
All human breast tumors	73/547 (13.3%)	44/547 (8.0%)	117/547 (21.4%)	2/88 (2.3%)	6/418 (1.4%)
Human breast HR+ [§]	48/232 (20.7%)	32/232 (13.8%)	80/232 (34.5%)	2/58 (3.4%)	6/232 (2.6%)
ER+PR+	39/186 (21%)	22/186 (11.8%)	61/186 (32.8%)	1/48 (2.1%)	6/186 (3.2%)
ER+PR–	9/41 (22%)	10/41 (24.4%)	19/41 (46.3%)	1/8 (12.5%)	0/41 (0%)
ER–PR+	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/2 (0%)	0/5 (0%)
Human breast HER2+	13/75 (17.3%)	4/75 (5.3%)	17/75 (22.7%)	0/10 (0%)	0/75 (0%)
Human breast TN	12/240 (5.0%)	8/240 (3.3%)	20/240 (8.3%)	0/20 (0%)	0/111 (0%)
All breast cancer cell lines	7/41 (17.1%)	9/41 (22%)	16/41 (39%)	8/41 (20%)	0/41 (0%)
Breast cancer cell lines HR+	1/12 (8.3%)	3/12 (25%)	4/12 (33.3%)	5/12 (41.7%)	0/12 (0%)
Breast cancer cell lines HER2+	2/10 (20%)	4/10 (40%)	6/10 (60%)	0/10 (0%)	0/10 (0%)
Breast cancer cell lines TN [¶]	4/19 (21%)	2/19 (10.5%)	6/19 (31.6%)	3/19 (15.8%)	0/19 (0%)

Abbreviations: HR+, hormone receptor positive; TN, triple negative.

* Catalytic domain *PIK3CA* mutations include H1047R, H1047L, H1047Y, and G1049R in order of frequency.

[†] Other *PIK3CA* mutations include E545K, E542K, N345K, E418K, and P539R in order of frequency.

[‡] Resequencing courtesy of Len Pennacchio and Jan-Fang Cheng of Lawrence Berkeley National Laboratory in breast cancers and by literature/internet (e.g., <http://www.sanger.ac.uk>) search in cell lines.

[§] Two distinct *PIK3CA* mutations were found together in four patients with hormone receptor-positive breast cancer (*PIK3CA*_H1047R with *PIK3CA*_E545K, *PIK3CA*_H1047Y with *PIK3CA*_E545K, *PIK3CA*_H1047R with *PIK3CA*_K111N, and *PIK3CA*_E545K with *PIK3CA*_E418K).

^{||} DNA extraction in these tumors courtesy of Mandy Madiredjo at The Netherlands Cancer Institute.

[¶] One triple-negative breast cancer cell line (BT20) had both a catalytic domain H1047R and a noncatalytic domain P539R mutation in *PIK3CA*.

that the former study overestimated the frequency of *AKT1* mutations in breast cancer (7, 8). Although the frequencies of these mutations have been explored in small breast cancer sets, there has been no comprehensive analysis of the mutational frequency of multiple PI3K pathway members in a large breast cancer series representing the three major subtypes of hormone receptor-positive, HER2-positive, and basal-like tumors. As the cell of origin, treatment, and outcomes are markedly different in the three major breast cancer subtypes, it is critical that the effects of PI3K pathway aberrations on pathophysiology and therapy responsiveness are analyzed independently in each subtype. Recent studies from us and others implicate *PIK3CA* mutations and *PTEN* loss in resistance of HER2-positive breast cancers to trastuzumab (9, 10). However, the role of PI3K pathway aberrations in the clinical behavior and therapy responsiveness of hormone receptor-positive tumors remains controversial, in part due to analysis of small and clinically heterogeneous sample sets.

We thus determined the frequency, breast cancer subtype specificity, and signaling effects of *PIK3CA*, *AKT*, and *PTEN* mutations in human breast tumors and breast cancer cell lines. Further, we examined the effects of *PIK3CA* mutations on hormone receptor-positive breast cancer patient outcomes after adjuvant tamoxifen treatment.

Materials and Methods

Human breast tumor samples. Human breast tumors were obtained from Tumor Banks following pathologist review under the auspices of Institutional Review Board–approved protocols at Clinic Hospital (Valencia, Spain; 306 tumors), the Netherlands Cancer Institute (34 tumors), and the M. D. Anderson Cancer Center. Frozen tissue from 547 tumors was used for

DNA extraction (Table 1); protein was extracted from 306 tumors. Tumors were collected and frozen in liquid nitrogen within 1 h of surgical excision after review of the tumor and a frozen section by a pathologist. Of the 547 cancers (Table 1), tumors were characterized for estrogen receptor- α (ER) and progesterone receptor (PR) status by immunohistochemistry ($n = 357$) or ligand-binding dextran-coated charcoal assay ($n = 190$). ER/PR positivity was designated when nuclear staining occurred in $\geq 10\%$ of tumor cells or with ligand binding of ≥ 10 fmol/mg. Hormone receptor positivity was designated when ER and/or PR were positive. HER2 status was assessed by immunohistochemistry and/or fluorescence *in situ* hybridization (FISH; $n = 357$) or by reverse-phase protein array (RPPA; $n = 190$). HER2 positivity was designated when 3+ membranous staining occurred in $\geq 10\%$ of tumor cells and/or with a *HER2* to *CEP17* ratio of >2.0 , or with a log₂ mean centered cutoff of $+0.82$ by RPPA (this cutoff predicts HER2 positivity by immunohistochemistry/FISH with a sensitivity of $\sim 80\%$ and a specificity of 96–100%). Tumors were designated as basal like (i.e., triple receptor negative) when they were negative for HER2, ER, and PR expression. DNA and protein from breast cancer cell lines (Supplementary Table S1) were obtained from Lawrence Berkeley National Laboratory at the University of California at San Francisco.

Mass spectroscopy-based approach evaluating single nucleotide polymorphisms. A mass spectroscopy-based approach evaluating single nucleotide polymorphisms (SNP) was used to detect the *AKT1*_E17K mutation, mutations in the equivalent sites of *AKT2* and *AKT3*, and 23 known mutations in *PIK3CA* (*PIK3CA*_A1046V, *PIK3CA*_C420R, *PIK3CA*_E110K, *PIK3CA*_E418K, *PIK3CA*_E453K, *PIK3CA*_E542K, *PIK3CA*_E545K, *PIK3CA*_F909L, *PIK3CA*_G1049R, *PIK3CA*_G451L456_V, *PIK3CA*_H1047L, *PIK3CA*_H1047R, *PIK3CA*_H1047Y, *PIK3CA*_H701P, *PIK3CA*_K111N, *PIK3CA*_M1043V, *PIK3CA*_N345K, *PIK3CA*_P539R, *PIK3CA*_Q060K, *PIK3CA*_Q546E, *PIK3CA*_R088Q, *PIK3CA*_S405F, and *PIK3CA*_T1025S; refs. 11, 12). PCR and extension primers for *AKT* and *PIK3CA* were designed using Sequenom, Inc. Assay Design. PCR-amplified DNA was cleaned using EXO-SAP (Sequenom), and primer was extended by IPLEX chemistry, desalted using Clean Resin (Sequenom), and spotted onto

Spectrochip matrix chips using a nanodispenser (Samsung). Chips were run in duplicate on a Sequenom MassArray MALDI-TOF MassArray system. Sequenom Typer Software and visual inspection were used to interpret mass spectra. Reactions where >15% of the resultant mass ran in the mutant site in both reactions were scored as positive. AKT1_E17K mutations were specifically confirmed with independent primers. All mutations were confirmed by Sanger sequencing in the M. D. Anderson Cancer Center Cancer Center Support Grant–supported sequencing core. Results were concordant in all cases.

PTEN sequencing. A high-throughput approach to the resequencing of *PTEN* and *PIK3CA* was performed on 88 breast cancers following whole-genome amplification (13). The resequencing protocol was as follows: oligonucleotide primers (sequences available on request) for amplifying the gene coding exons were designed to give a product size in the range of 200 to 700 bp with a minimum of 40 bp flanking the splice sites using the Exon Primer program, which is bundled with the University of California at Santa Cruz Genome Browser (build hg17). M13F and M13R tags were added to the forward and reverse primers, respectively. Five nanograms of genomic DNA from each breast tumor were amplified in an 8-L PCR using AmpliTaq Gold (Applied Biosystems) on PE 9700 machines and subsequently cleaned using a diluted version of the EXO-SAP–based PCR product presequencing kit (USB Corp.) dispensed by a nanoliter dispenser (Deerac Fluidics Equator, Inc.). All PCR set-up procedures were performed in a 384-well format using a Biomek FX workstation after optimization. Sequencing reactions were then performed using the M13 primers along with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and cleaned with BET before separation on an ABI 3730xl DNA Analyzer. Base calling, quality assessment, and assembly were carried out using the Phred, Phrap, Polyphred, Consed software suite. All sequence variants identified were verified by manual inspection of the chromatograms. Mutation frequencies determined using this approach should be considered lower estimates as all exon sequences were not covered in all subjects with perfect mutation capture. In contrast, the false-positive rate with this approach is low to nonexistent (13).

Reverse-phase protein lysate microarray. RPPA as performed in our laboratory has been described previously (14, 15) and was used to quantify PTEN expression and phosphorylation of AKT at Thr³⁰⁸ and Ser⁴⁷³, glycogen synthase kinase 3 (GSK3) at Ser²¹, mammalian target of rapamycin (mTOR) at Ser²⁴⁴⁸, and p70S6K at Thr³⁸⁹ as a ratio to total expression of each protein using antibodies from Cell Signaling (AKT, PTEN, mTOR and all phospho-specific antibodies), Epitomics, Inc. (total p70S6K antibody), and Santa Cruz

Biotechnology (total GSK3 antibody). Because of potential effects of differences in tissue handling on protein phosphorylation in particular, 306 tumors from a single-institution batch (Clinic Hospital) were used for this analysis.

Cell lines. Twelve hormone receptor–positive breast cancer cell lines were cultured in complete medium (RPMI 1640 supplemented with 5% fetal bovine serum) and treated with LY294002 to determine the concentration resulting in 50% growth inhibition (GI₅₀) in each cell line.

Statistical analysis. Group characteristics were tabulated and compared between groups with the χ^2 test or Kruskal-Wallis test as appropriate. One hundred and fifty-seven hormone receptor–positive breast cancers from patients treated with adjuvant tamoxifen were used for the outcome analyses. Overall survival (OS) was measured from the date of diagnosis to the date of death from any cause. Recurrence-free survival (RFS) was measured from the date of diagnosis to the date of breast cancer recurrence. Patients who died before experiencing a disease recurrence were considered censored at their date of death. Survival outcomes were estimated with the Kaplan-Meier method and compared between groups with the log-rank statistic. Multivariable Cox proportional hazards models were fit to determine the association of *PIK3CA* mutations with survival outcomes after adjustment for other patient characteristics.

Results

Frequency of *PIK3CA*, *AKT*, and *PTEN* mutations in different breast cancer subtypes. A mass spectroscopy–based approach using methods designed to detect SNPs (11, 12) was used to detect the *AKT1_E17K* mutation, mutations in the equivalent sites of *AKT2* and *AKT3*, and mutations in *PIK3CA* in 547 breast cancers, primarily from Caucasian and Hispanic patients, and 41 cell lines (Table 1). This approach is more sensitive than conventional Sanger sequencing, having the potential to detect mutations that are present in only a subset of tumor cells or in tumors with high levels of normal cell contamination, which is commonly the case in breast cancer (11, 12). The *AKT1_E17K* mutation (Fig. 1) was detected in only 6 of 418 breast cancers (1.4%). An additional two tumors returned equivocal results due to potential *AKT1_E17K* mutation peaks comprising <15% of the area of the wild-type peak that could represent possible mutations. Nevertheless, although this confirms the likely role of *AKT1_E17K* mutations in breast

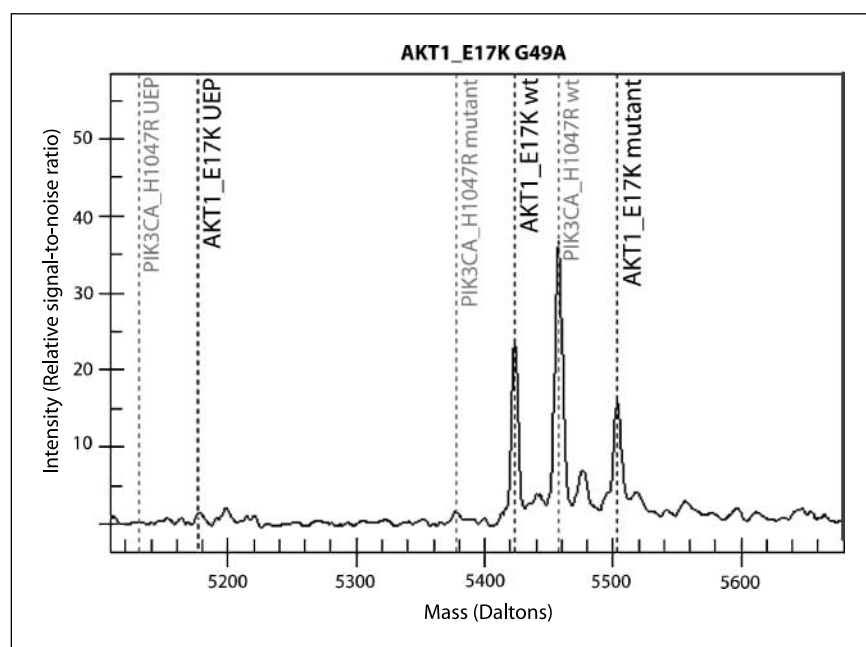
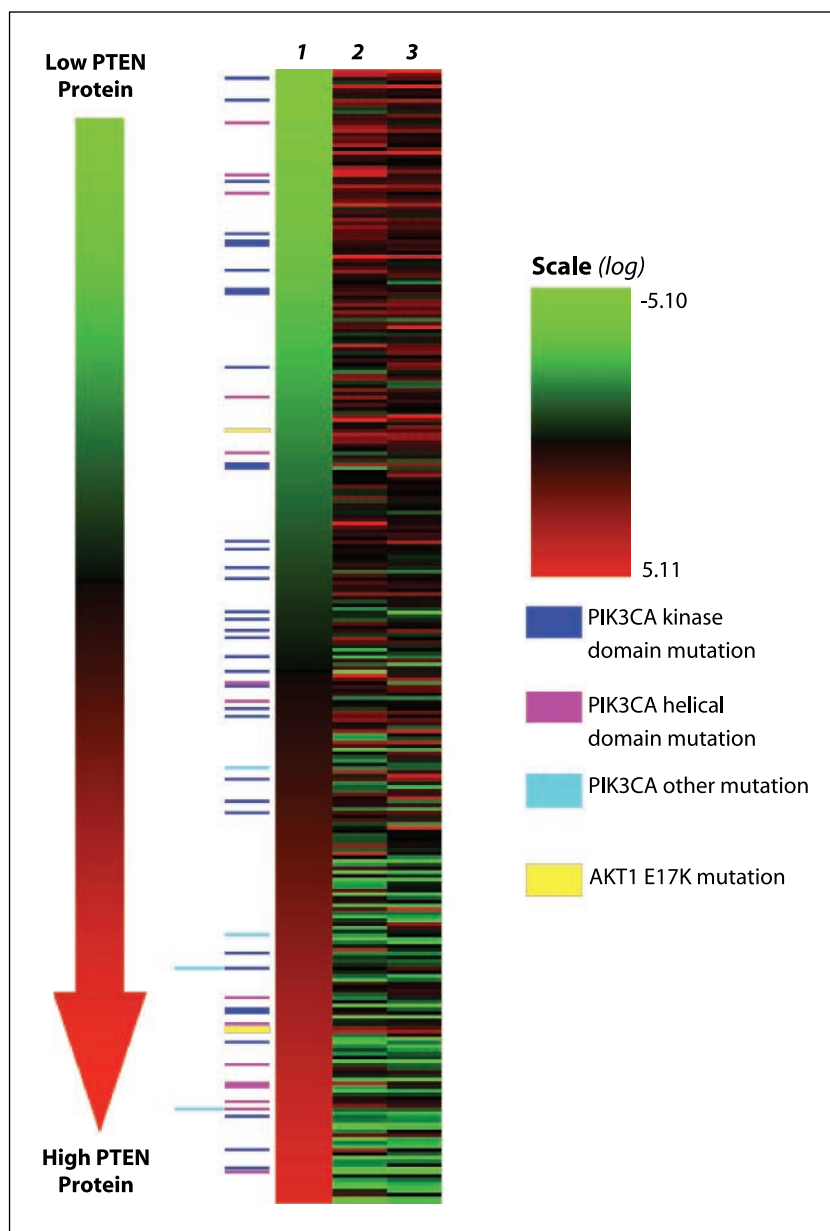


Figure 1. Detection of *PIK3CA* and *AKT1* mutations. A mass spectrometry tracing of an *AKT1* E17K-mutant SNP allele in a hormone receptor–positive breast cancer. wt, wild type.

Figure 2. Effects of PTEN loss and *PIK3CA* mutation on AKT activation in human breast tumors. PTEN and the two AKT phosphorylation sites (AKTp308 and AKTp473) were quantified using reverse-phase protein lysate array. The quantification data were then log transformed (base 2), mean centered, ordered by increasing PTEN expression level from above down, and plotted in the heat map shown. In the mean centering schema used, red indicates a relatively high level of (phospho)protein expression and green indicates a relatively low level of (phospho)protein expression. The level of PTEN expression is shown in lane 1 and the levels of AKT phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ are shown in lanes 2 and 3, respectively. AKT phosphorylation was expressed as a ratio to total AKT expression for presentation in this figure. Clearly, AKT phosphorylation is strongly inversely correlated with PTEN protein expression. However, there is no clear association between *PIK3CA* mutation and AKT phosphorylation at either amino acid site. Further, there was no significant difference ($P = 0.41$) in the frequency of tumors with *PIK3CA* mutations among those tumors with the highest and lowest quartiles of PTEN expression [17 of 77 (22.1%) and 12 of 77 (15.6%), respectively]. Tumors from one institution batch (i.e., from Clinic Hospital) were used for this analysis.



cancer pathophysiology, the frequency is significantly lower than the 8% previously reported in a set of 55 breast cancers (6). *AKT1_E17K* mutations were restricted to hormone receptor-positive breast cancers expressing both ER and PR (6 of 186, 3.2%). No *AKT1_E17K* mutations were detected in 75 HER2-positive or 111 hormone receptor-negative and HER2-negative (triple receptor negative or basal like) breast cancers. *AKT2_E17K* and *AKT3_E17K* mutations were not detected in any sample, suggesting that they do not contribute to breast cancer pathophysiology.

PIK3CA mutations were detected in 117 of 547 breast cancers (21.4%), significantly more frequently than *AKT1_E17K* mutations ($P < 0.0001$). Of the 23 sites in *PIK3CA* that were assessed, mutations were detected in exon 20 that encodes the catalytic domain of PI3K (68 *PIK3CA_H1047R*, 2 *PIK3CA_H1047L*, 2 *PIK3CA_H1047Y*, and 1 *PIK3CA_G1049R*), in exon 9 that encodes the PI3K helical domain (31 *PIK3CA_E545K* and 6 *PIK3CA_E542K*),

and at other sites (4 *PIK3CA_N345K*, 2 *PIK3CA_E418K*, and 1 *PIK3CA_K111N*). Four hormone receptor-positive breast cancers possessed two distinct *PIK3CA* mutations (*PIK3CA_H1047R* with *PIK3CA_E545K*, *PIK3CA_H1047Y* with *PIK3CA_E545K*, *PIK3CA_H1047R* with *PIK3CA_K111N*, and *PIK3CA_E545K* with *PIK3CA_E418K*). One triple-negative breast cancer cell line (BT20) also had both a catalytic domain (H1047R) and a noncatalytic domain mutation (P539R) in *PIK3CA*. Although *AKT1_E17K* mutations were restricted to hormone receptor-positive breast cancers, *PIK3CA* mutations were present in all breast cancer subtypes. However, *PIK3CA* mutations were more frequent in hormone receptor-positive (34.5%; $P < 0.0001$) and HER2-positive tumors (22.7%; $P = 0.001$) than triple receptor-negative or basal-like breast cancers (8.3%). There was no difference in the frequency of *PIK3CA* mutations between ER-positive and ER-negative/HER2-positive tumors (10 of 37 versus 7 of 37; $P = 0.58$). *PTEN* mutations were assessed in a subset of 88 patients by Sanger sequencing with only

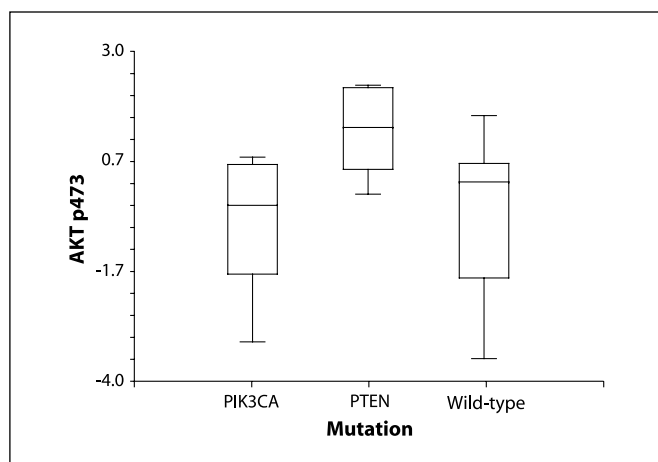


Figure 3. Effect of *PTEN* and *PIK3CA* mutations on AKT activation/phosphorylation at Ser⁴⁷³ in 40 breast cancer cell lines. AKT phosphorylation at Ser⁴⁷³ (AKTp473) was significantly higher in *PTEN*-mutant cell lines than in *PIK3CA*-mutant ($P = 0.005$) or *PTEN/PIK3CA* wild-type ($P = 0.001$) cell lines. In contrast, there was no significant difference ($P = 0.64$) in AKT phosphorylation at Ser⁴⁷³ between *PIK3CA*-mutant and *PTEN/PIK3CA* wild-type cell lines. AKTp473 was quantified using RPPA and expressed on the Y axis after logarithmic conversion and mean centering.

two mutations being found, both in hormone receptor-positive tumors.

Unlike *AKT1_E17K* mutations that were not detected in 41 breast cancer cell lines (Supplementary Table S1), *PIK3CA* (16 of 41, 39%) and *PTEN* mutations (8 of 41, 20%)¹² were more common in cell lines than patient tumors (Table 1). *AKT1_E17K*, *PIK3CA*, and *PTEN* (where assessed) mutations were mutually exclusive in all patient tumors and breast cancer cell lines assessed.

Effect of aberrations in the PI3K pathway on PI3K pathway activation in breast cancer. *PTEN* loss is well known to activate the core PI3K signaling pathway but the functional proteomic effects of *PIK3CA* mutations in human tumors are not well characterized. We thus applied RPPA to determine if *PTEN* loss and *PIK3CA* mutations have similar effects on PI3K pathway signaling in human breast tumors (Fig. 2). The tumors were split into two groups (“*PTEN* low” and “*PTEN* high”) using the median *PTEN* protein expression value. As expected, AKT phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ were both present at significantly higher levels in *PTEN*-low compared with *PTEN*-high breast cancers ($P = 1.7 \times 10^{-16}$ and 7.9×10^{-15} , respectively), as were phosphorylation of mTOR ($P = 0.0008$) and p70S6K ($P = 3.6 \times 10^{-15}$). As indicated in Fig. 2, almost all tumors with high levels of phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³ in AKT had low *PTEN* levels. In contrast, there were no significant differences in *PTEN* levels or in phosphorylation of AKT, GSK3, mTOR, or p70S6K between *PIK3CA*-mutant tumors, whether analyzed together or separately as catalytic and helical domain mutants, and *PIK3CA* wild-type breast tumors. To exclude the possibility of *PTEN* loss obscuring the effects of *PIK3CA* mutation on AKT and PI3K pathway activation, 153 breast cancers with *PTEN* protein levels below the median were removed and the analysis was repeated with *PTEN*-high tumors. However, no significant association was shown between *PIK3CA* mutation or mutation subtype and phosphorylation of AKT, GSK3, mTOR, or

p70S6K in the subsequent analysis (data not shown). In contrast, AKT phosphorylation was increased in the three *AKT1*-mutant tumors in this set (Fig. 2) despite two of these mutant tumors having high *PTEN* levels. Thus, despite the clear association between *PTEN* protein levels and PI3K pathway activation, no clear association was present between *PIK3CA* mutation (or mutation subtype) and PI3K pathway activation in either human tumors (Fig. 2) or breast cancer cell lines (Fig. 3). Therefore, *PTEN* protein loss and *PIK3CA* mutations have markedly different functional effects on the PI3K pathway in breast cancer.

***PTEN* but not *PIK3CA* mutations render cells sensitive to growth inhibition by the PI3K inhibitor LY294002.** The distinct functional proteomic effects of *PIK3CA* mutations and *PTEN* loss in hormone receptor-positive breast cancer suggest that these events may be associated with differential sensitivity to PI3K pathway-targeted therapies. Indeed, a low level of *PTEN* protein represented a major determinant of the sensitivity of 12 hormone receptor-positive breast cancer cell lines to the small-molecule PI3K inhibitor LY294002 (Fig. 4). In comparison with *PTEN* loss, *PIK3CA* mutations were associated with decreased sensitivity to LY294002.

Correlation of *PIK3CA* mutations with outcomes in tamoxifen-treated hormone receptor-positive breast cancer patients. Both the breast cancer subtype and the method of patient treatment influence patient outcomes, rendering it important to determine the effects of aberrations in the PI3K pathway on patient outcomes in well-characterized tumor sets with consistent treatment approaches. It has previously been reported that *PTEN* loss is associated with adverse outcomes in

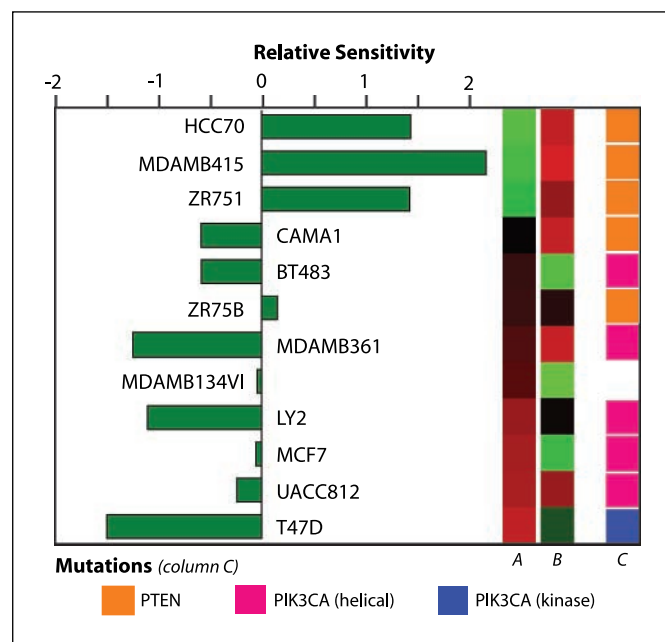


Figure 4. The relative sensitivity of 12 hormone receptor-positive breast cancer cell lines to the PI3K inhibitor LY294002. LY294002 was applied to the panel of hormone receptor-positive breast cancer cell lines and the concentration causing 50% growth inhibition (GI_{50}) was determined and presented as relative sensitivity ($-\log GI_{50}$). The cell lines are presented in order of increasing *PTEN* protein expression (lane A) as determined using RPPA. *PTEN*-low ($P = 0.0007$) and *PTEN*-mutant ($P = 0.02$) cell lines are significantly more sensitive to growth inhibition by LY294002 than *PIK3CA*-mutant cell lines. Lane B, AKT phosphorylation at Ser⁴⁷³ determined using RPPA [color coded as in lane A (i.e., green, low; black, medium; and red, high AKT phosphorylation)]. Lane C, cell line mutation status.

¹² <http://www.sanger.ac.uk>

breast cancer (9, 10, 16). A recent study by us showed that *PIK3CA* mutations predict adverse outcomes after treatment with trastuzumab for HER2-positive breast cancer (10). There are currently insufficient *PIK3CA* mutations in basal tumors to perform a comprehensive analysis (Table 1). We were able to identify a sample set of 157 early-stage hormone receptor-positive tumors from patients treated with adjuvant tamoxifen. *AKT* and *PTEN* mutation status were not included in the outcome analyses due to the low frequency of these aberrations.

PIK3CA mutation status was not significantly associated with any measured clinical variable apart from nodal status (Supplementary Table S2) nor was it significantly associated with differential OS or RFS times in the 157 patients with early-stage tamoxifen-treated hormone receptor-positive breast cancer (Fig. 5; Supplementary Table S3). The specific *PIK3CA* mutation type [kinase domain versus all other (largely helical domain)] was also not significantly associated with differential patient outcomes (Fig. 5; Supplementary Table S3). In multivariable models in the 157 tumors including *PIK3CA* mutation status, age and stage at diagnosis, age at diagnosis (as a continuous variable), and stage (II/III versus I) were found to be significant predictors of OS, and only stage at diagnosis was found to be a significant predictor of RFS. Tumor grade was not included in the multivariable models due to missing data (see Supplementary Table S2).

Discussion

We have shown that PI3K pathway aberrations are common in breast cancer, pointing to a critical role for this signaling pathway in breast carcinogenesis. *PIK3CA* oncogene mutations are particularly common, whereas *AKT* and *PTEN* mutations occur less frequently (Table 1). The *AKT1_E17K* mutation was detected in only 6 of 418 breast cancers (1.4%), confirming a role in breast cancer pathophysiology albeit in a limited number of breast cancers. Amplification of *PDK1*, *PIK3CA*, *PIK3CB*, *AKT1*, *AKT2*, and p70S6K is also among the extensive list of known aberrations that can activate PI3K pathway signaling in cancer (1). The frequency of PI3K pathway mutational aberrations was markedly different among the different breast cancer subtypes, being most common in hormone receptor-positive tumors and least common in basal-like cancers. This breast cancer subtype specificity suggests that *PIK3CA* mutations and other PI3K pathway aberrations may play a distinct role in the pathogenesis of these different diseases. Further, because genomic aberrations can predict responsiveness to targeted therapies, and because multiple PI3K pathway members are frequently aberrant in human breast tumors through mutation and other anomalies, this creates an expectation that targeting this pathway will provide an effective therapeutic approach in breast cancer (1, 3). Genomic aberrations such as those studied herein may facilitate identification of patients who will benefit from PI3K pathway-targeted therapies.

PIK3CA and *PTEN* mutations have been reported to be mutually exclusive in many cancers with a notable exception being endometrial tumors (17, 18). In the breast cancer cell lines and tumors analyzed herein, *AKT1_E17K*, *PIK3CA*, and *PTEN* mutations were also mutually exclusive. *PTEN* loss and *PIK3CA* mutation have not been studied extensively but where they have, they have not been reported to be mutually exclusive, an observation confirmed herein (19).

PI3K pathway activation has been reported to be associated with poor outcomes in certain cancers (9). We have shown that an

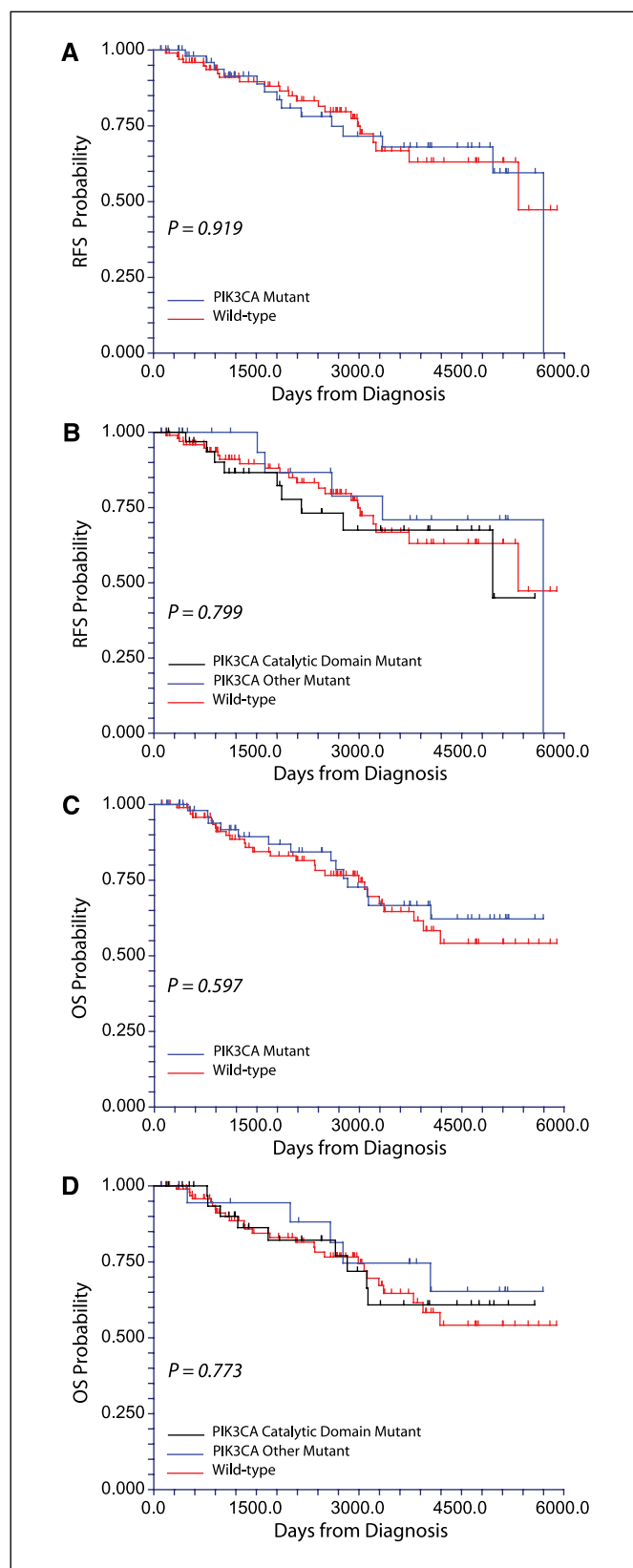


Figure 5. Correlations between *PIK3CA* mutations and patient survival. No significant differences in RFS (A and B) or OS (C and D) times were found after adjuvant tamoxifen therapy between patients with early-stage *PIK3CA* wild-type hormone receptor-positive breast tumors and those with *PIK3CA*-mutant breast tumors.

integrated signature of PTEN protein loss and *PIK3CA* mutation in HER2-positive breast cancer is an even stronger predictor of trastuzumab resistance than either *PIK3CA* mutation or PTEN loss alone (9, 10). Herein, *PIK3CA* mutations were not associated with a significant effect on hormone receptor-positive breast cancer patient outcome after adjuvant tamoxifen therapy, compatible with the results of a previous study (19). Another recent study found that, although *PIK3CA* mutation status overall was not prognostic, the presence of helical domain mutations predicted a poor outcome whereas the presence of kinase domain mutations predicted an improved outcome (20). However, unlike our study, this study was not confined to a homogeneous group of breast cancer patients as described herein. Our study is the largest study to date of the outcome implications of *PIK3CA* pathway deregulation in a homogeneous group of patients with early-stage hormone receptor-positive breast cancer who received adjuvant tamoxifen. In contrast, it has previously been reported that PTEN loss is associated with adverse outcomes in breast cancer (9, 10, 16).

Notwithstanding the lack of an outcome association with *PIK3CA* mutation status, there remains a high probability that appropriate PI3K pathway manipulation could alter outcomes for hormone receptor-positive breast cancer patients in response to hormonal manipulation or chemotherapy. However, a phase 3 trial of a mTOR inhibitor in combination with an aromatase inhibitor failed to show significant activity in an unselected hormone receptor-positive breast cancer patient population (21). Whether mTOR represents a suboptimal target for therapy in breast cancer, whether other combinations of therapies with mTOR inhibition will be effective, or whether feedback loops bypass the activity of mTOR inhibitors requires additional analysis (22). Novel PI3K- and AKT-targeted therapies are being introduced into trials [e.g., perifosine (Keryx), SF1126 (Semafore), PX166 (ProlX), BEZ256 (Novartis), and EX147 (Exelixis)] with the expectation that these compounds may bypass feedback loops and have more efficacy than mTOR inhibitors. It is clear that a systems biology approach to kinase signaling interconnections will facilitate the rational implementation of drugs and particularly drug combinations targeting the PI3K pathway in breast cancers with different genomic aberrations targeting this pathway.

Unlike *AKT1_E17K* mutations that were not detected in 41 cell lines, *PIK3CA* and *PTEN* mutations were more common in cell lines than patient tumors (Table 1). A higher frequency of *PIK3CA* and *PTEN* mutations could be due to a failure to detect mutations in tumors as a result of technical factors. However, this alone is unlikely to account for these differences because *AKT1* mutations should then be more readily identified in cell lines. Thus, there is likely to be a selection pressure for *PIK3CA* and *PTEN* but not *AKT1_E17K* mutations during adaptation to culture. Due to the low frequency of aberrations and the generally good outcome associated with hormone receptor-positive cancers, determining whether *AKT1_E17K* mutations contribute to patient outcomes and therapy responsiveness requires analysis of a large number of tumors. In our study, the breast tumors with

AKT mutations had high AKT phosphorylation levels, although the low number precluded this from reaching statistical significance. However, none of the six patients with *AKT1_E17K*-mutant hormone receptor-positive tumors in this study has recurred, suggesting that *AKT1* mutations may be associated with a good outcome. If confirmed in a larger series, this may indicate that AKT activation confers a selective advantage during early hormone receptor-positive tumorigenesis but inhibits tumor dissemination during progression. Consistent with this, although AKT1 is necessary for optimal initiation of tumorigenesis, it inhibits invasion and metastasis (23–25). AKT may thus be an initiating oncogene for hormone receptor-positive breast cancers but its anti-invasive properties may prevent disease progression contributing to a good prognosis.

As shown herein, PTEN protein loss and *PIK3CA* mutations have markedly different functional effects on activation of signaling through the PI3K pathway in human breast cancers and in breast cancer cell lines, likely leading to differential sensitivity to the pathway inhibitor LY294002. Thus, PI3K pathway activation by PTEN loss versus *PIK3CA* mutation could lead to different outcomes and is likely to have important implications for the use of pathway-targeted therapies in human tumors.

In summary, PI3K pathway aberrations are common in breast cancer, pointing to an important role for this signaling pathway in breast carcinogenesis and as a potential target for therapy. The clear breast cancer subtype specificity of these aberrations suggests that they may play a distinct role in the pathogenesis of different breast cancer subtypes. PI3K pathway aberrations are particularly common in hormone receptor-positive breast cancer. Despite the lack of an outcome association with common *PIK3CA* mutations, these mutations may have important implications for the clinical selection of targeted therapies in patients with hormone receptor-positive tumors that possess these aberrations.

Disclosure of Potential Conflicts of Interest

J.W. Gray: commercial research grants, GlaxoSmithKline, Cellgate, Affymetrix, and Cell Biosciences; consultant, Agendia, Cepheid, and Bristol-Myers Squibb. G.B. Mills: scientific/advisory committee member, Abbott Laboratories, Ambit Biosciences Corp., Lpath Therapeutics Inc., and Texas Institute for Genomic Medicine; consultant, GlaxoSmithKline, Semafore Pharmaceuticals Inc., and TAU Therapeutics; stock options, GLT, Inc.; royalty income, Upstate Biotechnology. The other authors disclosed no potential conflicts of interest.

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