Identification and Quantification of AKT Isoforms and Phosphoforms in Breast Cancer Using a Novel Nanofluidic Immunoassay*

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Breast cancer subtype-specific molecular variations can dramatically affect patient responses to existing therapies. It is thought that differentially phosphorylated protein isoforms might be a useful prognostic biomarker of drug response in the clinic. However, the accurate detection and quantitative analysis of cancer-related protein isoforms and phosphoisoforms in tumors are limited by current technologies. Using a novel, fully automated nanocapillary electrophoresis immunoassay (NanoPro™1000) designed to separate protein molecules based on their isoelectric point, we developed a reliable and highly sensitive assay for the detection and quantitation of AKT isoforms and phosphoforms in breast cancer. This assay enabled the measurement of activated AKT1/2/3 in breast cancer cells using protein produced from as few as 56 cells. Importantly, we were able to assign an identity for the phosphorylated S473 phosphoform of AKT1, the major form of activated AKT involved in multiple cancers, including breast, and a current focus in clinical trials for targeted intervention. The ability of our AKT assay to detect and measure AKT phosphorylation from very low amounts of total protein will allow the accurate evaluation of patient response to drugs targeting activated PI3K-AKT using scarce clinical specimens. Moreover, the capacity of this assay to detect and measure all three AKT isoforms using one single pan-specific antibody enables the study of the multiple and variable roles that these isoforms play in AKT tumorigenesis. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.023119, 3210–3220, 2013.

Activation of the PI3K-AKT signaling pathway is one of the most common events in cancer (1, 2). Pathway activation can confer a number of advantages to the cancer cells, including enhanced proliferation and survival (1, 2). Multiple mechanisms exist by which the pathway may become activated, including amplification or mutation in receptor tyrosine kinases (e.g. ERBB2 in breast and EGFR in lung tumors), mutation of the catalytic or regulatory subunits of PI3K (e.g. PIK3CA in colorectal and breast tumors), loss of the negative regulator PTEN (e.g. mutation in prostate and melanoma), and gain of function of AKT (e.g. amplification or mutation in breast and pancreatic tumors) (reviewed in Refs. 1 and 2).

AKT represents a central node in the PI3K signaling cascade (3). AKT is recruited to the cell membrane via its pleckstrin homology domain when PI3K phosphorylates PIP2 to form PIP3 (4, 5). Following recruitment, AKT is phosphorylated by PDK1 and the rictor-mTOR complex, resulting in conformational changes and activation of the protein (5–8). Multiple studies have shown that the phosphorylation of AKT leads to the phosphorylation and activation of downstream effectors of the signaling pathway, such as mTOR complex 1 and S6K (reviewed in Ref. 1). The central role of this pathway in cancer is further underscored by the efforts of multiple pharmaceutical companies that have developed inhibitors against AKT as potential anti-oncogenic therapeutics (9).

Despite the importance of AKT in growth and survival signaling in cancer, there are surprisingly few data that address the specific roles played in growth and survival by the multiple AKT family members (AKT-1, -2, and -3) and different phosphorylation and putative phosphorylation sites that can potentially activate the protein. Western blot analysis has been the foundation of most AKT studies, but in many cases pan-AKT antibodies have been employed that fail to distinguish between the different AKT isoforms. Recent siRNA silencing studies have indicated distinct functions for different AKT family members within a cell (10, 11). Moreover, there is evidence in breast cancer that the three isoforms exhibit different localizations and therefore must have at least partially distinct functions (12). Similarly, evidence is mounting for multiple phosphorylation sites in AKT beyond the two most studied phosphorylation events (Thr-308 and Ser-473) (5–8). Phosphorylation at serine and threonine residues at Thr-72 and Ser-246 may be required for the activation or regulation of kinase activity (13). The functional significance of constitutive phosphorylation of Ser-124 and Thr-450 is still unknown (14). Finally, there is evidence that phosphorylation of tyrosine residues at Tyr-315 and Tyr-326 is required for full kinase activity (15).
Analysis of such phospho- and isofrom-specific activation often requires complicated in-depth analyses using large quantities of proteins, purified recombinant protein, immunoprecipitation, incorporation of $^{32}$P isotopes, and/or mass spectrometry, which makes such studies more difficult to perform and not easily adaptable to clinical specimens. Thus, better methods are required for the accurate assessment of both phosphoform and isofrom usage in cells with an activated PI3K-AKT pathway and the effects of pathway inhibitors using relatively small amounts of starting material. We describe here the development of such an assay using nanocapillary-based isoelectric focusing (16). This approach allows the separation of AKT into distinct peaks that correspond to different iso- and phosphoforms using a small amount of starting material and a single pan-specific antibody. This approach should allow for more accurate determinations of isofrom usage in different cell types, as well as of changes in phosphorylation states in response to pathway inhibition, including in clinical specimens.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Lysate Preparation**—BT474 and MDAMB231 cells were cultured in DMEM with 10% FBS until 80% confluent. For harvesting, the medium was removed and the cells were washed with PBS twice and subsequently lysed in the appropriate amount of 0.5% Nonidet P-40 buffer containing 50 mM Tris, 120 mM NaCl, 1 mM EDTA, 1 mM NaF, 0.1 mM Na-orthovanadate, and 10 mM DTT, with supplemental protease and phosphatase inhibitors from Roche (Complete Mini Protease Inhibitor Tablets, catalog no. 11 836 153 001). The crude lysates were centrifuged at 14,000 × g for 20 min, and the cleared lysates were collected and processed for downstream applications or stored at -80°C. Protein was quantified using the Bio-Rad Protein Assay (catalog no. 500–0006), and protein concentration values were taken as the average of at least two technical replicates.

**NanoPro™ 1000 Assay Development Parameters**—The instrument used in this study (NanoPro 1000) was purchased from ProteinSimple™ (Santa Clara, CA). For AKT isofrom separation, we used a mix of 5–8 and 4–7 pH ampholyte gradients at a ratio of 80% to 20%.

**RESULTS**

**Assay Development**—To develop a sensitive and reliable assay measuring expression of the various AKT protein isoforms and phosphoforms, we employed a novel nanocapillary immunoassay technology based on isoelectric focusing (16). This platform (NanoPro™ 1000) employs the automated separation of proteins using isoelectric focusing performed in an ultrathin nanocapillary, with subsequent immobilization on the capillary’s specially coated wall using UV crosslinking. Solutions with primary antibody, HRP-conjugated secondary antibody, and ECL are then passed through the capillary, and the protein of interest is visualized and represented as peaks using specialized data acquisition and analysis software (Compass™). The protein separation is based on net charge, allowing the separation and detection of multiple isoforms of the same protein using a single primary antibody, even if the size difference among them is negligible.

An appropriate ampholyte gradient is critical when developing a NanoPro™ 1000 assay because the protein of inter-
est, along with all its post-translationally modified isoforms, must have a pI within the gradient’s pH range. In order to choose the appropriate ampholyte gradient for AKT assay development, we obtained the estimated pI values of all three AKT isoforms using the Expasy Proteomics database. AKT1 and AKT3 were predicted to have very similar pI values (5.75 and 5.72, respectively), whereas AKT2 was predicted to have a pI value of 5.98. We therefore chose a pH 5–8 ampholyte mix, nested with a 20% pH 4–7 gradient. Nesting the gradient with 20% pH 4–7 ampholyte mix stretches the pH buffering capacity at both capillary ends (experimental observation) and provides comfortable separation of molecules within a 4.5–8.5 pI range, which should allow most, if not all, of the possible PTM-induced changes in AKT protein to be detected.

AKT Isoform Identification—In order to facilitate the identification of all three AKT isoforms, given the very close pI values of AKT1 and AKT3, we decided to work with a cell line in which AKT3 is not expressed. Based on RNA expression data generated previously in our lab (17), luminal breast cancer cell lines such as BT474 and HCC2185 express very little or no AKT3. Using Western blotting, we confirmed that indeed BT474 cells do not express detectable levels of AKT3 protein (Fig. 1A), in contrast to basal cells such as MDAMB231 and BT549, which express AKT3. We therefore proceeded with BT474 cells for identification of the AKT1 and AKT2 isoforms.

We prepared BT474 lysates at various protein concentrations in NanoPro 1000 lysis buffer (20 mM Bicine 0.6% CHAPS). For AKT detection, we used an AKT antibody that recognizes all AKT isoforms (T-AKT antibody), as well as AKT1/2 isoform-specific antibodies. The T-AKT antibody generated an AKT profile with seven peaks within a pI range of 5.46–6.04 (Fig. 1B). Using the AKT isoform-specific antibodies, we then identified AKT1 and AKT2 in these cells. The four leftmost peaks (pI = 5.46, 5.54, 5.58, and 5.63) were recognized by the AKT1 antibody, whereas the three rightmost peaks (pI = 5.75, 5.88, and 6.04) were recognized by the AKT2 antibody.
As expected, we did not detect any peaks using the AKT3 antibody (data not shown). These results are consistent with the theoretical AKT pI calculations, as AKT1 is predicted to have a more acidic pI than AKT2, and therefore should be to the left of AKT2 in our gradient.

To confirm these results, we used RNAi in order to knock down the AKT1 and AKT2 isoforms specifically. For this experiment, we transfected BT474 cells with AKT1- or AKT2-specific RNAi (Qiagen) or with a nonsilencing control and ran lysates on the NanoPro 1000. Transfection with the AKT1 RNAi resulted in a 4-fold reduction of the four leftmost peaks, whereas the three rightmost peaks remained almost unaffected (Fig. 1C, middle panel). Similarly, in the samples transfected with the AKT2-specific RNAi, the three rightmost peaks were significantly reduced (Figure 1C, bottom panel), whereas the peaks identified as AKT1 remained unchanged.

Identification of Phosphorylated AKT—Because proteins in this system are separated based on charge, we should ideally be able to separate molecules with very similar sizes, which would otherwise be difficult or impossible to separate with methods based on molecular weight separation. Post-translational modifications on proteins (including phosphorylation and dephosphorylation events) pose many such difficulties, because they result in very minor, or negligible, changes in the molecule’s size that might be hard or impossible to detect using standard methodologies.

In order to identify phosphorylated AKT1 and AKT2, we treated lysates from BT474 cells with lambda phosphatase, which removes phosphate groups nonspecifically. Untreated BT474 lysates generated a peak profile with seven AKT peaks as expected. Samples treated with lambda phosphatase showed profiles “collapsed” into three major distinct peaks with pI values of 5.63, 5.75, and 6.04 and a minor peak at 5.88. The 5.63 and 5.75 peaks were detected by the AKT1 antibody, whereas the 5.88 and 6.04 peaks were detected by the AKT2 antibody (Fig. 2A). Nonphosphorylated AKT1 has an estimated pI = 5.75, without taking into consideration any other possible post-translational modifications, which ex-
Fig. 2. Identification of phosphorylated AKT isoforms. A, BT474 lysates were treated with lambda phosphatase and run on a NanoPro 1000. Total AKT, AKT1, and AKT2 were detected using the appropriate antibodies. Treatment with phosphatase collapsed the AKT profile into three major peaks and one minor peak (pI = 5.63, 5.75, 5.88, and 6.04; second top panel). The 6.04 peak was recognized by the AKT2-specific antibody and represents unphosphorylated AKT2, and the minor 5.88 peak represents a small amount of P-AKT2; the remaining two peaks were detected by the AKT1-specific antibody. Peak 5.75 represents unphosphorylated AKT1. The 5.63 peak is either a partially resistant phosphorylated AKT1 moiety or unphosphorylated AKT1 carrying a second post-translational modification. B, a pS473 antibody was used to identify the AKT1 isoforms phosphorylated at S473. Peaks at 5.46, 5.54, and 5.58 were detected by this antibody and likely represent P-AKT moieties phosphorylated at S473. A minor peak at 5.38 was also found to likely be phosphorylated at that site. DMSO, dimethyl sulfoxide.
though the AKT2 antibody reacted with the 5.75 peak (Fig. 1B) suggesting that it was phosphorylated AKT2. Moreover, even though the AKT2 antibody reacted with the 5.75 peak (Fig. 1B) in untreated samples, it failed to do so in the phosphatase-treated sample (Fig. 2A), which suggests a second phosphorylated AKT2 isoform at 5.75. Finally, the peak at 6.04 was the dominant AKT2 peak after phosphatase treatment and likely represents the completely unphosphorylated AKT2 isoform. In the case of AKT2, theoretical calculations regarding phosphorylation events on the molecule predict a shift of 0.17 pI units for the first two phosphorylation events and an additional shift of 0.13 pI units for the second two phosphorylation events. Given the detection of unphosphorylated AKT2 at 6.04, it is likely that the 5.88 phosphopeak carries two phosphorylation events, whereas the 5.75 peak carries four phosphorylation events.

Identification of pS473—Even though T308 phosphorylation has been shown to partially activate AKT, additional phosphorylation on S473 within the regulatory domain of the molecule is necessary for full activation (14, 18). Measurement of S473 phosphorylation is widely used to assess AKT activity, and it is especially useful in determining the effect of pharmacological inhibitors designed to inhibit AKT directly or through the PI3K pathway upstream. Therefore, identification of the AKT isoforms that are phosphorylated at S473 in our assay would provide an excellent tool for quantifying AKT activation in cancer cells or inhibition of the PI3K pathway upon drug treatment. In order to determine the phosphorylated AKT peaks that carry phosphorylation on S473, we used a pS473-specific antibody. Peaks 5.46, 5.54, and 5.58 were detected by the pS473 antibody, whereas the 5.63 peak was not (Fig. 2B). In addition, the pS473 antibody detected the minor peak detected by the T-AKT antibody to the leftmost part of the profile (pI = 5.58), which suggests that this is yet another AKT S473 phosphoform. To further validate the identity of these peaks as pS473, we treated BT474 cells with a PI3K inhibitor (GSK2126458A). As expected, this treatment completely abolished pS473 phosphorylation (Fig. 2B, lower panel), further confirming the identity of these peaks as pS473.

Identification of AKT Isoforms in Breast Cancer

AKT3 Identification—AKT1 and AKT2 isoforms are ubiquitously expressed across tissues, whereas AKT3 expression is more restricted. In breast, AKT1 and AKT2 are the predominant AKT isoforms, but AKT3 is up-regulated in several breast cancer cell lines, particularly those of the basal subtype (17). In order to have a complete AKT isoform profile in breast cancer cell lines, we also tested MDAMB231 cells, which show high expression of AKT3, unlike BT474 (Fig. 1A). In lysates from MDAMB231 cells, the pan-AKT antibody generated a profile with nine peaks, which included the seven peaks present in BT474 cells and two unique peaks at 5.67 and 5.83 (Figs. 3A and 3B). Both of these peaks were detected with an AKT3-specific antibody. Treatment of the MDAMB231 lysates with lambda phosphatase caused collapse of the 5.67 peak, whereas the 5.83 peak was not affected, suggesting that they are the phosphorylated and unphosphorylated AKT3 isoforms, respectively (Fig. 3A). Thus, in MDAMB231 cells, which express all three AKT isoforms, nine peaks are detected by the pan-AKT antibody, corresponding to all three AKT isoforms and some of their phosphorylated moieties, generating an AKT peak profile that can be used to quantify these proteins using a single detection reagent (Fig. 3B, top panel).

Assay Reliability and Sensitivity—In order to determine the reliability of our AKT assay, we prepared BT474 cell lysates and ran a NanoPro 1000 assay with 96 technical replicates. Samples were loaded on the assay plate at 0.05 µg/ml and were run for eight cycles, in all 12 capillaries of each cycle. We then measured total and phosphorylated AKT1 and AKT2, determined the fraction of phosphorylated AKT1 and AKT2 relative to total (P/T), and calculated the standard deviation and the coefficient of variation (cv) of these values within a cycle (across 12 capillaries) and across cycles (cycles 1–8) (supplemental Table S1). P/T values for AKT1 and AKT2 did not vary considerably within a cycle or across cycles. Specifically, the intracycle variation for AKT1 ranged from 0.7% to 1.5%, and for AKT2 from 0.6% to 1.5%. Similarly, the intercycle cv values for AKT1 ranged from 2.2% to 3.3%, and for AKT2 from 0.6% to 1.6% (supplemental Table S1). The cv values within this range are very acceptable and prove that the assay described here is stable and reproducible and can be used reliably to quantify AKT expression and activity across cycles, even though data from each cycle are obtained 2 to 3 h apart from the beginning to the end of a complete assay run.

In order to determine the sensitivity of the assay, we then titrated the amount of total protein needed in order to detect all seven AKT1/2 peaks. For this experiment, we counted BT474 cells before lysis (the total number of cells obtained was 4.7 × 10⁶) and then lysed the cells and determined the total amount of protein obtained (1.7 mg of protein). Therefore, on average each cell produced 0.36 ng of protein. We then processed the lysates for the NanoPro 1000 assay. Starting with an initial lysate concentration of 0.4 µg/µl, we
prepared seven 2-fold serial dilutions and ran these samples on the NanoPro 1000. The lowest lysate concentration needed in order to obtain a complete AKT profile is 0.05 μg/μl, and the minimum amount of lysate needed for a single run is 2 μl. Therefore, given that in this case each cell produced 0.36 ng of total protein on average, the theoretical number of BT474 cells required in order to obtain a complete AKT profile in our assay was 280. Moreover, because the capillaries used in NanoPro assays need only 400 nl in order to be filled, instead of the 2 μl we loaded, the lowest amount of total protein needed in order to detect AKT was 20 ng, a protein amount that is produced in theory by 56 cells. Even though this number will vary depending on the cell type, cell size, lysis efficiency, and other handling parameters, as well as AKT expression levels, it is clear that the AKT assay described here is extremely sensitive and can be used to measure AKT protein expression and levels of activation using only minimal amounts of total protein derived from scarce or limited samples, including rare cell populations and fine needle aspirate samples.
**AKT Isoform Expression in Breast Cancer Cell Lines**—Different AKT isoforms seem to have distinct, non-redundant functions in normal and cancer cells (19). Expression of these isoforms is variable across different cell types, with AKT1 and AKT2 more commonly expressed in epithelial cells and AKT3 having a more restricted expression pattern, mainly in the brain and testes (20). The AKT assay described here uses one single antibody to detect all three AKT isoforms. This made it possible for the first time to measure the relative protein levels of AKT1/2/3 within a cell and make quantitative comparisons, assuming the affinity of the antibody is equal against all three isoforms. Thus, using this assay we measured the expression of all AKT isoforms and identified phosphoforms across a panel of breast cancer cell lines comprising both luminal (12 cell lines) and basal (10 cell lines) subtypes (Fig. 4A). AKT1 and AKT2 isoforms were expressed in all cell lines tested, whereas only 8 out of 22 cell lines expressed AKT3. Of these, six cell lines were basal cells, and only two (UAC812 and ZR75–1) were of luminal subtype (Fig. 4B). Thus, AKT3 expression in breast cancer seems to be primarily within basal-type cells. In contrast, even though AKT1 and AKT2 were expressed in all cell lines tested, luminal cells expressed these isoforms at higher levels than basal cells (Fig. 4A).

In addition to the absolute expression of AKT1/2/3, we also measured the levels of the phosphorylated moieties we were able to identify earlier (Fig. 2 and Fig. 3) as a percentage of total AKT1, -2, or -3, accordingly (Fig. 4A). Phosphorylation levels of AKT1 at S473 did not vary considerably across cell lines, with 61% being the lowest and 89% the highest amount of phosphorylated AKT1 within a cell line. In contrast, 4P and 2P phosphorylation on AKT2 and phosphorylation on AKT3 showed higher variation among cell lines. 4P-AKT2 ranged from 14% to 52% of total AKT2, and 2P-AKT2 ranged from 23% to 85%. Similarly, pAKT3 levels ranged between 16% and 89% of total AKT3 in cell lines that expressed this isoform (Fig. 4B).

**Qualitative and Quantitative Assessment of AKT after Drug Treatment**—In order to assess qualitative and quantitative changes in AKT1 and AKT2 expression and phosphorylation with drug treatment, we treated BT474 cells with lapatinib and an AKT inhibitor (AKTi; GSK690693), performed Western blot and NanoPro assays, and used isoform-specific AKT antibodies, as well as antibodies against pS473 and pS474, to evaluate AKT changes after drug treatment. Treatment of BT474 cells with lapatinib and an AKT inhibitor (AKTi; GSK690693), performed Western blot and NanoPro assays, and used isoform-specific AKT antibodies, as well as antibodies against pS473 and pS474, to evaluate AKT changes after drug treatment. Treatment of BT474 cells with lapatinib did not result in major changes in AKT1 and AKT2 levels (Fig. 5A). In contrast, pS473 levels were reduced 4-fold relative to the dimethyl sulfoxide control. Similarly, pS474 levels were reduced 2-fold. Treatment with the AKT inhibitor caused a 3-fold decrease in AKT1, whereas AKT2 was slightly increased (Fig. 5A). Importantly, even though AKTi treatment reduced total AKT1 levels, there was a marked increase in S473 phosphorylation. Similarly, phosphorylation of S474 was noticeably higher in AKTi-treated cells. Very similar results were obtained when Western blotting was used (Fig. 5A, panels a and b), which shows that the NanoPro assay described here can be used reliably for the quantification of AKT, with the added benefit of very high sensitivity (10 ng of total lysate needed, compared with micrograms for Western blots), as well as a higher level of isoform and phosphoform resolution than obtained with traditional size-based separation methods.

Interestingly, the AKT2 peaks showed a dramatic shift toward the acidic end of the capillary and presented lower pI values, similar to pAKT1 peaks. pAKT2 peaks at 5.75 and 5.88 were diminished (Fig. 5B). These results suggest that in...
dition to quantitative changes in S474 phosphorylation, treatment with the AKT inhibitor causes extensive qualitative changes in AKT2 phosphoforms. This could be a result of additional modifications on AKT2 or hyperphosphorylation of the molecule. Additional experiments will be necessary in order to address these findings.

**DISCUSSION**

The PI3K-AKT pathway plays a pivotal role in tumorigenesis, with most of its elements found to be mutated or amplified in multiple human cancers (21–23). AKT is a central element in the PI3K pathway and is activated by most known growth factors involved in cancer (24–27). However, the role that each AKT isoform plays in health and disease is not completely understood, as most studies on AKT were performed using antibodies that detect all three isoforms, despite increasing evidence suggesting they might have distinct, non-overlapping functions in normal tissues and cancer. For example, recent siRNA silencing studies showed distinct functions for different AKT family members within a cell (10, 11), and in breast cancer it was shown that the three isoforms exhibit different localizations and therefore are thought to have at least partially distinct functions (12). Additionally, even though all three AKT isoforms can transform cells in vitro (28),

**Fig. 5. Changes in AKT isoform expression and phosphorylation with drug treatment.** A, Western blotting and NanoPro assay were used to measure total AKT1, total AKT2, pS473, and pS474 levels in BT474 cells. Similar results were obtained using both methods, suggesting that NanoPro assays can be reliably used for these experiments, with the added advantage of higher resolution and increased sensitivity relative to traditional Western blots. B, lapatinib- and AKTi-treated cells exhibit qualitative changes with respect to AKT phosphorylation. Lapatinib reduced overall pS473, but the S473 phosphopeak at 5.38 was increased (a and b). AKTi treatment increased phosphorylation at S473 and S474 but resulted in phospho-AKT2 peaks shifting to more acidic pIs, suggesting additional modifications or hyperphosphorylation of the molecule (c and d).
AKT2 is the isoform most frequently amplified or overexpressed in human malignancies (19, 29–31).

Thus, as a result of the lack of available methodologies, and despite the importance of AKT in growth and survival signaling in cancer, very little is known regarding the specific roles played in growth and survival by the three AKT family members (AKT1, -2, and -3) or by the different phosphorylation sites that can regulate and activate the protein.

Using a novel automated nanocapillary technology based on isoelectric focusing, we developed an assay to detect and quantify all three AKT isoforms (AKT1, -2, and -3) and multiple phosphorylated forms in breast cancer cells using minute amounts of total protein. This approach allows the separation of AKT into distinct peaks that correspond to different iso- and phosphoforms and the detection of these peaks using a single pan-specific antibody against AKT. This assay enabled us to accurately measure expression levels of all three AKT isoforms in a breast cancer cell line panel using protein derived from a very small number of cells. We were also able to measure levels of activated AKT by detecting and quantifying multiple AKT phospho-isoforms, including AKT phosphorylated at S473. Using this technology, we managed to quantify the effects of pharmacological inhibitors used to interfere with the PI3K-AKT pathway by determining quantitative and quantitative changes in AKT isoform and phosphoform levels in response to treatment.

We used phosphatase treatment to assist in assigning the peaks for phospho-AKT1 and -AKT2 isoforms. We noted that the peak at 5.63 corresponding to AKT1 was present in both phosphatase-treated and untreated samples but was reduced in the treated sample. There are two scenarios that could explain this result. In the first case, the 5.63 peak represents a phosphorylated AKT1 species that is partially resistant to phosphatase treatment, which explains its presence at reduced levels after lambda phosphatase. Alternatively, the 5.63 peak may represent two different AKT1 molecules: a phosphorylated AKT1 isoform, and an unphosphorylated second AKT1 isoform that carries a post-translational modification resulting in a more acidic pi than the that of the unmodified, unphosphorylated AKT1 that migrates at 5.75. In any case, the phosphorylated AKT1 at 5.63 is not phosphorylated at S473 (Fig. 2B), unlike the rest of the phospho-AKT1 peaks that show S473 phosphorylation.

We found that AKT1 and AKT2 were ubiquitously expressed across all the breast cell lines we tested, but that AKT3 expression was restricted almost exclusively to basal-like cell lines. Given that PI3K-AKT pathway inhibitors have been shown by us to be more effective in luminal and HER2 subtypes than basal cell lines (32), it will be interesting to study the dynamics of the response with respect to p-AKT3 activity in these cells to determine whether AKT3 plays a role in resistance.

Even though additional work is necessary in order to completely map each peak to a particular phosphorylation or other post-translational modification event, this assay can be used to measure the relative expression of AKT1/2/3 in breast cancer lines using very little starting material. Moreover, the identification of phosphorylated AKT1/2/3 allows us to quantify the activation of AKT in these cells and measure the effect of pharmacological inhibitors on AKT phosphorylation.

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