Reverse phase protein microarrays: Quantifying cell signaling proteins for clinical trial samples

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Breast cancer subtype classification, current and future therapies in a historical context

PROTEOMIC ANALYSIS

Challenge: How do you measure the state of the fluctuating activity of ongoing signal pathways and cellular circuits in lysed tissue cells?

Solution: The state of signal pathways minute to minute is recorded in the phosphorylated or activated state of protein nodes in the signal pathway.

Measure the phosphorylated or activated state of a kinase, a kinase partner, or a pathway.
The Laser Capture Microdissection Process

1. Place cap on tissue
2. Pulse laser at target cells
3. Remove cap with adhered target cells
4. Extract molecules from target cells

Arcturus
Life Technologies

mmi CellCut

UV cutting laser, membrane slide, adherent cap

Leica

tissue on glass slide with foil

Zeiss

Catapulting

objective

laser
tissue
gavity
tube
Core Needle Biopsy for LCM

Core needle biopsy, 16 gauge
2 cm x 0.5 cm tissue
$10^9$ cells in tissue section
10,000 tumor cells
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Methodology/assay</th>
<th>Cellular yield/area of microdissection</th>
<th>References</th>
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<tbody>
<tr>
<td>DNA</td>
<td>Loss of heterozygosity</td>
<td>100–1,000 cells</td>
<td>50, 53</td>
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<tr>
<td>DNA</td>
<td>Imprinting/DNA methylation</td>
<td>200 cells</td>
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<td>gDNA</td>
<td>Genetic mosaic analysis</td>
<td>2,000 cells</td>
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<tr>
<td>RNA</td>
<td>cDNA library construction</td>
<td>25,000 cells (93 ng total RNA)</td>
<td>54, 55</td>
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<td>Gene-expression arrays</td>
<td>5,000 cells (14.7–18.6 ng total RNA)</td>
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<td>Real-time RT-PCR</td>
<td>100 cells from FFPE</td>
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<td>200 and 1,000 cells</td>
<td>58</td>
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<tr>
<td>RNA</td>
<td></td>
<td>22,000 cells/37.5 ng RNA</td>
<td>81</td>
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<td></td>
<td>10,000 cells/40 ng RNA from maize</td>
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<td>RNA</td>
<td></td>
<td>single cell</td>
<td>82–84</td>
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<tr>
<td>Protein</td>
<td>Western blot</td>
<td>100 cells/1 reaction or 2,000 cells/200 μl</td>
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<td>4,000–5,000 cells</td>
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<tr>
<td>Protein</td>
<td>2D gel electrophoresis</td>
<td>500 cells (optimized blotting procedure)</td>
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<td>Protein</td>
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<td>2,500 cells</td>
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<td>Protein</td>
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<td>8,000–10,000 cells</td>
<td>62</td>
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<td>2D-DIGE</td>
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<td>5</td>
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<tr>
<td>Protein</td>
<td>Molecular profiling: reverse-phase protein microarray</td>
<td>3.7 mm^2 area</td>
<td>85</td>
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<tr>
<td>Protein</td>
<td></td>
<td>10,000 cells (100–200 μg in 350 μl)</td>
<td>86</td>
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<tr>
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<td>Mass spectrometry: MALDI or LC/MS-MS</td>
<td>20,000–25,000 cells</td>
<td>87</td>
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<tr>
<td>Protein</td>
<td></td>
<td>50,000 cells</td>
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<td>Mass spectrometry: SELDI</td>
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<td>Mass spectrometry: SELDI</td>
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<td>10,000–15,000 cells</td>
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<td>Protein</td>
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<td>300 microvessels</td>
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<tr>
<td>Protein</td>
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<td>1,500 cells</td>
<td>66</td>
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<tr>
<td>Protein</td>
<td></td>
<td>3,000–5,000 cells</td>
<td>67</td>
</tr>
</tbody>
</table>
H&E Staining Protocol

Frozen Sections

1. 70% ethanol fixative, 3–10 s
2. dH₂O, 10 s
3. Mayer’s hematoxylin, 15 s
4. dH₂O, 10 s
5. Scott’s Tap Water Substitute, 10 s
6. 70% ethanol, 10 s
7. Eosin Y (optional), 3–10 s
8. 95% ethanol, 10 s
9. 95% ethanol, 10 s
10. 100% ethanol, 30–60 s
11. 100% ethanol, 30–60 s
12. Xylene, 30–60 s
13. Xylene, 30–60 s
14. Air dry slide as quickly as possible
Skin, breast, cartilage – any tissue with strong intercellular adhesive forces
1. Remove cap from freezer, keep on dry ice.

2. Group caps from the same sample together.

3. Remove cap from tube.

4. Immediately add extraction buffer.

5. Use pipette tip to spread extraction buffer on cap surface.

6. Use a pipette to aspirate the lysed cells/buffer from the cap surface.

7. Place cell lysate in a clean tube.

8. Place the LCM caps on the original labeled tube, and spin at ~12,000rpm for 10 sec.

9. Remove any lysate from the tubes. Add the lysate to the original lysate.

10. Heat lysate at 100°C for 5 minutes.

11. Use a microscope to observe the surface of cap to ensure complete lysis. Discard LCM caps.
**Dish:** Whole Cell Lysate

**LCM Cap with microdissected cells**

**Optimal cell number >10,000 cells**

**Protein Extraction Buffer:**

- 450 μL T-PER™ Tissue Protein Extraction Reagent (Pierce)
- 10% v/v TCEP Bond Breaker (100 μL) (Pierce)
- 450 μL 2X SDS Tris-glycine Loading Buffer (Invitrogen)

  - Use 1.0 μL extraction buffer per 1000 cells.
  - 15 μL minimum volume to cover surface area of Macro CapSure™ cap.
  - Incubate cap with extraction buffer for 1 min. at room temperature. Transfer lysate to a clean microcentrifuge tube.
  - Boil lysate for 5 min. at 100°C prior to immunoblotting or microarray printing. Store whole cell lysate at -80°C.
Whole Cell Lysate for Mass Spec

LCM Cap with microdissected cells
Optimal cell number >5,000 cells

Mass Spectrometry Extraction Buffer:
8M Urea
TrisHCl Buffer pH 7.0 - 7.5

- Add extraction buffer to cells on the cap surface.
- Incubate 1-2 min. at room temp.
- Aspirate buffer up and down on surface of cap. Transfer lysate to clean microcentrifuge tube.
- Proceed with reduction, alkylation, and digestion per standard protocols.
Types of Protein Microarrays

Forward Phase Protein Microarray
- Capture
- Signal Generation
- Anti-A
- Anti-B
- Anti-A2
- Anti-B
- Label
- Analytes

Reverse Phase Protein Microarray
- Capture
- Signal Generation
- Anti-A
- Anti-B
- Analytes

Protein Microarrays
Mapping Signal Pathway States

- Data analyzed to a single value per patient.
- Data normalized across slide.
- Multiple patient samples and multiple antibodies analyzed.

Idea  Technology  Proof of principle  Basic science applications Pre-clinical/translational  Clinical trials

- Miniaturized ligand immunoassay
  Ekins R & Chu FW (1991)
- Molecular profiling
- Propose antigen-down protein microarray
  Liotta LA & Petricoin EF (2000)

- Robotic array
- Protein binding substratum
- Commercially available antibodies
- Phospho-specific antibodies
- Laser Capture Microdissection

Reverse Phase Protein Microarray
Prostate phosphoprotein signaling
Paweletz C, et al. (2001)

NCI-60 cell line profile
Follicular Lymphoma biomarkers

- Rhabdomyosarcoma, Breast cancer
  Lung cancer, Melanoma, Leukemia

Patent 6,969,614

Commercialization
Wide-spread adoption / Independent labs
Validation in leukemia
Tibes R, et al. (2006)

Enabling technologies
Phosphoprotein stabilization
Espina V, et al. (2008)

- Predict treatment response
- Design rationale therapy
- Correlate outcome with phosphoproteomic profile
Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front

Cloud P Paweletz\textsuperscript{1,2,3}, Lu Charboneau\textsuperscript{2}, Verena E Bichsel\textsuperscript{1,2}, Nicole L Simone\textsuperscript{2}, Tina Chen\textsuperscript{2}, John W Gillespie\textsuperscript{4}, Michael R Emmert-Buck\textsuperscript{3}, Mark J Roth\textsuperscript{6}, Emanuel F Petricoin III\textsuperscript{1} and Lance A Liotta\textsuperscript{*1,2}

Multiple samples/array

One antibody probe/array

Controls, standards, samples: all on the same array
depositions/feature of 0.5μg/μL total protein

Nitrocellulose coated slides (FAST slides):
High protein binding capacity
Uniform surface
Stable
Compatible with fluorescent, colorimetric, chemiluminescent detection
Reverse Phase Protein Microarrays

- Phosphorylated/activated state of signal proteins
- One antibody per analyte
- High sensitivity - femtomole range
- FNA/core needle biopsy
- Built-in positive controls and calibrators
- High precision and linearity - CAP/CLIA requirements

Internal dilution curve assures high dynamic range
Immunostaining of the Arrays
Catalyzed Signal Amplification

Application of Primary antibody

Application of biotinylated secondary Ab

Application of streptavidin-biotin-HRP complex

Application of biotinyl tryamide

Application of streptavidin/HRP
Which cell signaling pathway will be studied? Survival, death, motility, invasion…. Are antibodies to the proteins of interest available?
Validated Antibodies

Phosphospecific: 302  Total protein: 60

Validated antibody: single band at specified molecular weight, competition by phosphopeptide
Biopsy → Stabilize phosphoproteins → Laser capture microdissection

Reverse phase protein microarray
More sensitive than IHC

Signal pathway map
Molecular Protein Profile Report
MR #: 5847309
Dx: Invasive ductal carcinoma
Protein pathway activation:
Prosurvival/autophagy
Potential target nodes:
AKT Ser473
mTOR Ser2448

Individualized therapy report
I-SPY TRIAL: Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging And molecular analysis

192 Breast carcinoma biopsies collected pre neo-adjuvant therapy

7µM tissue section

Microdissection

Whole Tissue Lysate

Reverse Phase Protein Array

Eligibility Criteria:
- Breast cancer, Stage II-III (T4, any N, M0), >= 3cm largest diameter
- No prior chemotherapy or RT
- Any ER/PR/Her2 status

Collaborator: Laura Esserman, UCSF
Microdissection vs. Whole Tissue Lysates

*Unsupervised Clustering Analysis Reveals Clear Need for LCM:*

Whole slide lysates form unique clusters largely characterized by low signal levels.

Pathway information is lost using whole slide lysates.

Patient matched pairs: microdissected or whole tissue lysates

Lack of concordance in majority of endpoints and samples

Clinical research trials using phosphoprotein signal pathway profiling

A. Her2+ Breast Cancer: (US Onc/GSK sponsored trial) EGF/HER2 combination therapy

B. Multiple Myeloma: (Virginia Cancer Specialists, Inova) targeted ex vivo inhibitor screening
   Status: Started May 2007  Target Completion Dec 2011

C. Breast Cancer Carcinoma in Situ: (DOD funded) DCIS cancer stem cells
   Status: Started Sept 2007  Completed Dec 2009

D. Colon Cancer Liver Metastasis (NITMEC): Stratified combination therapy
   Status: Started Aug 2009  Target Completion Dec 2011

E. Breast Cancer Stage IV: (Side Out sponsored trial) Individualized therapy based on genomic and proteomic analysis
   Status: Started Dec 2009  Target Completion Dec 2011

F. Breast Cancer Carcinoma in Situ (PINC): (DOD funded) Chemoprevention trial
   Status: Started April 2011  Target Completion April 2013
Advantages for clinical trial research:

1. Assurance of a certain level of rigor to the scientific community regarding quality of the test result.

2. Defined realm of activity & licensed professional staff.

3. Pre-clinical studies do not need to be repeated prior to FDA filing.

4. Assay can be offered immediately as a home-brew test, without need for FDA approval.

5. Accredited labs attract pharmaceutical companies and investors.
Nitmec trial: Study design

PATIENT POPULATION: 30-50 STAGE IV CRC PATIENTS THAT
-- FAILED FIRST LINE CHEMOTHERAPY
-- PRESENT WITH HEPATIC METASTASIS ACCESSIBLE
   BY CT SCAN FOR BIOPSY

1ST LIVER BIOPSY:

-- RPPM analysis for the evaluation of
  PDGFR, cAbl and cKit activation
  Status (pgdpt score)
-- K-RAS mutation analysis

ARM 1
PGDT score ≥ 2.3
Escalating Imatinib dose administration

ARM 2
PGDT score < 2.3
Panitumumab monotherapy

2nd LIVER BIOPSY:
After 28 days treatment:

Imatinib plus Panitumumab

2nd LIVER BIOPSY:
After 2-3 months treatment:

Comparisons of EGFR and downstream pathway activation such as AKT, ERK are made between sequential pre and post treatment biopsies.

2 Arms

Gleevec® Dose
Escalation (4 weeks)

Gleevec® + Vectibix™
(27 weeks)

Vectibix™ (31 weeks)

Clinical PIs: Alex Spira and Kirsten Edmiston

Chip Petricoin and Mariaelena Pierobon

Nitmec is a prospective non-randomized clinical trial sponsored by Novartis
Side Out Clinical Trial: Personalized therapy for advanced breast cancer
Sponsored by the Side Out foundation

**PATIENT POPULATION: 25 STAGE IV BREAST PATIENTS THAT**
-- FAILED AT LEAST THREE LINES CHEMOTHERAPY
-- PRESENT WITH METASTASIS ACCESSIBLE
BY CT SCAN FOR BIOPSY

**BIOPSY:**

-- RPPM analysis for the evaluation of activation status of 11 key proteins
-- IHC plus eventual Fish analysis
-- mRNA microarray analysis

**Based on the results of the 3 analyses a Medical Committee establishes the most appropriate therapy for each patients**

**This is an open labeled trial in which all FDA drugs approved for cancer treatment can be selected as therapeutic agents**

**Phospho-protein measured at GMU**
- pAkt S473
- pcAbl T735
- pcKit Y719
- pEGFR Y1173
- pErb2 Y1248
- pErk 1-2
- pmTOR S2481
- pp70SKT389
- pPDGFR Y751
- PTEN TOTAL
- pVEGFR Y996

Nicholas Robert and Dan Van Hoff Clinical PIs
Testing the hypothesis that the elevated activity of a drug target predicts response

Compare new patient value to existing population data for phospho EGFR values

<table>
<thead>
<tr>
<th>PATIENT A</th>
<th>DRUG TARGET</th>
<th>ACTIVITY LEVEL</th>
<th>DRUG</th>
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<tr>
<td>Phospho-EGFR</td>
<td>3+</td>
<td>TARCEVA</td>
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<tr>
<td>Phospho-c-KIT</td>
<td>1+</td>
<td>GLEEVEC</td>
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<tr>
<td>Phospho-VEGF</td>
<td>3+</td>
<td>AVASTIN</td>
<td></td>
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<tr>
<td>Phospho-mTOR</td>
<td>0</td>
<td>TORISEL</td>
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<th>DRUG</th>
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<tr>
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<td>3+</td>
<td>GLEEVEC</td>
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<tr>
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<tr>
<td>Phospho-mTOR</td>
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<td>TORISEL</td>
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Malignant Precursor Cells Pre-Exist in Human Breast DCIS and Require Autophagy for Survival

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Abstract

**Background:** While it is accepted that a majority of invasive breast cancer progresses from a ductal carcinoma in situ (DCIS) precursor stage, very little is known about the factors that promote survival of DCIS neoplastic cells within the hypoxic, nutrient deprived intraductal microenvironment.

**Methodology and Principal Findings:** We examined the hypothesis that fresh human DCIS lesions contain pre-existing carcinoma precursor cells. We characterized these cells by full genome molecular cytogenetics (Illumina HumanCytoSNP profile), and signal pathway profiling (Reverse Phase Protein Microarray, 59 endpoints), and demonstrated that autophagy is required for survival and anchorage independent growth of the cytogenetically abnormal tumorigenic DCIS cells. Ex vivo spheroid forming cells from the organ culture, and abrogated xenograft tumor formation.

**Conclusions:** Cytogenetically abnormal spheroid forming, tumorigenic, and invasive neoplastic epithelial cells pre-exist in human DCIS and require cellular autophagy for survival.


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Ductal Carcinoma In Situ

When does invasion first emerge?
When and how do invasive carcinoma cells originate within the pre-malignant lesion?
When does the invasive phenotype arise?

Do breast cancer stem cells / progenitor cells first arise within the duct of pre-malignant lesions?

Are the hallmarks of invasiveness and aggressiveness predetermined early at the level of DCIS?

How do the malignant progenitor DCIS cells survive in the hypoxic, nutrient deprived microenvironment (niche)?
Ductal Carcinoma In Situ Microecology

Neoplastic epithelial cells confined within the duct

Myoepithelial cells

Immune cells

Blood vessels

Lymphatics

Basement membrane

ECM

Fat cells

Calcium spicule

Central necrosis

Stromal cells
DCIS cells must proliferate and survive in a high stress microenvironment.

DCIS lesions are non-vascularized. The radius of a typical DCIS lesion is 200-500 microns. Maximum distance between blood vessels for oxygen diffusion is 25-50 microns.
Clustered calcifications suspicious for DCIS

MAMMOGRAM

DX BIOPSY

BENIGN

DCIS

EX VIVO CULTURE

SPHEROID

Protein microarray for cellular signaling

Whole genome molecular cytogenetics

Ex vivo culture

Serum free media + EGF

Tumorigenicity NOD/SCID

LIVING HUMAN DCIS DUCT SEGMENT

SURGICAL THERAPY

MAMMOGRAM
THIS IS THE FIRST EVIDENCE FOR DCIS CANCER PROGENITOR CELLS

Number of patient cases transplanted = 56
Number of cases observed for > 3 months = 43/56
Number of tumors observed from cultured primary cells and/or spheroids = 21/27 transplanted (77.7%)
Number of pure DCIS tumors = 18/22 (81%)
Number of IDC tumors (positive controls) = 9/20 (45%)

Invasive and tumorigenic DCIS cells pre-exist in human DCIS lesions

**A**
Histomorphology of DCIS, grade III (H&E stain)

**B**
In vitro spheroid formation from human breast DCIS organoid culture.

**C**
3-D structure of cultured DCIS cells with formation of a lumen

**D**
NOD SCID murine xenograft tumor generated from human DCIS spheroid progenitor cells

DCIS progenitor cell invasion of autologous stroma in vitro
Characterization of Human Breast DCIS Cultured Tissue

RPMA characterization of *in vitro* cell types confirms structure and function relationship:

- **Spheroids** (CD44+, COX2+, MMP-14+, E-Cadherin -)
- **Epithelial cells** (EGFR+, CD44-, E-Cadherin+)
- **Cuboidal cells** (EGFR+, E-Cadherin+)
Human DCIS malignant progenitors are cytogenetically abnormal: The earliest genetic basis of breast cancer

Spheroid abnormal karyotype signature included loss of copy number on chromosome 5, 6, 8, and 13, and gain of copy number on chromosomes 1, 5, and 17.

- **RED**: homozygous deletion
- **ORANGE**: hemizygous deletion
- **GREEN**: copy-neutral loss of heterozygosity (usually benign)
- **BLUE**: duplication/amplification/trisomy

Illumina 300,000 CytoSNP beadchip
Molecular genotype
Elevation of stem cell, autophagy and survival related proteins in DCIS spheroids persist over time in culture.

Differences in cell signaling proteins were a stable characteristic of the observed phenotype, and were maintained in an independent verification analysis.
Autophagy (self eating) promotes survival of DCIS cells within the hypoxic nutrient deprived intraductal environment.

In order to survive the cell digests its own contents using internal phagosomes.

Autophagy upregulated *in vivo* and *in vitro* in DCIS.

Atg5 positive cells within duct *in vivo*.
1. Activation of autophagy may divert the hypoxic cells away from apoptosis and thereby support the survival and growth of DCIS neoplastic cells within the lumen.

2. Autophagy has been shown to be a key regulator of survival for cells deprived of an anchoring substratum.

3. Autophagy may facilitate cell movement through areas of degraded matrix by the phagocytic processing of matrix breakdown fragments.
- 28 frozen tissue biopsies were embedded in OCT and sectioned onto glass slides.
- DCIS, invasive, and stroma cells were isolated using laser capture microdissection.
- Printed lysates were stained with autophagy proteins: Beclin-1, ATG 5, ATG 12, and LC3B.
- Intensity values were analyzed using MicroVigene software.

<p>| Table 1. Antibodies used with RPMA |</p>
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
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<tbody>
<tr>
<td>ATG 5</td>
<td>1:100</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>ATG 12</td>
<td>1:50</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>1:100</td>
<td>Sigma</td>
</tr>
<tr>
<td>LC3B</td>
<td>1:100</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Her2 Y1248*</td>
<td>1:1000</td>
<td>Upstate</td>
</tr>
</tbody>
</table>
ATG 5: DCIS and invasive values were not significantly different. Invasive and stroma values were not significantly different. However, the intensity values of DCIS and stroma were significantly different.

One-way ANOVA Test (SAS 9.2)
Beclin-1: The intensity values of DCIS and invasive were not significantly different, but stroma values were significantly different when compared to DCIS and invasive.
Can we treat DCIS via the Autophagy pathway?

Chloroquine inhibits the degradative function of autolysosomes: causes loss of mitochondrial membrane potential, leaking of lysosomes, accumulation of toxic substances, and apoptosis.

3-D structure of cultured DCIS cells invading the stroma

Degeneration of the DCIS cell colony following chloroquine treatment (50uM, 2 days)
Chloroquine is a well tolerated oral drug that blocks autophagy. Can this therapy suppress spheroid survival and kill DCIS progenitors?

Degradation of cytoplasmic material = Energy generation in the face of oxygen and nutrient deprivation = Cell survival

Autophagosomal accumulation in the presence of chloroquine
Can we treat DCIS via the Autophagy pathway?

DCIS malignant progenitor cells exhibit multiple cytogenetic abnormalities in organ culture.

The surviving cells, after Chloroquine treatment, were found to be cytogenetically normal.
PINC Trial: Preventing Invasive breast Neoplasia with Chloroquine

Identify Patients, Screen for eligibility, Obtain consent

For patients with diagnosis of DCIS, Image guided core bx for baseline biomarker analysis, Breast MRI

DCIS ER+ Any Grade
Randomize patients to Chloroquine 500 mg/ week or 250 mg/week x 4 weeks

DCIS ER- Any Grade
Randomize patients to Chloroquine 500 mg/ week or 250 mg/week x 4 weeks

Follow up MRI
Surgery, Pathology for biomarkers
Radiation Tx for lumpectomy patients
Clinical Follow up

MRI before Tx
MRI post Tx

Patient enrollment

3 months of chemopreventive therapy

Whole genome molecular cytogenetics

Biopsy 1
Protein microarray for cellular signaling

Biopsy 2

in vitro growth

Surgical therapy
PINC Trial: Preventing Invasive breast Neoplasia with Chloroquine

Prevention is P.I.N.C.

Keep your ducts in line.

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