Regulation of inflammatory breast cancer cell invasion through Akt1/PKBα phosphorylation of RhoC GTPase

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Running Title: Akt1 Modulates Inflammatory Breast Cancer Invasion
Abstract

With a 42% and 18% 5- and 10-year respective disease-free survival rate, inflammatory breast cancer (IBC) is arguably the deadliest form of breast cancer. IBC invades the dermal lymphatic vessels of the skin overlying the breast and as a consequence nearly all women have lymph node involvement and ~1/3 have gross distant metastases at the time of diagnosis. One year after diagnosis ~90% of patients have detectable metastases, making IBC a paradigm for lymphovascular invasion. Understanding the underlying mechanisms of the IBC metastatic phenotype is essential for new therapies. Work from our laboratory and others show distinct molecular differences between IBC and non-inflammatory breast cancers. Previously we demonstrated that RhoC GTPase is a metastatic switch responsible for the invasive phenotype of IBC. In the current study we integrate observations made in IBC patients with in vitro analysis. We demonstrate that the PI3K/Akt signaling pathway is crucial in IBC invasion. Key molecules involved in cytoskeletal control and cell motility are specifically upregulated in IBC patients compared with stage and cell-type-of-origin matched non-inflammatory breast cancer patients. Distinctively, RhoC GTPase is a substrate for Akt1 and its phosphorylation is absolutely essential for IBC cell invasion. Further our data show that Akt3, not Akt1 has a role in IBC cell survival. Together our data demonstrate a unique and targetable pathway for IBC invasion and survival.
Introduction

Inflammatory breast cancer (IBC) is a phenotypically distinct and exceptionally lethal form of breast cancer characterized by rapid progression and poor prognosis. The term IBC was first coined in 1924 to describe a locally advanced breast cancer with unique clinical features including, skin erythema and thickening, nipple retraction and peau d' aurange (1). IBC is clinically distinguished by rapid onset of primary skin changes, typically occurring within twelve weeks and progression to Stage IIIb/IV disease within 6 months (2-4). By definition, IBC is a T4d tumor at diagnosis and typically affects younger women, often during their childbearing years (5).

Although the actual numbers are disputed by the IBC community, current SEER data suggests that IBC accounts for 1% to 5% of all newly diagnosed breast cancers in the United States annually (6). Despite significant improvements in the disease-free survival rates of non-inflammatory breast cancer (nIBC) patients, IBC patient survival rates remain low, with a 5-year disease-free survival rate of less than 40%, in comparison to the ~90% of nIBC (4, 7-9). The poor prognosis is due to infiltration of tumor emboli that metastasize within the dermal lymphatic vessels of the skin overlying the breast (3, 4).

Although notable progress has been made in the last decade in the study of IBC, elucidating the molecular mechanisms involved in driving IBC metastasis is essential for improved treatment. It is suggested that IBC disseminates passively through the dermal lymphatic vessels (10). However, work by our
laboratory and others suggest that this is not the case. Our laboratory has previously identified genes involved in inflammatory breast cancer invasion; RhoC GTPase has an essential role in driving the metastatic phenotype of IBC, being overexpressed in >90% of IBC tumors as compared to stage-matched nIBC tumors (11, 12).

RhoC GTPase dynamically reorganizes the actin cytoskeleton and controls nearly all aspects of cellular motility (13-16). RhoC GTPase overexpression leads to transformation of immortalized human mammary epithelial cells with an invasive phenotype similar to that of IBC (17). RhoC also promotes metastasis in other invasive cancers, including pancreatic ductal adenocarcinoma (18), lung cancer (19), and melanoma (20).

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays a role in enhancing cancer cell motility, invasion, and metastasis (21-23). Akt encompasses three highly conserved homologues: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). The specific functional roles of Akt isoforms continue to be under investigation, particularly their roles in metastasis. Although each Akt kinase is activated through similar mechanisms, their different tissue-specific expression patterns suggest distinct roles. Recent studies have shown that Akt1 inhibits, while Akt2 promotes non-inflammatory breast cancer cell migration and invasion (22, 24). In the current study, we examine the roles of Akt1, -2 and -3 in IBC and demonstrate that Akt1 is responsible for IBC cellular motility and invasion through phosphorylation of RhoC GTPase.
Materials and Methods

Cell culture.

Cell lines were authenticated by the Johns Hopkins Genomic Resource Center by STR analysis and maintained under defined culture conditions (25). SUM149 IBC cells grown in Ham’s F12 medium (Mediatech, Inc., Manassas, VA, USA) with 5% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 1% Penicillin/Streptomycin, Antibiotic/Antimycotic, L-glutamine (Mediatech), Hydrocortisone (Invitrogen, Carlsbad, CA) and an insulin/transferring/selenium cocktail (Gibco, Carlsbad, CA). MDA-MB-435 cells were grown in MEM medium (Mediatech) with 5% FBS, 1% Penicillin/Streptomycin, L-glutamine, sodium pyruvate and MEM non-essential amino acids (Mediatech). The MDA-MB-231 cells were grown in DMEM medium (Mediatech) with 5% FBS, 1% Penicillin/Streptomycin, and 750 µg/ml Insulin (Invitrogen, Carlsbad, CA). MCF7 were grown in DMEM medium with 10% FBS and 1% Penicillin/Streptomycin. All cell lines were grown at 37°C in 95%:5% air:CO2.

Patient samples.

Tumor samples were retrieved from the tissue bank of the General Hospital Sint-Augustinus (Antwerp, Belgium). Clinical and pathological data are stored in a database in accordance with hospital privacy rules and local IRB approval. Specimens were brought to the pathologists immediately after resection and part of the tissue was placed in liquid nitrogen and subsequently stored at -180°C.
In vitro transfection experiments.

SUM149 cells were transfected with wildtype (WT) RhoC3XHA, RhoCS73A, PTEN, RhoCS73D, RhoAS73D, or pcDNA6-His-LacZ using FuGene HD transfection reagent (Roche, Branchburg, NJ). Transfection efficiency was 38-74% as determined by β-galactosidase staining of the LacZ-transfected cells after a 16 h incubation at 37°C with X-gal staining solution (20 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride-hexahydrate in 1X PBS, pH 7.4 (Life Technologies, Inc., Carlsbad, CA). Transfected cells were allowed to incubate at 37°C for 48 h. RhoCS73A, RhoCS73D, and RhoAS73D constructs were developed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

SUM149, MDA-MB-435, MDA-MB-231, and MCF7 cells were transfected with siRNA directed against either pan-Akt, Akt1, Akt2, Akt3 or a siRNA control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 48 h using GeneSilencer siRNA transfection reagent (Genlantis, Inc., San Diego, CA).

For pharmacologic inhibition of Akt, cells were treated with 10 µM of Akt Inhibitor II (Calbiochem, Gibbstown, NJ) for 24 h at 37°C.

RNA extraction and gene expression analysis.

To comparatively analyze the PI3K/Akt pathway in a set of human IBC and nIBC samples, we used the Human PI3K/Akt Signaling Pathway RT² Profiler™ PCR Array (SA Biosciences, Frederick, MD, USA). This small-scale array profiles the expression of 84 genes including members of the Akt and PI3K families and their
regulators as well as 5 housekeeping genes. Prior to analysis, RNA was extracted from 34 IBC samples and 48 non-stage matched nIBC samples using protocols described previously (26). The data set represents the same samples as reported on in earlier studies (26, 27). Clinicopathological data are provided in Supplemental Table 1. RNA, 2 µg, was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Preparation of the PCR-master mix and PCR-reactions on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) were carried out according to the manufacturer’s instructions. Two samples were run in duplicate to assess assay reproducibility. RNA obtained from WT SUM149 cell lines and transfected SUM149 cell lines were processed in a similar manner. For the cell lines, PCR assays were done in duplicate. CT-values were normalized relative to the median expression level of all 5 housekeeping genes using the $2^{-\Delta \text{C}T}$ method. Relative gene expression (RGE) data were subsequently scaled as such that, for each sample, the median relative gene expression level is 0 and the standard deviation is 1. PCR data generated on the WT and transfected cell lines were processed in a similar manner but independent from the data generated on the human samples.

In addition to the PCR data set, we analyzed a partially independent Affymetrix data set consisting of 41 IBC samples, 55 non-stage matched nIBC samples and 8 normal breast samples. Clinicopathological data are provided in Supplemental Table 1. For this data set, RNA was analyzed and processed as described before (26). RNA was hybridized onto HGU133plus2 gene chips in
collaboration with the VIB Nucleomics Core (UZ Gasthuisberg, O&N, Leuven, Belgium). Raw array data are publicly available on Array Express (accession number E-MTAB-1006). Microarray data were background corrected, normalized, summarized and log2-transformed using the GCRMA-algorithm (27). 22,419 Probe sets with fluorescence intensities above log2(100) in at least 25% of the arrays were filtered in and were considered informative for further analysis. In case of multiple probe sets corresponding to a single gene, the probe set with the highest standard deviation was selected for further analysis.

**Western blot and immunoprecipitation analysis.**

Proteins were harvested from cell cultures using RIPA buffer and 5 µl/ml protease inhibitor cocktail (Calbiochem) and/or phosphatase inhibitor (Thermo Scientific, Waltham, MA, USA). Protein concentration was evaluated using a BCA Protein Assay kit (Pierce Scientific, Rockford, IL) at a wavelength of 562 nm. For western blot analysis, aliquots of 30 µg were mixed with Laemelli buffer. For immunoprecipitation, whole cell lysates (300 µg) were incubated ON at 4°C with primary antibodies specific for RhoC GTPase (developed in house), Akt1, Akt2, or Akt3 (Cell Signaling). Antibody-bound proteins were incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) or donkey anti-chicken IgY-agarose (Gallus Immunotech, Inc., Cary, NC) at 4°C for 3 h. Samples were washed 5X with PBS.

All protein samples were heat denatured, separated by SDS-PAGE on pre-cast 4-20% Tris-HCl gels (BioRad, Hercules, CA), transferred to
nitrocellulose, blocked with 3% powdered milk (Nestle Carnation) in PBS with 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO). For western analysis, immobilized proteins were probed using antibodies specific for pan-Akt, Akt1, Akt2, or Akt3 (Cell Signaling) proteins and the membranes were then stripped and probed for phosphorylated Akt protein. Anti-Akt1, anti-Akt2, or anti-Akt3 immunoprecipitate blots were incubated with a phospho-Akt antibody (Santa Cruz Biotechnology). Anti-RhoC GTPase blots were incubated with a phospho-Serine antibody (Cell Signaling). Protein bands were visualized by ECL (Millipore, Co., Billerica, MA).

**Akt kinase assay.**

Akt kinase activity was measured using the Cellular Activation of Signaling ELISA (CASE) kit (SA Biosciences, Frederick, MD), directly measuring serine 473 Akt phosphorylation on cultured cells. SUM149, MDA-MB-435, MDA-MB-231, and MCF7 cells were seeded at 13,000 cells per well of a 96-well tissue culture plate, serum starved ON and stimulated 30 min with 10 ng/ml EGF, 10 ng/ml IGF, or FBS. Half of the samples were treated with an anti-phospho-Akt primary antibody and the other half with an anti-pan-Akt primary antibody. After incubation with primary and secondary antibodies developing solution was added for 10 min at RT followed by stop solution and absorbance measured at 450 nm.

**$^{32}$P Akt protein kinase assay.**
The Akt kinase assay was performed by incubating RhoC oligopeptide containing an Akt phosphorylation consensus sequence, WT RhoC, RhoCS73A mutant protein, $^{32}$P-gamma-ATP (MP Biomedicals, Solon, OH) and protein kinase reaction mixture (20 mM HEPES, pH 7.2, 10 mM MgCl$_2$, 10 mM McCl$_2$, 1 mM DTT, 0.2 mM EGTA, 20 μM ATP, 1 μg phosphatidylserine, and protein kinase activator; Sigma). Alkaline phosphatase (Sigma) was added to the proteins as a control and a scrambled peptide sequence was used as a comparative control for the RhoC peptide. Reactions were stopped after 30 min by heating at 95°C for 10 minutes. The level of phosphorylated substrate measured by liquid scintillation.

**Matrigel™ invasion assay.**

Invasion assays were performed using a Matrigel™ Invasion Chamber (BD Biosciences, San Jose, CA) with 8μm pore filters as previously described (28). Briefly, 1.25x10$^5$ cells in serum-free medium were added to rehydrated invasion chambers and allowed to migrate toward normal growth medium for 24 hr at 37°C. The media was aspirated and inserts gently wiped with a cotton swab. Methylene blue was added into each insert for 30 min, washed with water and allowed to dry at RT for 16 h. Cells were counted in continuous (10x) magnification fields. Data are expressed as the percent invasion of the treated cells relative to the untreated control cells.
To assess reproducibility of the PCR assays, replicate samples were analyzed comparatively using Spearman correlation analysis. Gene-wise correlation coefficients for the genes commonly identified in the PCR microarray data set and the Affymetrix data set (N=79) were calculated similarly. Differential gene expression analysis comparing IBC to nIBC, tumor samples to normal samples or WT SUM149 cells to transfected SUM149 cells was performed using a non-parametric Mann-Whitney U-test. P-values were corrected for multiple testing using the Benjamini and Hochberg step-up false discovery rate controlling procedure and adjusted P-values less than 0.05 were considered significant, unless explicitly stated.

To determine if the PI3K/Akt pathway is differentially regulated between human samples from patients with IBC and nIBC, a global test for the PI3K/Akt pathway genes was performed. The result of the global test was corrected for the cell-of-origin subtype classification, which we reported on in previous studies (27, 29). The set of 84 genes can be further subdivided into eight biological processes. Each of these biological processes was further analyzed in a similar manner on the PCR data set and appropriate corrections were carried out. The above outlined strategy was then repeated on the Affymetrix data set, with the exception that the gene subset analysis was performed only for those terms identified as significant in the PCR data set and that the gene subset lists were expanded using corresponding Gene Ontology terms.
To perform a comparative analysis of the PI3K/Akt-specific gene expression profile of the cell lines and the human tissue samples we adopted the following strategy. Using Principal Component Analysis (PCA) we compared the expression profiles of the wild-type and PTEN- and RhoCS73A-transfected SUM149 cell lines. Next, gene-wise regression coefficients for the first (capturing the difference between wild-type and RhoCS73A-transfected SUM149 cells) and the second (capturing the difference between the wild-type and the PTEN-transfected SUM149 cells) principal components were used to calculate the corresponding first and second metagene expression levels in the Affymetrix data set. Resulting metagene expression values were compared between IBC and nIBC using a Mann-Whitney U-test.

*In vitro* data were analyzed using a GraphPad software package for Windows (Prism 4.0). A *p* value of ≤0.05 was considered statistically significant.
Results

Expression of active Akt in IBC cells

We began by determining differences in expression of Akt1, -2, -3 and phosphatase and tensin homology deleted on chromosome 10 (PTEN) between IBC and nIBC patient samples. Gene analysis was initiated using a PCR data set, including IBC and nIBC samples identified according to TNM status, stage, histological grade, hormone receptor status, and subtype classification (Supplemental Table 1). When evaluating the replicate experiments, significant correlation coefficients (range: 0.955 – 0.999; P<0.0001) were achieved, indicating good overall reproducibility of our assays. We found that neither Akt1, -2, -3 or PTEN are differentially expressed between IBC and nIBC at an FDR less than 0.05. When examining at nominal P-values, both Akt1 (P=0.037; FDR=0.15) and PTEN (P=0.007; FDR=0.07) demonstrate overexpression in IBC.

In Figure 1a we analyzed the PI3K/Akt gene set on our PCR data set, comparing IBC with nIBC patient samples. Out of 84 PI3K/Akt genes, 26 (31%) are differentially expressed between IBC and nIBC, from which 18 (11%) remain significant after correction for multiple testing (FDR<0.05). Global test analysis of the PI3K/Akt pathway genes reveals a significant difference between IBC and nIBC for the entire gene set (P=0.037). These data suggest that the genes related to the PI3K/Akt signaling pathway are differentially regulated in IBC compared with nIBC.

As shown in Figure 1b, differences in the PI3K/Akt pathway are associated with the basal-like molecular breast cancer subtype (Global test,
Since the basal-like breast cancer subtype is frequently observed in IBC (27, 29), we performed a global test for the PI3K/Akt pathway genes, comparing IBC with nIBC, and correcting for the influence of the molecular breast cancer subtypes. After correction, the global test comparing IBC to nIBC turned out to be inconsequential, indicating that, in general, the differential PI3K/Akt regulation associated with molecular subtype is not IBC-specific.

More in depth analyses were performed on 8 specific subgroups of the PI3K/Akt gene set, which are listed in Table 1. Global tests were performed on these gene lists comparing IBC with nIBC and using the same correction for the molecular subtype, after which only genes involved in PI3K subunit p85-dependent regulation of actin organization and cell migration remain significantly associated with the distinction between IBC and nIBC tumors (P=0.045). This suggests that the PI3K/Akt pathway in IBC is specifically associated with tumor cell motility and actin reorganization.

To corroborate the data obtained using PCR, we repeated the above outlined analysis using a more elaborate data set of 104 Affymetrix profiles of 41 IBC samples, 55 nIBC samples and 8 normal breast samples, containing 68 samples in common with the PCR data set. After filtering non-informative genes, we identified 79 common genes for which a gene-wise correlation analysis between both data sets was performed. The latter demonstrated a platform-independent reproducibility of the gene expression profiles (median correlation coefficient: 0.597, 5th percentile: 0.233, 95th percentile: 0.881). Correlation coefficients are shown in a heatmap format in Supplemental Figure 1.
comparing IBC to nIBC, again no statistical difference was observed for Akt1, -2, -3 or PTEN at an FDR less than 0.05 (Figure 1c). In comparison to normal breast epithelium, Akt1 is overexpressed in tumor samples, whereas Akt2, and PTEN are higher in the normal samples (all Ps<0.003; all FDRs<0.05). Out of 79 genes, 33 (42%) were differentially expressed between IBC and nIBC from which 21 (27%) remained significant after correction for multiple testing (FDR<0.05). In line with these results, global testing revealed a significant difference between IBC and nIBC for the PI3K/Akt gene set (P=0.003), but this difference was even more pronounced when testing the molecular subtypes (P<0.001).

Sample plots and gene plots are shown in Figure 1d. Correcting the global expression differences for the PI3K/Akt gene set between IBC and nIBC for the influence of the molecular subtypes abolished the significant result (P=0.179). Given the results for the gene subset analysis on the PCR data set, we reiterated the gene subset analysis for an expended list of motility-related genes (GO:0048870) only and found significant differences between IBC and nIBC (P<0.001) even after adjusting for the effect of the molecular subtypes (P=0.049). Of note, results obtained on the entire Affymetrix data set were strongly comparable to those obtained on the subset of IBC and nIBC samples not contained in the PCR data set (N=28) suggesting our results are data set independent (data not shown).

To determine if Akt has a specific role in IBC invasion we compared the SUM149 IBC cell line with the basal cell-type of origin matched nIBC cell lines, MDA-MB-231 and MDA-MB-435 and the luminal cell-type of origin, ER+ MCF7
cells. The SUM149s are a reliable model of IBC, often reflecting what is observed in patient samples (11, 12, 17, 26-31). Figure 2a is western blot analysis of individual Akt isoform protein expression and phosphorylation. Shown are total Akt1, -2, and -3 protein expressed in all cell lines by immunoblotting using antibodies specific for each isoform (Figure 2a). Also shown is an immunoprecipitation for Akt1, -2, and -3 followed by immunoblotting with a phospho-Akt antibody. These data show that Akt1 and -2 are phosphorylated both in the IBC and the nIBC cell lines. Akt3 is phosphorylated in the IBC cells and at a lower level in the MCF7 nIBC cells. Since phosphorylation is indicative of Akt activity, we determined the activity of Akt using a kinase assay. SUM149 IBC cells have a constitutively active Akt (Figure 2b). In contrast, Akt activation of the nIBC cells occurs only upon growth factor stimulation with epidermal growth factor (EGF) or insulin-like growth factor (IGF).

Akt affects breast cancer cell invasion

Akt-regulated signaling plays a significant role in numerous processes including cell invasion (32). Since comparison of IBC and nIBC patient samples implicated PI3K/Akt pathways related to cytoskeletal reorganization and migration, we next determined if Akt has a role in breast cancer invasion. Figure 2c demonstrates treatment of the SUM149 and MDA-MB-435 cells with a pharmacological Akt inhibitor, which inhibits Akt phosphorylation and causes a significant 75% decrease in the invasive capabilities of the IBC cells in an
invasion assay. In contrast, inhibition of Akt in the MDA-MB-435 cells did not affect cell invasion.

Similar results were observed with a pan-Akt small inhibitor RNA. In comparison to mock transfected and scrambled controls, the siRNA depleted Akt expression in both the SUM149 and MDA-MB-435 cells by ~90%. When placed in an invasion assay a significant 83% decrease in the invasive capabilities of the SUM149 IBC cells was observed, with no effect on the MDA-MB-435 cells (Figure 2d).

Akt is negatively regulated through PTEN, which directly inactivates the PI3K/Akt pathway (33). Corresponding to the highly phosphorylated and active levels of Akt, as shown in Figure 3a, protein expression of PTEN is lost in the SUM149 IBC cells but not the nIBC cells. Analysis of cytoplasmic and nuclear PTEN expression in IBC and nIBC patient samples using tissue microarrays and tumor sections demonstrates no clear trend of expression in IBC tissue (data not shown).

PTEN re-expression in the SUM149 cells leads to a significant 40% decrease in invasion (Figure 3b). There was a slight, non-significant increase in the number of apoptotic cells when PTEN was re-expressed (Figure 3c). Taken together, these data support a role for active Akt in IBC migration.

Using the PI3K/Akt PCR-array, we determined how re-expression of PTEN in the SUM149 cells would alter the expression of PI3K/Akt gene set. At a FDR less than 0.05, none of the genes were differentially expressed between WT SUM149 cells and PTEN-transfected SUM149 cells. However, when taking into
account the small sample size of the data set (N=4) and readjusting the FDR to 0.1, differential expression was identified for 45 (54%) genes, corresponding to the same set of genes with nominal P-values smaller than 0.05. Seventeen (20%) genes were overexpressed in the WT SUM149 cells and 28 (33%) genes were overexpressed in the PTEN-transfected SUM149 cells. These data suggest differences in expression for genes involved in the PI3K/Akt pathway upon PTEN re-expression. Several of these gene products, including Rac1, RhoA and Cdc42, are implicated in cell motility and suggest an altered state of cell motility with PTEN re-expression. The PI3K/Akt pathway gene expression profile comparing the WT SUM149 IBC cells with the SUM149 PTEN transfectants is displayed in Figure 3d. Interestingly, we observed that re-expression of PTEN in SUM149 cells is paralleled by BAD overexpression.

Akt1 mediates IBC cell invasion

Classically opposing molecular characteristics between IBC and nIBC, including differences in E-cadherin and caveolin-1 and -2 expressions (34), cautioned us not to assume that the effects seen on the invasive capabilities of the IBC cells with Akt inhibition were a result of Akt2 activity, as demonstrated in previous nIBC studies (22, 24). Using specific SmartPool siRNAs we selectively depleted each isoform of Akt (Figure 4a) and determined the effect on invasion. Figure 4b is a comparison of Akt1 and Akt2 depletion in all breast cancer cell lines and demonstrates that specific inhibition of Akt1 results in a significant 72% decrease in IBC cell invasion, with no effect on the nIBC cells. In contrast,
inhibition of Akt2 significantly reduces (75%-92%) the invasive capabilities of each of the nIBC cell lines without affecting the IBC cells. Proliferation assays (Supplemental Figure 2a) were performed and demonstrate no difference in cell proliferation upon depletion of Akt1 or Akt2. Similarly, apoptosis assays (Supplemental Figure 2b) demonstrate that depletion of either Akt1 or Akt2 in the IBC cells does not affect cell viability, indicating the observed invasion assay results were not due to cell death.

Depletion of Akt3 does not affect invasion of any of the IBC or nIBC cell lines (data not shown). However, we found that Akt3 has an effect on IBC cell survival. Specific depletion of Akt3 leads to a significant 21% increase in apoptosis after serum starvation as compared to untreated and scrambled control samples (Figure 4c). No effect on cell survival of the MDA-MB-231 and MDA-MB-435 nIBC cells is observed, however, there is a slight decrease in MCF7 survival upon Akt3 depletion. Taken together these data suggest specific roles for Akt1 and Akt3 in IBC cell motility and survival, respectively.

Phosphorylation of RhoC GTPase in inflammatory breast cancer cells

Our previous work demonstrated that RhoC GTPase is overexpressed in IBC cell lines and patient samples (11, 26), and is required for IBC cell invasion and metastasis (28). Studies suggest that Rho proteins can act as Akt substrates (35). Supplemental Table 2 is a comparison of putative Akt phosphorylation consensus sequences from known Akt effector proteins and Rac1, RhoC and RhoA GTPases. To determine if Akt phosphorylation regulates
RhoC-mediated invasion, we created a RhoCS73A mutant that cannot be phosphorylated. Figure 5a are the results of an in vitro $^{32}\text{P}$-based kinase assay demonstrating that RhoC is an Akt substrate. Wild-type (WT) RhoC or mutant RhoCS73A protein, as well as a peptide containing the Akt phosphorylation consensus sequence, were tested as substrates for active Akt1. Alkaline phosphatase was added as a control to the proteins and the peptide, and compared to a scrambled peptide control. WT RhoC has significantly ($p<0.001$) higher levels of $^{32}\text{P}$ incorporation compared to the RhoCS73A mutant. These levels were nearly abrogated with the addition of alkaline phosphatase. Similarly, the RhoC peptide containing the Akt phosphorylation consensus sequence has significantly higher levels of $^{32}\text{P}$ incorporation compared to the scrambled peptide control. These data suggest that RhoC GTPase could act as a substrate for Akt1.

To determine if RhoC GTPase is phosphorylated in breast cancer cells we performed immunoprecipitation of RhoC followed by immunoblotting for phospho-serine. Although this is not specific for serine 73 we postulated that increased Akt1 activity in the IBC cells would result in higher levels of serine-phosphorylated RhoC as compared to the nIBC cell lines. Figure 5b demonstrates that the SUM149 cell line has comparatively higher levels of serine phosphorylated RhoC GTPase.

To discern if Akt phosphorylation of RhoC GTPase plays a role in IBC cell invasion, we transfected the SUM149 cells with the RhoCS73A mutant. Expression of RhoCS73A leads to a significant 85% decrease in the invasive
capacity of the IBC cells (Figure 5c) without affecting the viability of the cells (Supplemental Figure 3a). This suggests that Akt1 phosphorylation of RhoC is required for invasion of inflammatory breast cancer cells.

Since RhoC GTPase has high homology to other Rho GTPases, particularly RhoA GTPase (91% on the protein level), we next wanted to demonstrate that Akt phosphorylation of RhoC specifically was responsible for increased invasiveness of IBC cells. We transfected the SUM149 cells with empty vector, RhoCS73D or RhoAS73D phosphomimetic mutants, and then depleted Akt1 or Akt2. As control, we treated cells with C3 exotransferase, a potent inhibitor of RhoA, -B and -C GTPases. Figure 5d demonstrates that depletion of Akt1 in the SUM149 cells transfected with the vector control or RhoAS73D mutant leads to an ~65% decrease in invasion. Expression of the RhoCS73D phosphomimetic mutant rescues the invasive capabilities of the Akt1-depleted SUM149 IBC cells. In all cases, treatment with C3 exotransferase leads to an ~60% decrease in cellular invasion, while depletion of Akt2 had no significant effects. Expression of either RhoCS73D or RhoAS73D does not affect the viability of the SUM149 cells (Supplemental Figures 3b and 3c, respectively).

To see the effect of RhoC GTPase phosphorylation on gene expression we comparatively analyzed the PI3K/Akt-specific expression profiles of the RhoCS73A transfected SUM149 IBC cells with the wild-type (WT) SUM149 cells. Again, at a FDR less than 0.05, none of the genes were differentially expressed between WT SUM149 cells and RhoCS73A-transfected SUM149 cells. However,
when taking into account the small sample size of the data set (N=4) and readjusting the FDR to 0.1, differential expression was identified for 57 (68%) genes, corresponding to the same set of genes with nominal P-values smaller than 0.05. Forty-five (54%) genes were overexpressed in the WT SUM149 cells and 12 (14%) genes were overexpressed in the RhoCS73A-transfected SUM149 cells. These data suggest that expression differences for genes involved in the PI3K/Akt pathway are RhoC-dependent in SUM149 cells. Again, Bad, Rac1, RhoA and Cdc42, were among the genes with significantly altered expression profiles. The PI3K/Akt pathway gene expression profile comparing the WT SUM149 IBC cells with the RhoCS73A transfectants is displayed in Figure 6a.

**Integrated analysis of cell line and patient sample PI3K/Akt expression data**

To investigate if the differences for the PI3K/Akt-specific expression profiles observed in the cell line data correspond to expression differences observed in the patient samples we performed following analysis. Using PCA, we compared the expression profiles of the WT SUM149 cells with the PTEN- and RhoCS73A-transfected SUM149 cells. We observed that the influence of PTEN overexpression on the PI3K/Akt-specific expression profile was relatively limited as compared to the effect of transfecting SUM149 cells with the RhoCS73A mutant. These data agree with the more pronounced expression differences observed between WT SUM149 cells and RhoCS73A-transfected SUM149 cells as compared to PTEN-transfected SUM149 cells and suggest that RhoC, more than PTEN, is a key player for the behavior of the SUM149 cells. A 2D-scatter
plot representation of the PCA is provided in Figure 6b. The first metagene of this analysis, essentially capturing the variation in PI3K/Akt-specific gene expression introduced by the RhoCS73A mutant, was applied onto the Affymetrix data of 41 IBC and 55 nIBC samples. We observed a borderline significant overexpression of this metagene in nIBC (IBC: -19.283, nIBC: -19.119, P=0.060), suggesting that the PI3K/Akt-specific expression profiles of human nIBC samples more closely resemble those of the SUM149 cells transfected with the RhoCS73A mutant. The second metagene, which more thoroughly captures the differences in PI3K/Akt-specific gene expression downstream of PTEN-overexpression, is not significantly associated with the tumor phenotype in our series of human samples (IBC: -14.874, nIBC: -15.035, P=0.610). Taken together these data demonstrate that phosphorylation of RhoC by Akt leads to a unique IBC-specific PI3K/Akt gene signature.
Discussion

Primary IBC tumors present as diffuse sheets or cords of cells, which rapidly invade the dermal lymphatic vessels of the skin overlying the breast (3). Several years ago we postulated that because of the rapidity of the disease, IBC was metastatic nearly upon inception. Although IBC remains understudied, current evidence suggests that this may be the case.

It has been suggested that the tumor emboli disseminate by a process of “passive metastasis”, referring to emboli floating freely in the dermal lymphatics (10). Due to the anatomy and physiology of lymphatic vessels, the pattern and rapidity of dissemination, IBC cells need to undergo active metastasis. Our early work identified RhoC GTPase as being overexpressed and responsible for the invasive and metastatic IBC phenotype (11, 26, 28). In the current study we demonstrate that RhoC GTPase is a substrate for Akt1 and phosphorylation of RhoC by Akt1 is required for IBC cell invasion.

We found no significant difference in expression of individual Akt isoforms in either IBC or nIBC patient samples. However, the PI3K/Akt signaling pathways related to actin cytoskeletal reorganization and cellular motility are significantly increased in IBC patient samples. Interestingly, expression of a mutant RhoC with a substitution of an alanine for a serine in the Akt phosphorylation consensus sequence exhibits a gene expression profile similar to nIBC patient samples. Although PTEN appears to have a role in the SUM149 IBC cell line, our evidence suggests that it does not have a major role in IBC patients.
Together our data suggests a downstream effect of phosphorylated RhoC on the expression of PI3K/Akt1-associated genes. As predicted, our data also demonstrate an effect of phosphorylated RhoC on motility genes. Rac1 and RhoA GTPases were overexpressed within the WT SUM149 cells, agreeing with the influence of PI3K/Akt1 perturbation on SUM149 cell motility.

The frequent aberrant activation of Akt/PKB in cancer has made it an attractive target for treatment, making the potential specific roles of each of the three Akt isoforms even relevant as treatments become individualized (36). Akt1 was first implicated in human cancer shortly after its discovery when amplification of an \textit{Akt1} allele was found in a primary gastric adenocarcinoma and recent studies have shown the involvement of Akt1 in breast, ovarian, and colorectal carcinomas through an activating mutation or loss of PTEN (37-39). To date we have no evidence of activating mutations in IBC and as shown in the present study, no clear link between PTEN loss and IBC.

Studies suggest that Akt1 decreases the migratory capabilities of nIBCs (38, 40). We did not observe any changes in the migratory potential of the nIBC cells with Akt1 depletion perhaps due to experimental design. Akt2 was first reported to be involved in ovarian cancer tumorigenesis (41); more recently it has been shown to promote migration of nIBC cells (22, 24). We found that depletion of Akt2 had a significant effect on nIBC cell invasion without affecting IBC cells. Interestingly, we found that Akt3 has an effect on IBC cell survival, while Akt1 does not. Also of interest is the observation that PTEN re-expression led to a concurrent increase in BAD expression. Our data demonstrates that in patient
samples BAD is overexpressed in nIBC. The differences in Akt function between IBC and nIBC are not surprising. Research over the past decade has repeatedly demonstrated that IBC has a unique molecular profile and uses alternate signaling pathways (27, 29, 42). This research has also previously implicated the PI3K pathway in IBC patient samples (43). The unique molecular profile of IBC helps explain the distinct phenotype of the disease and its resistance to conventional breast cancer therapies.

Serine 73 of RhoC GTPase lies within the switch II region between the effector protein-binding and GTP-binding domains (35, 44). RhoC GTPase uniquely undergoes two conformational changes during its conversion from a GDP-bound-signaling-inactive form to a GTP-bound-signaling-active form (44). Conformational changes could lead to distinctive interactions with downstream effector proteins. Alternatively, phosphorylation of the switch II region could lead to a conformational change altering interaction of RhoC with Rho-regulatory proteins. Currently we are testing if phosphorylation of RhoC by Akt1 affects interactions with upstream or downstream effector molecules.

RhoC GTPase expression and activation is associated with advanced cancers and is limited in normal tissue, making it an extremely attractive therapeutic target (45). However, the high homology of RhoC to RhoA- and RhoB GTPases, which are involved in numerous normal cellular functions, prevents direct targeting of RhoC. Thus, targeting Akt1 in IBC cells may lead to a way to inhibit RhoC GTPase.
Acknowledgements

The authors would like to thank Kate Groh, M.D. and Galina Radunsky, M.D. for expert technical assistance. This work was supported in part by the Congressionally Directed Medical Research Program, Breast Cancer Research Program, DAMB-17-03-1-0728 (KLvG), W81WXH-06-1-00495 (KLvG) and W81XWH-08-1-0356 (KLvG). This project was completed as a collaborative effort of the IBC International Consortium.
References


Figure legends

Table 1. PI3K/Akt gene set subgroups analysis.

Figure 1. Akt expression in breast cancer patients. (a) A gene plot representing a global test analysis comparing the PI3K/Akt gene set between patient samples from IBC and nIBC in the PCR data set. Each gene is represented by a coloured bar, a red bar denoting overexpression in IBC and a green bar denoting overexpression in nIBC. The height of the bar corresponds to the standardized gene expression level (Z-score; (x-µ)/σ, with x being the raw unstandardized gene expression level for the corresponding gene, and µ and σ being respectively the mean and standard deviation of a reference population for the same gene estimated using random class label permutations). A standardized gene expression level of 3 corresponds to a gene significantly overexpressed at a FDR less than 5%. The current gene plot shows that the PI3K/Akt gene set is differentially expressed between IBC and nIBC (P=0.037). (b) A gene plot representing a global test analysis comparing the PI3K/Akt gene set according to the molecular subtypes in the PCR data set. Again, each gene is represented by a colored bar, with red, green, dark blue, light blue and purple representing respectively the Basal-like, ErbB2+, Luminal A, Luminal B and the Normal-like molecular subtypes. The current gene plot indicates that the expression of the PI3K/Akt gene set is associated with the molecular subtypes (P=0.022). The
Table in the top right corner summarizes the number of significant genes (S; FDR<5%) and non-significant genes (NS; FDR>5%) per molecular subtype. It can be observed that the majority of genes in both categories (S and NS) are overexpressed in the Basal-like molecular subtype. (c) Comparison of Akt1, -2, -3, and PTEN gene expression in patient samples of the Affymetrix data set reveals that when comparing to normal breast epithelial tissue, Akt1 was overexpressed in the cancerous samples, whereas Akt2, and PTEN were overexpressed in the normal breast epithelium samples (p<0.003; FDR<0.05). For Akt3, no difference is observed. (d) A similar gene plot as panel (a), but for the Affymetrix data set. Again, the PI3K/Akt gene set is differentially expressed between IBC and nIBC (P=0.003).

Figure 2. Akt/PKBα activity is higher in inflammatory breast cancer cells. (a) Immunoprecipitation and immunoblot analysis for phosphor- and total Akt1, -2 and -3 expression, respectively. SUM149 IBC cells compared to MDA-MB-231, MDA-MB-435, and MCF7 nIBC cells. (b) A cell based ELISA used to directly measure serine 473 phosphorylation of Akt comparing subconfluent SUM149 IBC cells with nIBC cells unstimulated or stimulated with EGF (10 ng/ml), IGF (10 ng/ml), or FBS for 30 min. (c) Matrigel™ invasion assay showing the effect of a pan-Akt pharmacologic inhibitor (iAkt) on SUM149 and MDA-MB-435 nIBC cells. Cells were treated with 10 µM of iAkt for 24 h. (i) Western blot analysis confirms inhibition of active Akt in the cells. (ii) Results of a Matrigel™ invasion assay. Data are combine from 3 separate experiments and represented as mean ± SEM.
(*p<0.05). (d) Inhibition of Akt with a SmartPool siRNA directed against all Akt isoforms (siAkt) in SUM149 IBC and MDA-MB-435 nIBC. (i) Western blot densitometry confirms the depletion of Akt by siRNA. (ii) The effect on invasion was seen in siAkt treated cells compared to mock transfected and scrambled control transfected cells. Data are from 3 separate experiments and represented as mean ± SEM (*p<0.05).

**Figure 3.** PTEN is lost in SUM149 inflammatory breast cancer cells. (a) Western blot analysis showing expression levels of the phosphatase and tensin homolog (PTEN) protein. Total cellular protein (30 μg) was loaded into each lane of the immunoblot. (b) Matrigel™ invasion assay demonstrating invasion of the SUM149 IBC cells when PTEN is re-expressed. Cells were transfected with a PTEN construct or mock transfected (UT). Invasion was measured after 24 h. Data are from 3 separate experiments and expressed as mean ± SEM. Representative images of cellular invasion in the chambers are demonstrated above (*P=0.0018). (c) Percent of apoptotic cells after re-expression of PTEN in SUM149 cells. Cells were PI stained and fixed in 70% ethanol prior to FACS analysis. Data in both panels are from 3 separate experiments and represented as mean ± SEM. (d) A gene plot representing a global test comparing the PI3K/Akt signaling pathways genes between WT SUM149 cells and PTEN-transfected SUM149 cells. Each gene is represented by a coloured bar with green denoting overexpression in the WT SUM149 cell line and red denoting overexpression in the PTEN-transfected SUM149 cell line. The height of the bar
corresponds to the standardized gene expression level as described before (see legend Figure 1a). Due to the low sample size, genes with a standardized gene expression level greater than 2 (P<0.05; FDR<10%) are considered significant.

**Figure 4.** Akt1 is required for SUM149 inflammatory breast cancer cell invasion while Akt3 is implicated in IBC apoptosis. (a) Western blot analysis for siRNA depletion of Akt1, -2 and -3. (b) A Matrigel™ invasion assay was performed after specific depletion of Akt1, Akt2 and scrambled control in SUM149 IBC cells compared to MDA-MB-231, MDA-MB-435, and MCF7 nIBC. Akt isoforms were specifically depleted using SmartPool siRNAs. Images are representative areas of cells that have invaded through the membrane. (c) Depletion of Akt3 in IBC and nIBC cells with Akt3-specific SmartPool siRNA for 48 h. Cells were PI stained and fixed in 70% ethanol prior to FACS analysis. Data in both panels are from 3 separate experiments and represented as mean ± SEM. All p values <0.0001.

**Figure 5.** RhoC GTPase is an Akt1 substrate and phosphorylation of RhoC by Akt1 is key in IBC cellular invasion. (a) In vitro kinase assay analysis of 32P incorporation by wild-type (WT) RhoC GTPase, a peptide containing the Akt phosphorylation consensus sequence or S73A phosphorylation mutants of both the RhoC protein and peptide. Data are from 3 separate experiments and represented as mean and ± SEM (p<0.001). (b) Immunoprecipitation of RhoC GTPase followed by an immunoblot with a phospho-serine specific antibody. (c)
Matrigel™ invasion assay comparing the effect of the RhoCS73A Akt phosphorylation mutant with WT RhoC GTPase in the SUM149 IBC cells. Data are from at least 3 separate experiments and expressed as mean ± SEM. (*p<0.0001). (d) Transfection of SUM149 IBC cells with a RhoC GTPase phosphomimetic mutant (RhoCS73D) and RhoA GTPase phosphomimetic mutant (RhoAS73D). IBC cells were either untreated or co-transfected with either RhoC- or RhoAS73D and C3 exotransferase, scrambled siRNA control, siAkt1, or siAkt2 for 48 h and placed in a Matrigel™ invasion assay. The line represents the level of invasion in C3 exotransferase-treated cells (an inhibitor of RhoA, -B and –C). Data are from 3 separate experiments and represented as mean ± SEM (p=0.021).

Figure 6. Perturbation of RhoC signaling in SUM149 cells and its effect on PI3K/Akt-specific gene expression profile. (a) A gene plot representing a global test comparing the PI3K/Akt signaling pathways genes between WT SUM149 cells and RhoCS73A-transfected SUM149 cells. Each gene is represented by a coloured bar with green denoting overexpression in the WT SUM149 cell line and red denoting overexpression in the RhoCS73A-transfected SUM149 cell line. The height of the bar corresponds to the standardized gene expression level as described before (see legend Figure 1A). Due to the low sample size, genes with a standardized gene expression level greater than 2 (P<0.05; FDR<10%) are considered significant. (b) A PCA plot generated using the PI3K/Akt-specific gene expression profiles for the WT and the PTEN- and RhoCS73A-transfected
SUM149 cells (respectively green, red and blue). The X- and Y-axes represent respectively the first and second principal components, which capture respectively the greatest and the greatest but one amount of variation in gene expression in the cell line data set. It can be observed that perturbation of RhoC in the SUM149 results in more pronounced differences in PI3K/Akt-related gene expression relative to PTEN-transfection in SUM149 cells. The first and second principal component models the variation in PI3K/Akt-related gene expression downstream of respectively RhoC perturbation and PTEN transfection.
Figure 1.

- **Expression higher in NIBC**
- **Expression higher in IBC**

Global Test P-Value = 0.037

Relative Expression

Threshold for significance
Figure 1.

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Global Test P-value = 0.022
Figure 2.
Figure 2.

i. 

<table>
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SUM149  MDA435

ii.

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<tr>
<th></th>
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*
Figure 2.

i. 

- Bar graph showing the ratio of Akt/Actin (A.U.) for SUM149 and MDA-MB-435 cells.
  - Mock (black bars)
  - Scr Control (gray bars)
  - siAkt (light gray bars)

ii. 

- Bar graph showing the percent of untreated control for SUM149 and MDA-MB-435 cells.
  - Significant difference indicated by an asterisk (*)
Figure 3.
b.

![Image of two micrographs and a bar graph showing percent invasion of untreated control between UT and PTEN groups.](Image_url)
Figure 3.
Figure 4.
Figure 4.
Figure 4.
Figure 5.

a.

Average Counts per Minute (CPM)

RhoC wt  RhoC wt + AP  RhoC ST73A  RhoC ST73A + AP  RhoC Scr Peptide  RhoC Peptide

*  *

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Figure 5.

C.
Figure 5.

d.

![Graph showing percent control with bars for different treatments compared to control. The bars are labeled Vector, RhoCS73D, and RhoAS73D. There is a significant difference indicated by an * between the treated and control groups.](image-url)
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| % Total Variance after correction | 1.00 | 1.00 | 0.750 |

- Process 1: AKT and PI3K family members and their receptors
- Process 2: IGF-1 Signaling Pathway
- Process 3: Inactivation Gsk3 and accumulation of beta-Catenin
- **Process 4: PI3K Subunit p85 genes and their regulation of actin organization and cell migration**
- Process 5: PTEN-dependent cell cycle arrest and apoptosis
- Process 6: BAD-phosphorylation and anti-apoptotic pathways
- Process 7: Genes involved in the mTOR signaling pathway
- Process 8: Regulation of eIF4e and p70 S6 kinase
Molecular Cancer Research

Regulation of inflammatory breast cancer cell invasion through Akt1/PKB α phosphorylation of RhoC GTPase

Heather L Lehman, Steven J Van Laere, Cynthia M van Golen, et al.

Mol Cancer Res  Published OnlineFirst August 15, 2012.

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