TITLE: An integrative genomic and proteomic analysis of PIK3CA, PTEN and AKT mutations in breast cancer

Running title: PI3K pathway mutations in breast cancer

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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 (33.8%) and HER2-positive (24.6%) than in basal-like tumors (8.3%). AKT1 (1.4%) and PTEN (2.3%) mutations were restricted to hormone receptor-positive cancers with PTEN protein levels also being significantly lower in hormone receptor-positive cancers. Unlike AKT1 mutations, 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 impact on outcome in 166 hormone receptor-positive breast cancer patients after adjuvant tamoxifen. PIK3CA mutations, in comparison with PTEN loss and AKT1 mutations, were associated with significantly less and indeed inconsistent activation of AKT and of downstream PI3K/AKT signaling in tumors and cell lines, and PTEN loss and PIK3CA mutation were frequently concordant, suggesting different contributions to pathophysiology. PTEN loss but not PIK3CA mutations rendered cells

sensitive to growth inhibition by the PI3K inhibitor LY294002. Thus, PI3K pathway aberrations likely play a distinct role in the pathogenesis of different breast cancer subtypes. The specific aberration may have implications for the selection of PI3K-targeted therapies in hormone receptor-positive breast cancer.

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, germline mutations in PTEN, LKB1, and TSC result in hamatomatous cancer predisposition syndromes (Cowdens, Peutz Jaeghers, and tuberous sclerosis, respectively). Additional pathway components including PIK3CA, PIK3CB, AKT1 and AKT2, PDK1, p70S6 Kinase (p70S6K) and IKBKE are frequently amplified in tumors.[1] Since genomic aberrations can predict responsiveness to targeted

therapies, and since 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 hot spots in exons 9 and 20 that encode portions of the helical and kinase domains of PI3K, 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 (approximately 30%).[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 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 547 well-characterized human breast tumors and 41 breast cancer cell lines. Using 166 uniformly treated early stage hormone receptor-positive tumors from a single center, we examined the effects of common PIK3CA mutational aberrations on patient outcomes after adjuvant tamoxifen.

Materials and Methods

Human Breast Tumor Samples

Five hundred and forty-seven frozen human breast tumors were obtained from Tumor Banks following pathologist review under the auspices of Institutional Review Board (IRB)-approved protocols at Clinic Hospital (Valencia, Spain-306 tumors), the Netherlands Cancer Institute (NKI-34 tumors) and the M. D. Anderson Cancer Center (MDACC). Tumors were collected and frozen in liquid

nitrogen within one hour of surgical excision after review of the tumor and a frozen section by a pathologist. Tumors were characterized for estrogen receptor alpha (ER) and progesterone receptor (PR) status by immunohistochemistry (IHC) or ligand-binding dextran-coated charcoal assay. ER/PR positivity was designated when nuclear staining occurred in ≥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 IHC and/or fluorescent in situ hybridization (FISH). HER2 positivity was designated when 3+ membranous staining occurred in ≥10% of tumor cells or with a HER2/CEP17 ratio of >2.0. Tumors were designated as basal-like (i.e. triple receptor-negative) when they were negative for HER2, ER and PR expression. Frozen tissue was used for DNA (Table 1) and protein extraction. DNA and protein from breast cancer cell lines (supplemental table 1) was obtained from Lawrence Berkeley National Laboratory (LBNL) at University of California San Francisco (UCSF).

Mass spectroscopy-based approach evaluating single nucleotide polymorphisms

A mass spectroscopy-based approach evaluating single nucleotide polymorphisms (SNP) was used to detect the E17K-AKT1 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).[11,12] Polymerase chain reaction (PCR) and extension primers for AKT and PIK3CA were designed using Sequenom, Inc. (San Diego, CA) Assay Design. PCR-amplified DNA was cleaned using EXO-SAP (Sequenom), primer 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 more than 15% of the resultant mass ran in the mutant site in both reactions were scored as positive. E17K-AKT1 mutations were specifically confirmed with independent primers. All mutations were confirmed by Sanger sequencing in the MDACC CCSG-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 upon request) for amplifying the gene coding exons were designed to give a product size in the range of 200–700 base pairs (bp) with a minimum of 40 bp flanking the splice sites using the Exon Primer program, which is bundled with the UCSC 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-μl PCR reaction using AmpliTaq

Gold (Applied Biosystems, Foster City, CA) on PE 9700 machines and subsequently cleaned using a diluted version of the EXO-SAP-based PCR product pre-sequencing kit (USB Corporation, Cleveland, OH) dispensed by a nanoliter dispenser (Deerac Fluidics Equator, Inc., Cambridge, MA). All PCR set-up procedures were performed in a 384-well format using a Biomek FX (Fullerton, CA) workstation after optimization. Sequencing reactions were then performed using the M13 primers along with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) 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 non-existent.[13]

Reverse Phase Protein Lysate Microarray (RPPA)

RPPA as performed in our laboratory has been described previously[14,15] and was used to quantify PTEN expression, and phosphorylation of AKT at threonine 308 and serine 473, glycogen synthase kinase (GSK) 3 at serine 21, mammalian target of rapamycin (mTor) at serine 2448 and p70S6 kinase (p70S6K) at threonine 389 as a ratio to total expression of each protein using antibodies from Cell Signaling, Inc. (Danvers, MA), Epitomics, Inc. (Burlingame, CA-total p70S6K

antibody) and Santa Cruz (Santa Cruz, CA-total GSK3 antibody). Because of potential effects of differences in tissue handling on protein phosphorylation in particular, only tumors from the largest single institution batch (i.e. from Clinic Hospital) were used for this analysis.

Cell Lines

Twelve hormone receptor-positive breast cancer cell lines were cultured in complete medium (RPMI1640 supplemented with 5% fetal bovine serum (FBS)) 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 chi-square test or Kruskal-Wallis test as appropriate. One hundred and sixty-six hormone receptor-positive breast cancers from patients treated with adjuvant tamoxifen were used for the outcomes 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 single nucleotide polymorphisms (SNP)[11,12] was used to detect the E17K-AKT1 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 E17K-AKT1 mutation (Figure 1) was detected in only 6 of 418 breast cancers (1.4%). An additional two tumors returned equivocal results due to potential E17K-AKT1 mutation peaks comprising less than 15% of the area of the wild-type peak that could represent possible mutations. Nevertheless, while this confirms the likely role of E17K-AKT1 mutations in breast cancer pathophysiology, the frequency is significantly lower than the 8% previously reported in a set of 55 breast cancers.[6] E17K-AKT1 mutations were restricted to hormone receptor-positive breast cancers expressing both ER and PR (6/190, 3.2%). No E17K-AKT1 mutations were detected in 65 HER2-positive or 113 hormone receptor- and HER2-

negative (triple receptor-negative or basal-like) breast cancers. E17K-AKT2 and –AKT3 mutations were not detected in any sample suggesting that they do not contribute to breast cancer pathophysiology.

PIK3CA mutations were detected in 117/547 breast cancers (21.4%), significantly more frequently than AKT1-E17K mutations (p=0.00001). 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, PIK3CA_E545K_with PIK3CA_E418K). Although E17K-AKT1 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 (33.8%, p=0.00001) and HER2-positive tumors (24.6%, p=0.0007) 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 –negative/HER2-positive tumors (9/29 vs. 7/35, p=0.39). PTEN mutations were assessed in a subset of 88 patients by Sanger sequencing with only two mutations being found, both in hormone receptor-positive tumors. Concordant with the subtype

specificity of mutations, mean PTEN protein levels assessed by RPPA were also significantly lower among hormone receptor-positive tumors (Table 1).

Unlike E17K-AKT1 mutations that were not detected in 41 breast cancer cell lines (supplemental table 1), PIK3CA (16/41, 39%) and PTEN mutations (8/41, 20%) (http://www.sanger.ac.uk) were more common in cell lines than patient tumors (Table 1). E17K-AKT1, 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 hormone receptor-positive 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 hormone receptor-positive breast tumors. The tumors were split into two groups ('PTEN low' and 'PTEN high') using the median PTEN protein expression value. As expected, AKT phosphorylation at threonine 308 and serine 473 were both present at significantly higher levels in 'PTEN low' as 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 figure 2, almost all tumors with high levels of phosphorylation of both threonine

308 and serine 473 in AKT had low PTEN levels. In contrast, there were no significant differences in PTEN levels, 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 hormone receptor-positive human breast tumors. To exclude the possibility of PTEN loss obscuring the effects of PIK3CA mutation on AKT and PI3K pathway activation, 153 human hormone receptor-positive breast cancers with PTEN protein levels below the median were removed and the analysis repeated with high PTEN tumors. However, no significant association was demonstrated 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 (Figure 2) despite two of these mutant tumors having high PTEN levels. Thus, despite the clear association between protein PTEN levels and PI3K pathway activation, no clear association was present between PIK3CA mutation (or mutation subtype) and PI3K pathway activation in either human tumors (Figure 2) or breast cancer cell lines (Figure 3). Thus, 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 twelve hormone receptor-positive breast cancer cell lines to the small molecule PI3K inhibitor LY294002 (Figure 4). In comparison to PTEN loss, PIK3CA mutations were associated with relative resistance 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 the outcome of patients, rendering it important to determine the effects of aberrations in the PI3K pathway on patient outcomes in well characterized tumor subtypes with consistent treatment approaches. It has previously been reported that PTEN loss is associated with adverse outcomes in breast cancer.[9,10,16] A recent study by us demonstrated 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 166 early stage hormone receptor-positive tumors from patients treated solely with adjuvant tamoxifen. AKT and PTEN mutation status were not included in the outcomes analyses due to the low frequency of these aberrations.

PIK3CA mutation status was not significantly associated with any measured clinical variable (Supplemental Table 2) nor was it significantly associated with differential overall survival (OS) or recurrence-free survival (RFS) times in the 166 patients with early stage tamoxifen-treated hormone receptor-positive breast cancer (Figure 5 and Supplemental Table 3). The specific PIK3CA mutation type (kinase domain versus all other (largely helical domain)) was also not significantly associated with differential patient outcomes (Figure 5 and Supplemental Table 3). In multivariable models in the 166 tumors including PIK3CA mutation status, age and stage at diagnosis, age at diagnosis (as a continuous variable) and stage (II/III vs. 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 Supplemental Table 2).

Discussion

We have shown that PI3K pathway aberrations are very common in breast cancer pointing to a critical role for this signaling pathway in breast carcinogenesis. PIK3CA oncogene mutations are particularly common, while AKT and PTEN mutations occur less frequently (Table 1). The E17K-AKT1 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 are also among the extensive list of known aberrations that can activate PI3K 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. Consistent with a selective role for the PI3K pathway in hormone receptor-positive tumors, PTEN protein expression was significantly lower in hormone receptor-positive than in other human breast tumors (Table 1). Consistent with this, when PI3K pathway activation status is estimated by AKT phosphorylation, the pathway is activated in up to 44% of hormone receptor-positive, 26% of HER2-positive and 16% of basal-like tumors.[17,18] 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, since genomic aberrations can predict responsiveness to targeted therapies, and since 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.[19,20] In the breast cancer cell lines and tumors analyzed herein, E17K-AKT1, PIK3CA, and PTEN mutations were also mutually exclusive.

Using RPPA, which allows PTEN levels to be assessed as a continuous variable, PTEN protein levels were significantly lower in hormone receptor-positive cancers than in other human breast cancers. Consistent with this, levels of AKT phosphorylation have been reported to be higher in hormone receptor positive tumors than in other breast cancer tumor lineages.[17,18] Recent studies suggest that breast cancers in mice engineered to lack PTEN have characteristics most closely related to basal-like tumors [21]. However, in humans, there does not appear to be an excess of basal-like tumors in women with Cowden's syndrome which is caused by a germline mutation in PTEN (Charis Eng personal communication).[22,23]

PI3K pathway activation has been reported to be associated with poor outcomes in certain cancers.[9] We have demonstrated that an 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 herein were not associated with a significant impact on hormone receptor-positive breast cancer patient outcome after adjuvant tamoxifen therapy, compatible with the results of a previous study.[24] Another recent study found that, while PIK3CA mutation status overall was not prognostic, the presence of helical domain mutations predicted a poor outcome while the presence of kinase domain mutations predicted an improved outcome.[25] However, unlike our study, this study was not confined to a homogeneously treated group of breast cancer patients with a specific subtype of

breast cancer 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 treated only with 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 an mTor inhibitor in combination with an aromatase inhibitor failed to demonstrate significant activity in an unselected hormone receptor-positive breast cancer patient population. [26] 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.[27] Novel PI3K- and AKT-targeted therapies are being introduced into trials (e.g. perifosine (Keryx), SF1126 (Semafore), PX166 (Prolx), BEZ256 (Novartis), EX147 (Exelixis)) with the expectation that these compounds may bypass feedback loops and have more efficacy than mTor inhibitors. It is clear that a comprehensive understanding of kinase signaling interconnections is important for the rational implementation of drugs and particularly drug combinations targeting the PI3K pathway in breast cancers with different genomic aberrations targeting this pathway.

Unlike E17K-AKT1 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 since 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 E17K-AKT1 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 E17K-AKT1 mutations contribute to patient outcomes and therapy responsiveness requires analysis of a large number of tumors. However, none of the six patients with E17K-AKT1 mutant hormone receptor-positive tumors in this study have recurred, suggesting that AKT1 mutation 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, while AKT1 is necessary for optimal initiation of tumorigenesis, its expression actually inhibits invasion and metastasis.[28-30] 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 demonstrated herein, PTEN protein loss and PIK3CA mutations have markedly different functional effects on signaling through the PI3K pathway

in hormone receptor-positive breast cancers and in breast cancer cell lines, likely leading to the demonstrated 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 mutational aberrations and low PTEN protein expression are particularly prominent 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.

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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.

Figure 2. Effect of PTEN loss and PIK3CA mutation on AKT activation in hormone receptor-positive human breast tumors. PTEN and the two AKT phosphorylation sites (AKTp308, AKTp473) were quantified in hormone receptor-positive breast cancers 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 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 threonine 308 (lane 2) and serine 473 (lane 3) are shown in lanes 2 and 3, respectively. 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 (17/77 (22.1%) and 12/77 (15.6%), respectively).Only tumors from the largest single institution batch (i.e. from Clinic Hospital) were used for this analysis.

Figure 3. Effect of PTEN and PIK3CA mutations on AKT activation/ phosphorylation at serine 473 in 40 breast cancer cell lines. AKT phosphorylation at serine 473 (AKTp473) was significantly higher in PTEN mutant cell lines than in PIK3CA-mutant (p=0.004) or PTEN/PIK3CA-wild type (p=0.0002) cell lines. In contrast, there was no significant difference (p=0.49) in AKT phosphorylation at serine 473 between PIK3CA-mutant and PTEN/PIK3CA-wild type cell lines. AKTp473 was quantified using reverse phase protein array and expressed on the Y axis after logarithmic conversion and mean centering.

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 (GI50) determined and presented as relative sensitivity (-Log GI50). The cell lines are presented in order of increasing PTEN protein expression (Lane A) as determined using reverse phase protein array (RPPA). PTEN-low (p=0.0065) 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 Serine 473 determined using RPPA (color coded as in lane A i.e. green low, black mean and red high AKT phosphorylation); Lane C-cell line mutation status.

Figure 5. Correlations between PIK3CA mutations and patient survival.

No significant differences in recurrence-free (RFS (A, B)) or overall survival (OS (C, D)) times were found between patients with early stage PIK3CA-wild type hormone receptor-positive breast tumors and those with PIK3CA-mutant breast tumors after adjuvant tamoxifen therapy.

Table 1. Frequency of mutations in the PIK3CA, AKT1 and PTEN genes along with PTEN protein expression in human breast cancers and breast cancer cell lines. PTEN protein expression was determined by reverse phase protein array independently for tumors and cell lines and is presented as mean centered log2 arbitrary units for comparison between breast cancer subgroups.

^ Resequencing courtesy of Len Pennacchio and Jan-Fang Cheng of Lawrence Berkeley National Laboratory in breast cancers and by literature/internet (e.g. www.sanger.ac.uk) search in cell lines.

^^ DNA extraction in these tumors courtesy of Mandy Madiredjo at NKI.

* 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.

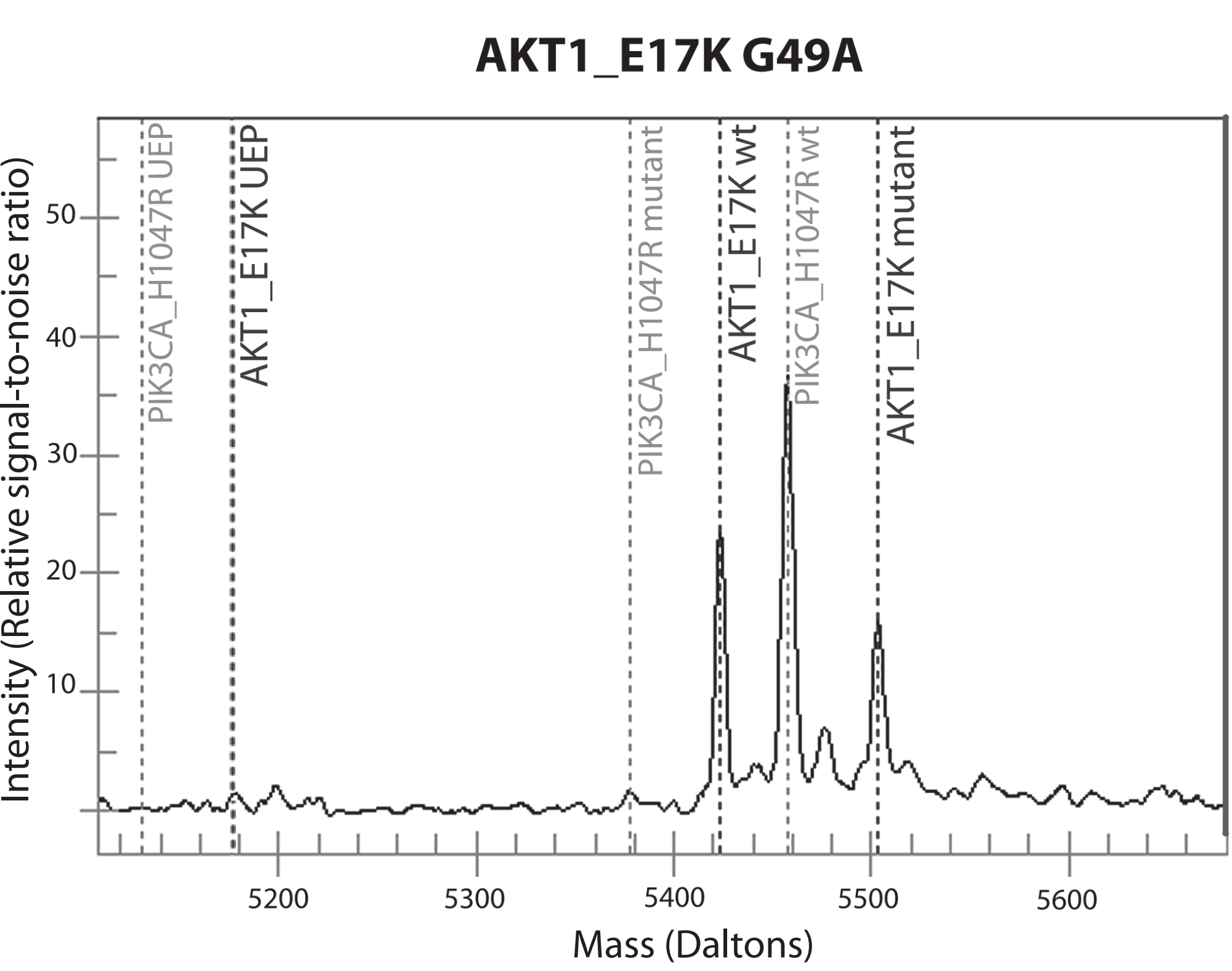
*** Two distinct PIK3CA mutations were found together in four patients with HR+ breast cancer.

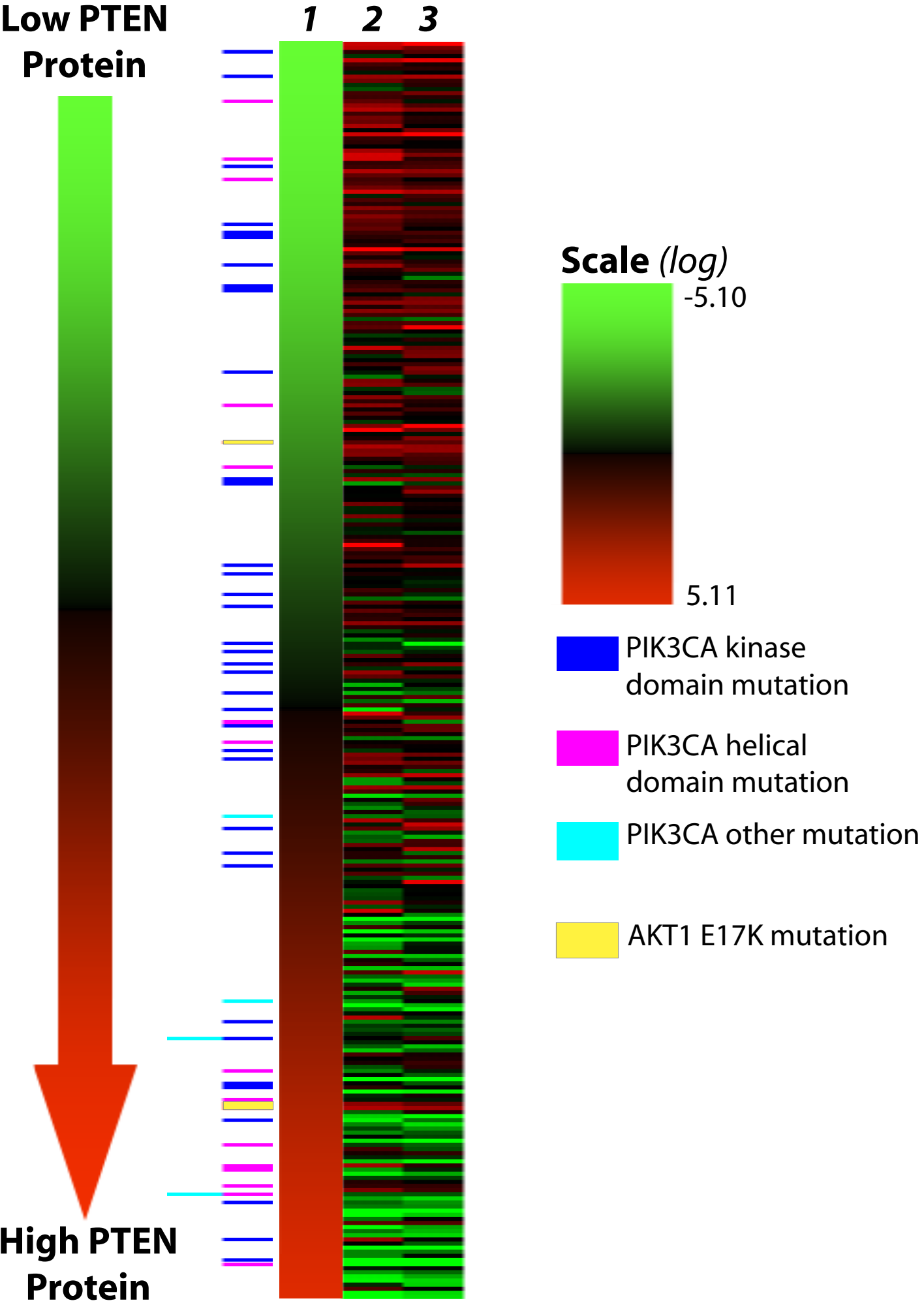
**** One TN breast cancer cell line (BT20) had both a catalytic domain H1047R and non catalytic domain P539R mutation in PIK3CA.

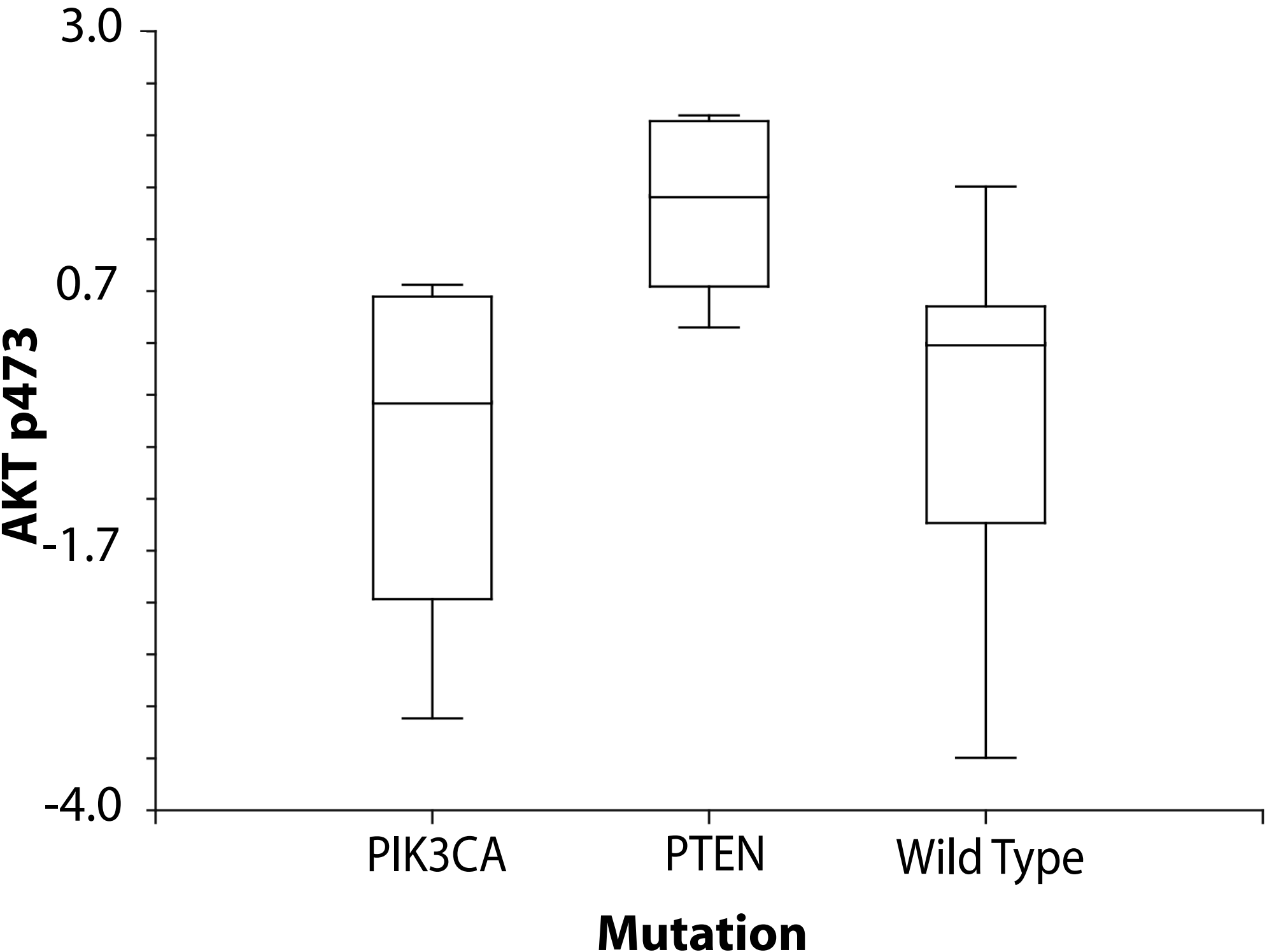
Supplemental Table 1. Breast cancer cell lines and their estrogen receptor status, clinical subtype and PIK3CA/PTEN mutational status.

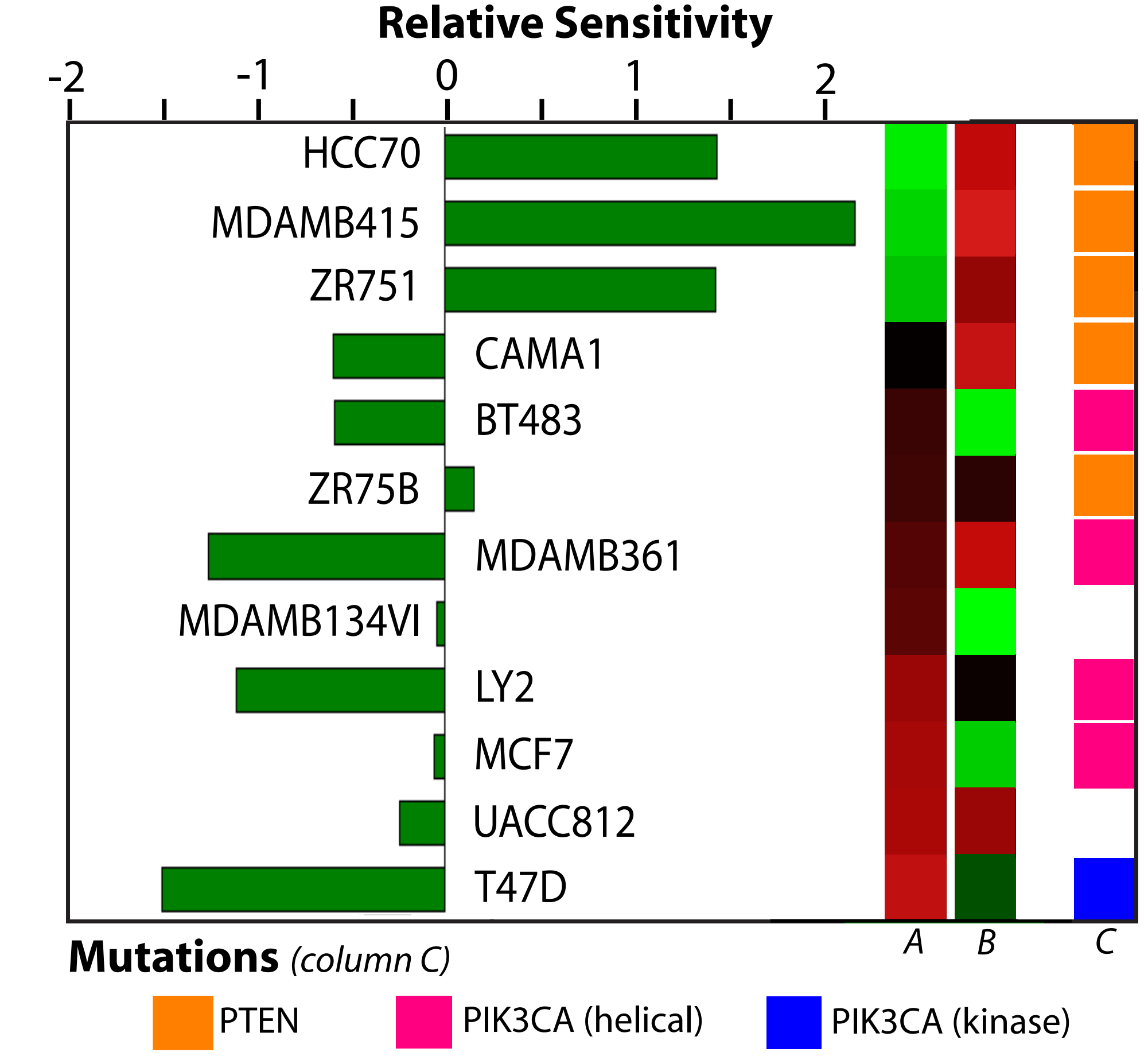
Supplemental Table 2. Associations between clinical variables and PIK3CA mutation status in 166 early stage hormone receptor-positive breast cancers. In some categories, numbers may not add up to 166 due to missing data.

Supplemental Table 3. Associations between PIK3CA mutation status and outcomes in 166 early stage hormone receptor-positive breast cancer patients treated only with adjuvant tamoxifen.


AKT1_E17K G49A
PIK3CA_H1047R UEP
AKT1_E17K UEP
PIK3CA_H1047R mutant
AKT1_E17K wt
PIK3CA_H1047R wt
AKT1_E17K mutant
Intensity (Relative signal-to-noise ratio)
50
40
30
20
10
5200
5300
5400
5500
5600
Mass (Daltons)


Low PTEN Protein
1
2
3
High PTEN Protein
Scale (log)
-5.10
5.11
PIK3CA kinase domain mutation
PIK3CA helical domain mutation
PIK3CA other mutation
AKT1 E17K mutation


3.0
0.7
-1.7
-4.0
AKT p473
PIK3CA
PTEN
Wild Type
Mutation


Relative Sensitivity
-2
-1
0
1
2
HCC70
MDAMB415
ZR751
CAMA1
BT483
ZR75B
MDAMB361
MDAMB134VI
LY2
MCF7
UACC812
T47D
A
B
C
Mutations (column C)
PTEN
PIK3CA (helical)
PIK3CA (kinase)

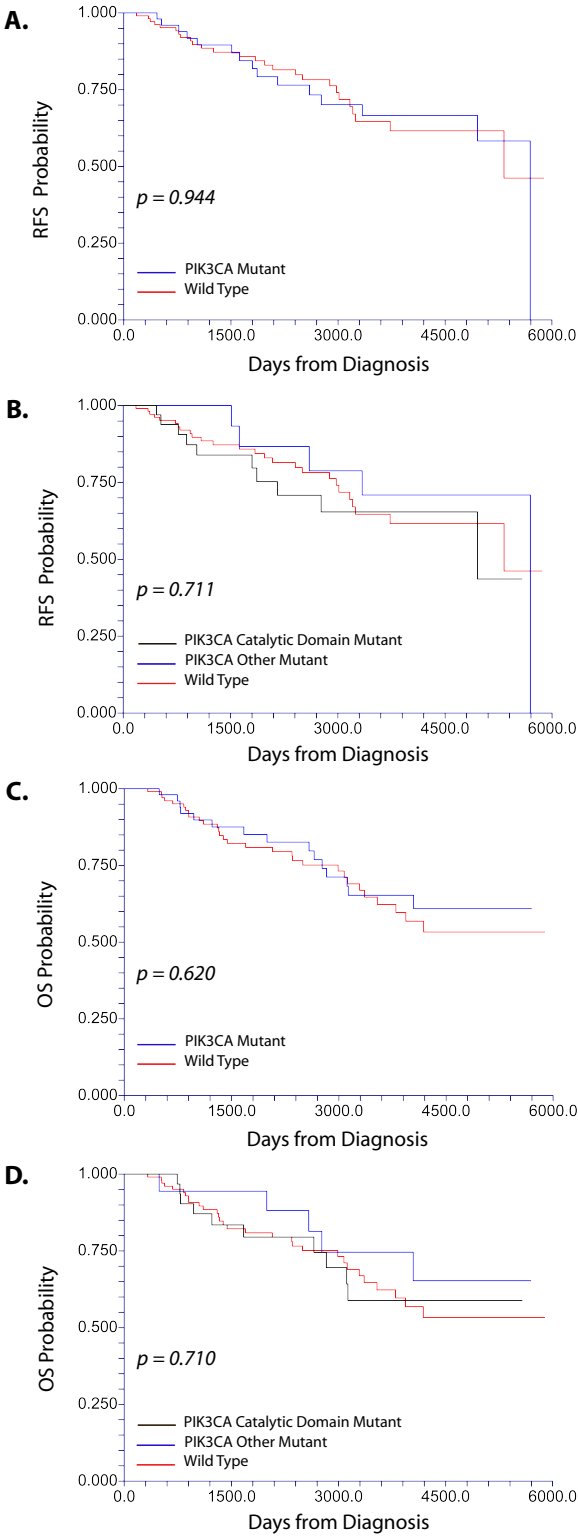

A.
RFS Probability
1.000
0.750
0.500
0.250
0.000
p = 0.944
PIK3CA Mutant
Wild Type
0.0
1500.0
3000.0
4500.0
6000.0
Days from Diagnosis
B.
RFS Probability
1.000
0.750
0.500
0.250
0.000
p = 0.711
PIK3CA Catalytic Domain Mutant
PIK3CA Other Mutant
Wild Type
0.0
1500.0
3000.0
4500.0
6000.0
Days from Diagnosis
C.
OS Probability
1.000
0.750
0.500
0.250
0.000
p = 0.620
PIK3CA Mutant
Wild Type
0.0
1500.0
3000.0
4500.0
6000.0
Days from Diagnosis
D.
OS Probability
1.000
0.750
0.500
0.250
0.000
p = 0.710
PIK3CA Catalytic Domain Mutant
PIK3CA Other Mutant
Wild Type
0.0
1500.0
3000.0
4500.0
6000.0
Days from Diagnosis

Table 1. Frequency of mutations in the PIK3CA, AKT1 and PTEN genes along with PTEN protein expression in human breast cancers and breast cancer cell lines. PTEN protein expression was determined by reverse phase protein array independently for tumors and cell lines and is presented as mean centered log2 arbitrary units for comparison between breast cancer subgroups.

Tumor subtype	**Mutation**					**PTEN protein expression (mean +/- SD)**
	PIK3CA catalytic domain*	**PIK3CA other****	**PIK3CA total**	**PTEN^**	**AKT1 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%)**	**Mean centered log2 expression (ANOVA p < 0.0001)**:
Human breast HR+***	49/240 (20.4%)	32/240 (13.3%)	81/240 (33.8%)	2/58 (3.4%)	6/240 (2.5%)	-0.25 (+/- 1.19)
ER+PR+	39/190 (20.5%)	22/190 (11.6%)	61/190 (32.1%)	1/48 (2.1%)	6/190 (3.2%)	-
ER+PR-	10/45 (22.2%)	10/45 (22.2%)	20/45 (44.4%)	1/8 (12.5%)	0/45 (0%)	-
ER-PR+	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/2 (0%)	0/5 (0%)	-

Human breast HER2+^^	12/65 (18.5%)	4/65 (6.2%)	16/65 (24.6%)	0/10 (0%)	0/65 (0%)	+0.58 (+/- 0.72)
Human breast TN	12/242 (5.0%)	8/242 (3.3%)	20/242 (8.3%)	0/20 (0%)	0/113 (0%)	+0.51 (+/- 0.82)
All breast cancer cell lines	**7/41 (17.1%)**	**9/41 (22%)**	**16/41 (39%)**	**8/41 (20%)**	**0/41 (0%)**	**Mean centered log2 expression (ANOVA p = 0.36):**
Breast cancer cell lines HR+	1/12 (8.3%)	3/12 (25%)	4/12 (33.3%)	5/12 (41.7%)	0/12 (0%)	+0.16 (+/- 1.33)
Breast cancer cell lines HER2+	2/10 (20%)	4/10 (40%)	6/10 (60%)	0/10 (0%)	0/10 (0%)	+0.40 (+/- 0.64)
Breast cancer cell lines TN****	4/19 (21%)	2/19 (10.5%)	6/19 (31.6%)	3/19 (15.8%)	0/19 (0%)	-0.28 (+/- 1.27)

ER-estrogen receptor alpha; PR-progesterone receptor; HR-hormone receptor; HER2-human epidermal receptor 2; TN-triple negative; SD-standard deviation.

^ Resequencing courtesy of Len Pennacchio and Jan-Fang Cheng of Lawrence Berkeley National Laboratory in breast cancers and by literature/internet (e.g. www.sanger.ac.uk) search in cell lines.

^^ DNA extraction in these tumors courtesy of Mandy Madiredjo at NKI.

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Supplemental Table 1. Breast cancer cell lines and their estrogen receptor status, clinical subtype and PIK3CA/PTEN mutational status.

Breast Cancer Cell line	Estrogen receptor status	Clinical Subtype	Mutation
MCF7	Positive	Hormone receptor +	PIK3CA E545K
T47D	Positive	Hormone receptor +	PIK3CA H1047R
BT483	Positive	Hormone receptor +	PIK3CA E542K
ZR751	Positive	Hormone receptor +	PTEN
ZR75b	Positive	Hormone receptor +	PTEN
ZR7530	Positive	Hormone receptor +	
MDAMB134v1	Positive	Hormone receptor +	
MDAMB415	Positive	Hormone receptor +	PTEN
CAMA1	Positive	Hormone receptor +	PTEN
LY2	Positive	Hormone receptor +	PIK3CA E545K
600MPE	Positive	Hormone receptor +	
HCC70	Positive	Hormone receptor +	PTEN
BT474	Positive	HER2-positive	PIK3CA K111N
HCC1569	Negative	HER2-positive	
MDAMB361	Positive	HER2-positive	PIK3CA E545K
MDAMB453	Negative	HER2-positive	PIK3CA H1047R
SKBR3	Negative	HER2-positive	
AU565	Negative	HER2-positive	
SUM225	Negative	HER2-positive	
HCC202	Negative	HER2-positive	PIK3CA E545K
UACC812	Positive	HER2-positive	PIK3CA N345K
UACC893	Positive	HER2-positive	PIK3CA H1047R
MDAMB468	Negative	Triple negative	PTEN
HBL100	Negative	Triple negative	
HCC38	Negative	Triple negative	
HCC1187	Negative	Triple negative	
HCC1954	Negative	Triple negative	PIK3CA H1047R
HCC2185	Negative	Triple negative	PIK3CA E545K
MDAMB231	Negative	Triple negative	
BT20	Negative	Triple negative	PIK3CA H1047R/P539R
BT549	Negative	Triple negative	PTEN
MDAMB157	Negative	Triple negative	PTEN
HS578T	Negative	Triple negative	
HCC1500	Negative	Triple negative	
HCC3153	Negative	Triple negative	
MCF10A	Negative	Triple negative	
MCF10F	Negative	Triple negative	
MCF12A	Negative	Triple negative	

SUM149PT	Negative	Triple negative	
SUM159PT		Triple negative	PIK3CA H1047L
SUM185PE	Negative	Triple negative	PIK3CA H1047R

Supplemental Table 2. Associations between clinical variables and PIK3CA mutation status in 166 early stage hormone receptor-positive breast cancers.

	All Tumors		PIK3CA Wild Type		PIK3CA Mutation		
	N	Percent	N	Percent	N	Percent	P-Value
N	166		109		57		
Histology							
Other	36	21.7	28	25.7	8	14.0	
Ductal	130	78.3	81	74.3	49	86.0	0.112
Pathologic T							
T1	59	35.5	42	38.5	17	29.8	
T2	81	48.8	48	44.0	33	57.9	
T3/4	26	15.7	19	17.4	7	12.3	0.308
Pathologic N							
N0	106	63.9	75	68.8	31	54.4	
N1-N3	60	36.1	34	31.2	26	45.6	0.089
ER							
Negative	3	1.8	3	2.8	0	0.0	
Positive	163	98.2	106	97.2	57	100.0	0.320
PR							
Negative	36	21.7	19	17.4	17	29.8	
Positive	130	78.3	90	82.6	40	70.2	0.076
Summary stage							
I	47	28.5	35	32.4	12	21.1	
II	95	57.6	56	51.9	39	68.4	
III	23	13.9	17	15.7	6	10.5	0.148
Nuclear Grade							
I	43	41.3	29	39.7	14	45.2	
II	49	47.1	37	50.7	12	38.7	
III	12	11.5	7	9.6	5	16.1	0.666

* In some categories, numbers may not add up to 166 due to missing data.

Supplemental Table 3. Associations between PIK3CA mutation status and outcomes in 166 early stage hormone receptor-positive breast cancer patients treated only with adjuvant tamoxifen.

Overall survival:	N	N Events	3 Year Estimate	95% C.I.	4 Year Estimate	95% C.I.	5 Year Estimate	95% C.I	P-Value
All	166	45	88.9%	(83.8%, 94%)	84%	(77.8%, 90.2%)	82.3%	(75.9%, 88.8%)	
PIK3CA									
Wild Type	109	30	88.5%	(82%, 94.9%)	82.2%	(74.2%, 90.2%)	80.9%	(72.6%, 89.2%)	
Mutation	57	15	89.8%	(81.4%, 98.3%)	87.5%	(78.2%, 96.9%)	85.1%	(74.8%, 95.3%)	0.620
PIK3CA									
Wild Type	109	30	88.5%	(82%, 94.9%)	82.2%	(74.2%, 90.2%)	80.9%	(72.6%, 89.2%)	
Catalytic	36	10	87.1%	(75.3%, 98.9%)	83.5%	(70.2%, 96.8%)	79.5%	(64.7%, 94.3%)	
Other	21	5	94.4%	(83.9%, 100.0%)	94.4%	(83.9%, 100.0%)	94.4%	(83.9%, 100.0%)	0.710
Recurrence-free survival:									
All	166	41	88.9%	(83.7%, 94.1%)	88.1%	(82.7%, 93.4%)	85.4%	(79.4%, 91.4%)	
PIK3CA									
Wild Type	109	26	88.5%	(82.1%, 94.9%)	87.2%	(80.4%, 94%)	85.8%	(78.6%, 93.1%)	
Mutation	57	15	89.5%	(80.9%, 98.2%)	89.5%	(80.9%, 98.2%)	84.4%	(73.7%, 95.1%)	0.944
PIK3CA									
Wild Type	109	26	88.5%	(82.1%, 94.9%)	87.2%	(80.4%, 94%)	85.8%	(78.6%, 93.1%)	
Catalytic	36	10	83.9%	(70.9%, 96.9%)	83.9%	(70.9%, 96.9%)	83.9%	(70.9%, 96.9%)	
Other	21	5	100.0%		100.0%		86.7%	(69.5%, 100%)	0.711