Targeting HER2 signaling in Invasive Lobular Cancers to improve responses to Antiestrogen Therapy

Undergraduate Research Thesis

Presented in Partial Fulfillment of the Requirements of Graduation with Research Distinction in Biomedical Science in the College of Medicine at The Ohio State University

by

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Abstract

Introduction: Invasive Lobular Carcinoma (ILC) is a distinct histological and molecular subtype of breast cancer that is often estrogen receptor (ER) positive. ILC patients suffer from late recurrences and unfavorable long-term outcomes, suggesting resistance to antiestrogen therapies[1]. Cross talk between growth factor signaling pathways such as EGFR/HER2 and ER along with HER2 activating mutations have also been reported at the time of recurrence[2]. We believe that HER2 is a viable target in endocrine resistant ILC and targeting HER2 using monoclonal antibody (trastuzumab) and tyrosine kinase inhibitors (TKI) along with antiestrogen therapy (ET), would be a novel alternative treatment strategy for endocrine resistant ILC patients.

Methods: We used ILC cell lines SUM44PE and MDA-MB-134-VI, along with Long Term Estrogen Deprived (LTED) and tamoxifen resistant (TamR) derivatives of each cell line in this study. We determined IC50 values of tamoxifen for each line using the MTT [[3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay. We have determined the effect of TKIs (Neratinib and Tucatinib) and Trastuzumab in combination with Tamoxifen on cell viability in *in vitro* cell culture system using MTT assay. We performed western blot analysis to study the effect of the drugs on downstream growth kinases such as AKT and MAPK signaling and Receptor Tyrosine Kinase (RTK) arrays to identify novel downstream signaling kinases responsive to these treatments.

Results: Prolonged exposure of parental ILC cell lines to tamoxifen rendered the cells tamoxifen resistant as demonstrated by higher IC50 for tamoxifen. Specifically, IC50-TAM for MDA-MB-134-VI Parental and TamR cells are 2.27μM and 29.3μM respectively; for SUM44PE Parental and TamR cells are 7.1μM and 9.6μM respectively. Amongst the LTED cell lines, every line except for MDA-MB-134-VI LTED D has a lower IC50 value for tamoxifen. Across MDA-MB-

134-VI cell lines, we observed increases in HER2 protein level in $TamR^{100}$ (4.3-fold), LTED A (2.6-fold), LTED D (1.1-fold) in comparison to the parental cell line. In SUM44PE lines, we observed HER2 protein level increases in $TamR^{100}$ (1.7-fold) and in LTED B (1.1-fold). ERα expression was decreased by at least 60% across all MDA-MB-134-VI LTED lines except LTED D when compared to parental cells, in addition to decreases in SUM44PE LTED A (8% decrease) and LTED B (32% decrease) compared to SUM44PE parental. The combination of tamoxifen, trastuzumab and neratinib (TTN) significantly reduced cell viability in SUM44PE TamR ($p = 0.0046$) and SUM44PE LTED B ($p = 0.0076$), but not in SUM44PE parental or LTED A when compared to tamoxifen monotherapy. Similarly, the combination of tamoxifen, trastuzumab, and tucatinib (TTT) significantly reduced cell viability in MDA-MB-134-VI-TamR cells ($p = 0.047$), LTED A ($p = 0.026$), LTED D ($p = 0.011$), and LTED E ($p = 0.007$), and TTN combination reduced cell viability in LTED A ($p = 0.001$) and LTED E ($p = 0.035$) compared to tamoxifen treatment alone. Levels of phosphoAKT and phosphoMAPK was reduced upon tamoxifen and tucatinib treatment of SUM44PE cells (82% decrease in pAKT and 41% decrease in pMAPK levels). Using RTK array we identified ACK1 as a target downstream of HER signaling in ILC cells that was inhibited upon treatment with tamoxifen or TKI.

Discussion: Targeting HER2 kinase activity with small molecule inhibitor along with anti- HER2 monoclonal antibody could improve efficacy of anti-estrogen therapy in endocrine therapy resistant invasive lobular cancer. Additionally, use of broad spectrum TKI, such as neratinib is more effective than specific HER2 inhibitors such as tucatinib. ACK1 is a novel protein that could be a therapeutic target in these cancers. Further *in vitro* studies are underway to determine the efficacy of these drug combinations.

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Introduction

Breast Cancer

Breast cancer is the most commonly diagnosed cancer among women (excluding skin cancer) with 276,480 new diagnoses in 2020 expected in the United States alone[3]. One in eight women will be diagnosed with breast cancer in their lifetime[4]. In Ohio, breast cancers accounts for the highest proportion of new cancer cases and incidence of breast cancer has increased by 6% from 2007 to 2016 [5]. Breast cancer is heterogeneous and can be classified into 3 main molecular subtypes based upon hormone receptor expression and the presence of growth factor receptors. The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are the targetable receptors used to delineate breast cancer subtypes. The subtypes are as follows: Hormone receptor positive/Luminal A/B (ER+, PR+, HER2-), Triple-negative/basal-like (ER-, PR-, HER2-), and HER2-positive (ER-, PR-, HER2+)[6]. The two major histological subtypes of invasive breast cancer are Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC). IDC and ILC are distinct entities that differ in their location of origin. IDC originates in the mammary milk ducts while ILC originates in the milk producing lobules[1]. IDC accounts for approximately 75-80% for all breast cancer cases, and ILC accounts for approximately 10-15% of breast cancer in women[1, 7, 8]. Even though ILC accounts for such a large portion of breast cancer patients, it remains a relatively understudied disease.

Invasive Lobular Carcinoma: features of a unique subtype of breast cancer

Invasive lobular carcinoma (ILC) is the second most common histological subtype accounting for 10-15% of all invasive breast cancer[1]. ILC is overall a slow-growing cancer as evidenced by low histological grade, low proliferative index, hormone receptor positivity

(ER+/PR+), HER2- and normal expression of p53, a tumor suppressor gene. The low expression of HER2 and absence of p53 mutation are associated with a good prognosis, and the cancer should be responsive to endocrine therapy, yet this stealthy cancer is known to be highly metastatic and often not diagnosed early[3]. The classical form of ILC is characterized as small discohesive neoplastic cells invading the stroma in single filed fashion caused by the hallmark deletion of cell adhesion gene E-cadherin (*CDH1*)[9]. Currently, ILC patients are routinely treated with endocrine therapies and drugs of choice include selective estrogen receptor modulators (Tamoxifen) for pre-menopausal women, aromatase inhibitors (such as Letrozole) for post-menopausal women, and estrogen receptor dowregulators in patients with metastatic disease (fulvestrant)[10].

Although the high hormone receptor positivity, HER2 negativity and normal p53 are favorable features[1, 11], ILC patients are still at high risk for late recurrence suggesting resistance to therapy[12]. The single file growth pattern of ILC results in tumors that are difficult to detect due to the lack of a palpable mass, leading to the delayed and late-stage diagnosis[12] (Figure 1). In addition, a combination of resistance to therapies and dormancy results in delayed recurrence and poor overall outcomes for patients[13]. Hence, there are several unique challenges in managing ILC: ***1. Delayed initial diagnosis[13], 2. higher stage at diagnosis[14], 3. lack of response to conventional systemic therapies[1], 4. worse overall long-term outcomes[11].*** Thus, identifying novel therapies that target pathways that induce resistance to conventional therapies is key to improving outcomes for patients with this cancer.

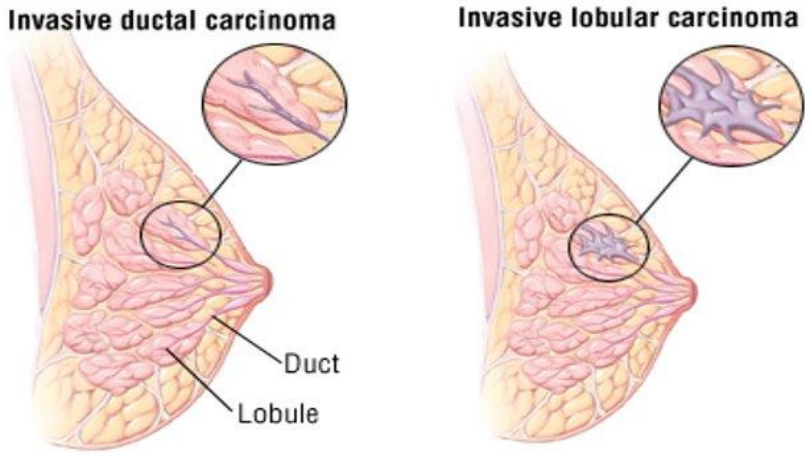

Figure 1: The distinct growth pattern results in "web-like" growth that is difficult to detect via mammogram (Harvard Health Publishing)[15]

Hallmark molecular signature in ILC

The loss of E-cadherin is the key molecular characteristic feature of ILC. The E-cadherin gene, *CDH1*, is located on human chromosome 16q22.1 and codes for a transmembrane glycoprotein [16] that supports cell-cell adhesion, regulates motility, and its loss leads to Epithelial-to-Mesenchymal transition (Figure 2A). Loss of E-cadherin in ILC is predominantly attributed to *CDH1* mutation, which in 89% of cases occurred concurrently with heterozygous deletion in chromosome 16q[17]. Analysis of invasive breast carcinoma cases in the TCGA dataset reveals that 66% (107/162) of ILC harbor mutations in *CDH1* compared to only 3% (22/741) of IDC (Fig 2B)[18-20]. The key pathways that are downstream of E-cadherin include Hippo, WNT, TGF-ß, NFkB, and growth signaling pathways[8]. The loss of E-cadherin initiates the cytoplasmic accumulation of p120 catenin, which plays an integral role in tumor invasion and initiates anoikis resistance[8, 21].

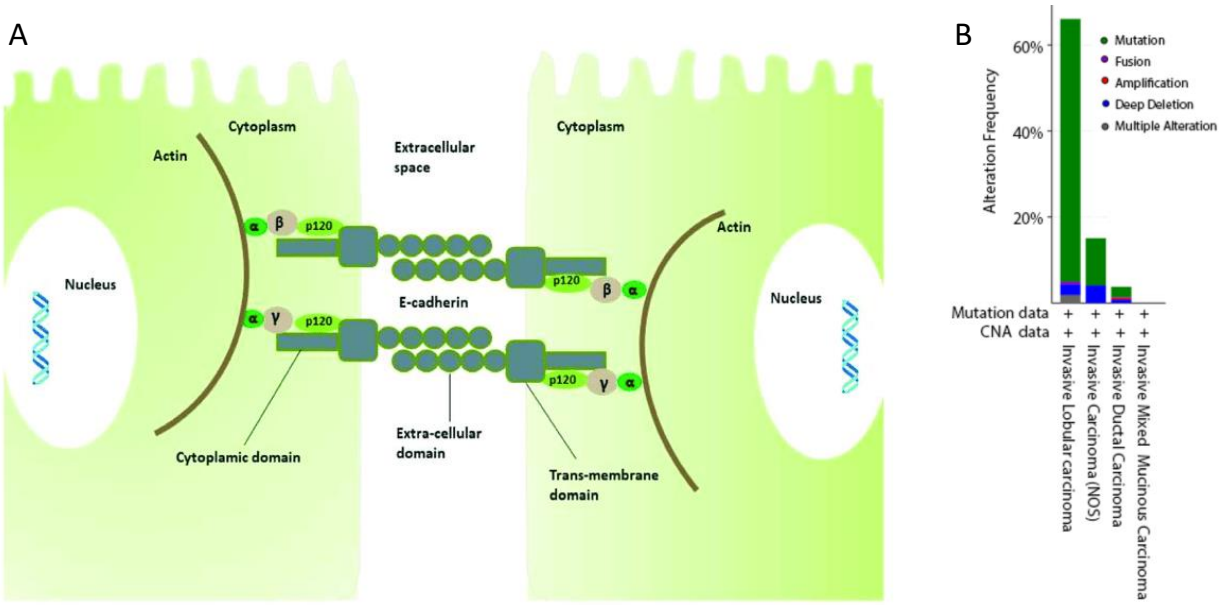

Figure 2: **A.** The intact E-cadherin junction [22] is essential for regulating cell-cell adhesion. **B.** *CDH1* mutation frequency across breast cancer subtypes based on TCGA dataset[8, 20].

Resistance to antiestrogen therapies

As previously mentioned, patients with ILC are routinely treated with endocrine (antiestrogen) therapy but one–third of patients fail to respond to treatment and develop endocrine resistance in the long term. The underlining mechanism behind acquired endocrine resistance in ILC is multifactorial. Previous studies have indicated that in ILC resistant cells, an upregulation of orphan nuclear receptor ERRγ occurs, thereby activating AP1 dependent transcription, which has been demonstrated to confer tamoxifen resistance [23, 24]. Stires *et.al.* reported upregulation of MAPK/ERK pathway and glutamate receptor in Sum44/LCCTam cells and their inhibition restored tamoxifen sensitivity[23]. Additional work has implicated WNT4, the ligand in the WNT signaling pathway, to be a driver for estrogen-induced growth and a potential mediator of endocrine resistance in ILC[12].

Another important factor in the endocrine resistance of ILC is *FOXA1*, which is an ER-transcription modulator known to play a key role in cancer progression and development of

endocrine resistance[25]. Through comprehensive genomic analysis, Ciriello *et.al*. observed spatially clustered mutations of *FOXA1* in 7% of ILC samples (n=127), leading to increased *FOXA1* expression and activity[17]. Higher rate of *FOXA1* mutations was observed in ILC compared to IDC (7% versus 2%). Stires *et.al.* showed amplification of *FOXA1* in tamoxifen resistant SUM44PE/LCCTam cell lines, which supports the notion that FOXA1 could be critical for endocrine resistance in ILC[23]. Another important pathway that has been implicated in imparting therapy resistance is the cross-talk between HER2 signaling pathway and hormone receptors (Figure 3)[26]. The HER2 signaling pathway can be targeted using monoclonal antibodies or small molecule tyrosine kinase inhibitors[26]. We have recently published a comprehensive review on the resistance mechanisms involved in ILC tumors and the clinical challenges[8].

HER2 as a target to overcome endocrine therapy resistance

HER2 is an oncogene and a key biomarker in breast cancer, that when overexpressed is an excellent therapeutic target. HER2 is a receptor tyrosine kinase (RTK) that is a member of the HER family (EGFR or HER1/HER3/HER4) of membrane bound receptors[27]. There is no known ligand of HER2, on the contrary HER2 is known to dimerize with itself and other members of the HER family. Dimerization results in autophosphorylation of the tyrosine residues (Y1023 & Y1248)[28] within the receptors, initiating multiple signaling cascades. The pathways activated by HER2 promote cell proliferation and survival (Figure 3) primarily through the PI3K/ATK and RAS/RAF/MAPK pathway[29].

Clinically, immunohistochemistry (IHC) and Fluorescence in situ hybridization (FISH) are the two methods used commonly in detecting HER2 in breast tumors[30]. While HER2 may not be commonly overexpressed (i.e, HER2-IHC: 3+) in ILC, studies have revealed HER2 activating mutations occur in 27% of ILC cases, and these mutations combined with E-cadherin loss results

in significantly worse prognosis[26, 31]. Patients with IHC score of 3+ are considered HER2 positive and such patients receive HER2 targeted therapies. However, these tests will not detect HER2 activating mutations, as seen in ILC[27, 32]. In addition, some of these tumors may be deemed HER2 indeterminate or equivocal with IHC score of 2+. Additionally, HER2/Epidermal Growth Factor Receptor (EGFR) cross talk with ER is shown to induce endocrine therapy resistance in breast cancers[31, 33, 34] and **recurring cancers show increased HER2 mutations and HER2 enrichment**. **These findings highlight the importance of testing the efficacy of HER2 targeted therapies in ILC patients[35].**

Hence, multiple data suggests the role of increased HER2 signaling in inducing resistance to antiestrogen therapies. HER2 is not often overexpressed in ILC at the time of diagnosis, but increased HER2 signaling (HER2 enrichment and activating mutations) have been reported in recurrent and metastatic tumors that are resistant to endocrine therapy. Trastuzumab (Herceptin), a monoclonal antibody against HER2, is only approved for tumors that overexpress HER2 (IHC 3+ or amplified by FISH) as the clinical trial NSABP B-47, demonstrated that trastuzumab had no benefit for low HER2 (HER2- 1+ or 2+ by IHC) expressing breast cancer [27]. Small molecule tyrosine kinase inhibitors of HER2 (such as tucatinib and neratinib) may present a therapeutic option in cases where there are HER2 activation mutations and intermediate expression of HER2.

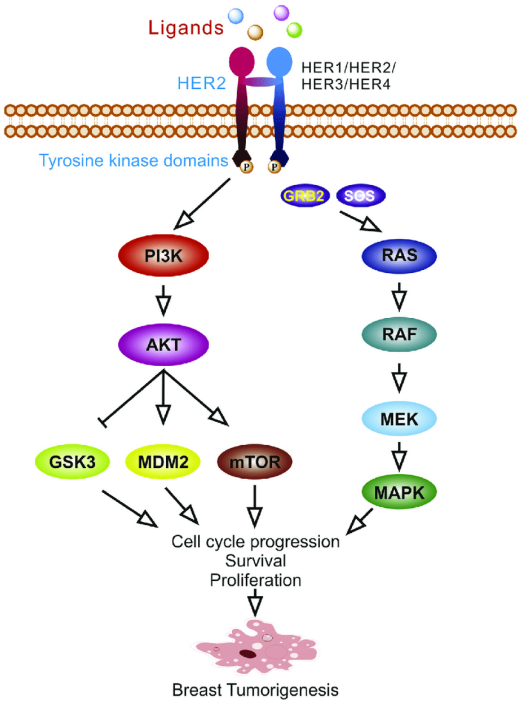

Figure 3: HER2 signaling pathway, which is essential to cancer progression, survival, and proliferation, is a targetable pathway[29].

Significance

There is increasing evidence that ILC differs from the more common Invasive ductal carcinoma (IDC) in clinical, pathological and genomic characteristics, yet treatment of this cancer follows the principles of IDC. One of the main challenges faced by patients with ILC is the higher risk of late recurrences due to resistance to endocrine therapies, which leads to significant morbidity. The inherent slow proliferative rate of these cancers and resistance to adjuvant endocrine therapy is recognized as one of the reasons for late recurrence. Activated HER2 signaling could lead to this resistance and worse outcomes, and targeted HER2 therapies in ILC may prove to delay cancer progression. Combination therapies have emerged as favorable treatment options in patients to curtail drug resistance and identifying alternative druggable targets is of importance to improve patient outcomes.

Hypothesis

ILC is a subtype of breast cancer that is often hormone receptor positive but plagued with overall worse long-term outcomes due to resistance to antiestrogen therapies. Activated HER2 signaling is one of the pathways by which these tumors escape from antiestrogen approaches. Although HER2 is not often overexpressed in ILC, activating mutations have been found to occur 27% of the time [31]. While trastuzumab (HER2 monoclonal antibody) as a single agent may not be an effective treatment option, a newer class of small molecule tyrosine kinase inhibitors (TKI) may be effective, as they would not require overexpression of HER2 to be effective. Two such TKI's are tucatinib (ONT-380), a small molecule HER2 TKI currently approved in metastatic HER2+ breast cancer, and neratinib, a small molecule inhibitor of HER2/EGFR that is FDA approved for early and metastatic breast cancer. **We hypothesize that combining trastuzumab, TKI, and antiestrogen therapy will be a viable alternative treatment for endocrine resistant ILC patients.**

We sought to prove our hypothesis through the following aims:

Aims

1. Determine the efficacy of HER2 inhibitors in combination with tamoxifen in endocrine resistant ILC using *in vitro* cell culture model
    a. Generate tamoxifen resistant ILC cell lines by long term exposure of parental ILC cell lines to tamoxifen
    b. Determine the efficacy of HER2 inhibitors as a single agent and in combination by studying the impact on cell proliferation
2. Elucidate the key downstream signaling events affected by HER2 inhibition in ILC that leads to growth inhibition

Study Design

Aim 1. Determine the efficacy of HER2 inhibitors in combination with tamoxifen in endocrine resistant ILC using *in vitro* cell culture model

1a. *Generate tamoxifen resistant ILC cell lines by long term exposure of parental ILC cell lines to tamoxifen.*

There are two commercially available human ILC cell lines, MDA-MB-134-VI and SUM44PE. Tamoxifen resistant lines will be generated through exposure of parental cell lines to low dosage of tamoxifen (100nM) for 6 months or more. Additional cell lines exposed to higher doses of tamoxifen (200nM, 500nM) will also be generated. Tamoxifen dose response will be measured using MTT [[3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] cell proliferation assay and IC50 (the half maximal inhibitory concentration) values will be calculated using GraphPad Prism 9.

1b. *Determine the efficacy of HER2 inhibitors as a single agent and in combination by studying the impact on cell proliferation.*

ILC Cell Lines (parental and TAM resistant) will be treated with combinations of either i) Tamoxifen alone, ii) TKI (Tucatinib or Neratinib) alone, iii) Trastuzumab alone, iv) Tamoxifen+TKI, or v) Tamoxifen + TKI + Trastuzumab. Proliferation will be measured using MTT assay.

Aim 2: Elucidate the key downstream signaling events affected by HER2 inhibition in ILC

2a. *HER2 protein levels of ILC parental and endocrine therapy resistant cell lines will be determined by western blot analysis.*

2b. *Downstream signaling events will be tested by western blot and a human receptor tyrosine kinase array, where phosphorylation of 71 different receptor tyrosine kinases will be tested.*

Methods

Cell Culture

MDA-MB-134-VI was obtained from the American Type Culture Collection (ATCC) and SUM44PE was obtained from Asterand, USA. MDA-MB-134-VI was maintained in 1:1 Dulbecco's Modified Eagle Media (DMEM):Leibovitz-15 (L-15) containing 10% heat inactivated fetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Gibco). SUM44PE was maintained in Ham's F-12 containing 2% FBS, 1g/L bovine serum albumin, 5mM ethanolamine, 10mM HEPES, 1μg/mL hydrocortisone, 5μg/mL insulin, 50mM sodium selenite, 5μg/mL apo-transferrin, 10nM triiodo thyronine, and 1% penicillin/streptomycin. Cell lines were incubated at 37°C in a 5% CO_2 incubator. MDA-MB-134-VI LTED Cell Lines (A, B, D, E) an SUM44PE LTED cell lines (A and B) were obtained from the lab of Dr. Steffi Oesterreich from the University of Pittsburgh Medical Center (UPMC) [12] and maintained in Improved Minimum Essential Media (IMEM) containing 10% Charcoal Stripped FBS (Gibco) and 1% Penicillin/Streptomycin. Cell lines were tested intermittently for mycoplasma contamination and authenticated by short tandem repeat profiling by the Ohio State Sequencing Core.

Generation of Tamoxifen Resistant Cell Line

Exponentially growing MDA-MB-134-VI and SUM44PE Parental cells were supplemented with 100nM 4-hydroxy-tamoxifen (Cayman Chemicals) in growth media. Cells are cultured in 100nM Tamoxifen for ~ 6 months before any experiments were performed and maintained in tamoxifen for further studies ($TamR^{100}$). Additional lines were generated which are cultured in 200nM tamoxifen ($TamR^{200}$) and 500nM tamoxifen ($TamR^{500}$) as well.

MTT Cell Proliferation Assay

To study effect of the drugs on cell proliferation, the MTT (3-(4,5-dimthylthiazol-yl)-2,5-diphenyltetrazolium bromide) assay is used. MTT assay (Sigma) utilizes the yellow tetrazolium MTT [[3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] as an indicator, where reduction of MTT by metabolically active cells reduces it to purple formazan. Solubilized formazan is quantified by measuring absorbance at 595nM using a spectrophotometer. Cells (15,000/well) are seeded into a 96-well plate. After 24 hours of cell seeding, complete media from the wells is replaced with estrogen stripped media and incubated overnight. The next day (~16hours), we add MTT reagent to cells in triplicate wells for 4 hours and then add solubilizing buffer (0 hour control). All other wells received a single drug or a combination of drugs and are incubated for 5 days in a 37^0C incubator at 5% CO_2. MTT reagent is added to all the wells after the 5 days of incubation, solubilizing agent is added after 4hours, and optical density is measured the next day. The fold change in cell proliferation after 5 days is calculated with respect to 0-hour control based on formazan produced (absorbance at 595nM). The percent of viable cells is calculated as the ratio of treated cells to the untreated control (100%). Effect of the drugs is assessed comparing percentage viability in untreated vs. treated cells. 4-hydroxy-tamoxifen, tucatinib (Cayman Chemicals), neratinib (Cayman Chemicals), and trastuzumab (Selleck Chemicals) were all tested using the cell proliferation assays.

Whole Cell Lysate preparation and protein estimation

Whole cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH8.0, 1% Triton-X-100, 0.05% Na-DOC, 0.1%SDS). Cells in culture dishes are washed twice with 1x PBS (phosphate-buffered saline, National Diagnostics), and incubated in RIPA buffer (RIPA

buffer, 0.1M PMSF, 1mM NaF, 1mM $NaVO_3$, and 0.1μM MG-132) for 30 minutes on ice, followed by centrifugation at 14000 rpm for 10 minutes at 4^0C. The supernatant was collected and stored at -80^0C. The total protein concentration was determined using the Pierce BCA assay kit (ThermoFisher). Whole cell lysates (30ug) were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gel and transferred on to a PVDF membrane. The membrane was then blocked in Rockland buffer (Rockland Antibodies & Assays, USA), and incubated with primary antibody over night at 4^0C. Primary antibodies used: HER2 (Cell Signaling #4290), Estrogen Receptor alpha (Abcam ab75635), phospho- AKT (Ser473) (Cell Signaling #4060) , phospho- p44/42 MAPK (Thr202/Tyr 204) (Cell Signaling #9101), β-actin (Cell Signaling #3700), AKT (Cell Signaling #4691), p44/42 MAPK (Cell Signaling #4695), and GAPDH (Cell Signaling #2118) at 1:1000 dilution in Rockland blocking buffer. The membranes were next washed with 0.1% TBS-T (Tris-buffered saline containing 0.1% Tween-20) twice followed by incubation of the membranes in anti-mouse secondary antibody (Licor) for 1 hour at room temperature or anti-rabbit HRP-linked secondary antibody (Cell Signaling). The membranes were then washed in 1% TBS-T, 5 min each, and fluorescent signals captured in Odyssey-CLX machine or chemiluminescence signal obtained via autoradiography using horseradish peroxidase substrates (ThermoFisher). Signal for each protein band was quantified using ImageStudio (for fluorescent signals) and ImageJ (for autoradiographic images).

Downstream Signaling Analysis

Cells (600,000) were seeded in 60mm tissue culture dishes and allowed to attach overnight in serum containing media. The next day, the existing media is replaced with serum free media, and cells were incubated in serum-free media for 48 hours. Cells were next treated with individual

drugs or a combination for 8 hours. After drug treatment, cells are stimulated with 0.1μg/mL of EGF for 15 minutes, washed 2x with PBS and then flash frozen on dry ice. Whole cell lysates are subsequently prepared, protein estimated and subjected to western blot analysis.

Phospho-Receptor Tyrosine Kinase Array

To determine the basal level of phosphorylated RTKs and their alteration upon drug treatment, we used Phospho RTK array kit (Abcam). Each of the membranes in the kit has antibody against 71 RTKs imprinted on it in duplicates. Whole cell lysates from exponentially growing cells, drug treated or control cells were incubated with the membrane, followed by washing and incubation with biotinylated anti-phosphotyrosine antibody to detect phosphorylated tyrosines on activated receptor kinases. This was followed by incubation with HRP-streptavidin, and signal was detected using Licor Odyssey-Fc imager. ImageStudio software was used to measure the signals of phosphorylated proteins.

Results

ILC Cell Lines demonstrate differences in their response to Tamoxifen

ILC Tamoxifen Resistant (TamR) and Long Term Estrogen Deprived (LTED) cell lines are distinct tools for modeling endocrine resistant lobular cancer in an *in vitro* setting. One method relies on the use of continues tamoxifen exposure, modeling the prolonged period of anti-estrogen treatment after diagnosis of ILC. The other method models the depleted estrogen environment that occurs during prolonged exposure to antiestrogen therapies. Dose response of tamoxifen varies across the TamR, parental, and LTED cell lines (Figure 4). The LTED cell lines that were obtained from the lab of Dr. Oesterriech at UPMC were intended to be the lines that we would use to model tamoxifen treatment resistant, but after conducting dose response experiments with tamoxifen, it

was clear that not all the LTED cell lines were consistently resistant to tamoxifen compared to the parental ILC cell lines. Thus, the tamoxifen resistant cell lines were generated in our lab through long term exposure to low doses of tamoxifen. Through clonal expansion, new cell lines were generated that required tamoxifen supplemented in the media in order to grow. MTT assays were conducted using all cell lines in order to determine sensitivity of each cell line to tamoxifen (Figure 4). IC50 values were computed using Graphpad Prism curve fitting, specifically the log(inhibitor) vs response – variable slope equation (Table 1).

Based on the results, we showed that both the MDA-MB-134-VI TamR and SUM44PE TamR cell lines are more resistant to tamoxifen treatment when compared to the parental cell lines. The MDA-MB-134-VI TamR^{100} cell line has a tamoxifen IC50 of 29.3μM compared to 2.27μM for the parental line. In SUM44PE TamR^{100}, the increase in IC50 was less compared to parental (9.6μM vs 7.1μM). Across LTED cell lines, the tamoxifen IC50 values were lower than parental, with the exception of MDA-MB-134-VI LTED D (8.2μM vs 2.27μM). MDA-MB-134-VI LTED A (IC50: 1.2μM), LTED B (IC50: 1.15μM), and LTED E (IC50: 1.6μM) were all more sensitive to tamoxifen monotherapy when compared to parental cells (IC50: 2.27). Among the different SUM44PE cell lines, LTED A (IC50: 5.1μM) and LTED B (IC50: 1.7μM) were both more sensitive to tamoxifen than parental SUM44PE (IC50: 7.1μM) (Table 1).

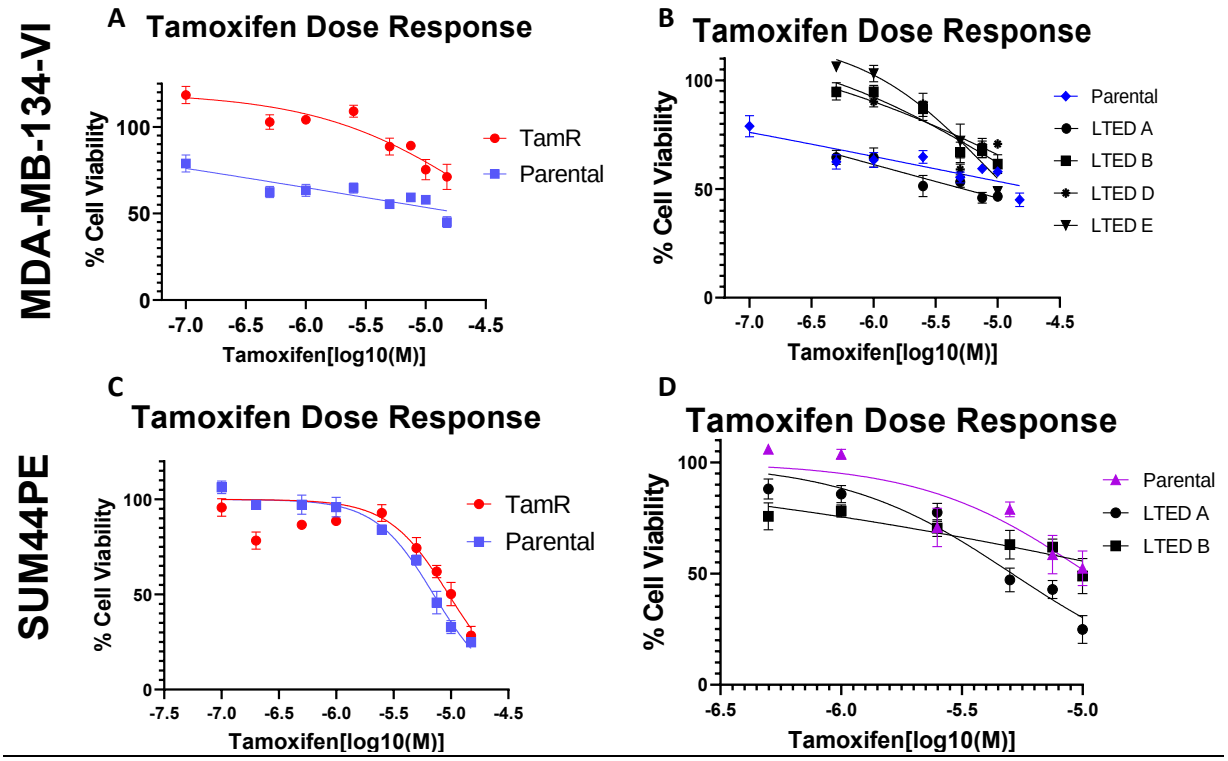

Figure 4: Freshly seeded cells (15,000) were treated with increasing concentration of tamoxifen (0μM -20μM). Cell viability was determined after 5 days using MTT reagent. **A**. MDA-MB-134-VI parental against MDA-MB-134-VI TamR^{100}, **B**. MDA-MB-134-VI parental and LTED, **C.** SUM44PE parental and SUM44PE TamR^{100}., **D**. SUM44PE Parental and LTED. All figures are an average of N = 3 experiments.

Cell Line	Tamoxifen IC 50
MDA-MB-134-VI Parental	2.27µM
MDA-MB-134-VI LTED A	1.2µM
MDA-MB-134-VI LTED B	1.15µM
MDA-MB-134-VI LTED D	8.2µM
MDA-MB-134-VI LTED E	1.6µM
MDA-MB-134-VI TamR^{100}	29.3µM
SUM44PE Parental	7.1µM
SUM44PE LTED A	5.1µM
SUM44PE LTED B	1.7µM
SUM44PE TamR^{100}	9.6µM

Table 1: List of ILC cell line and the corresponding IC50 value for tamoxifen monotherapy

Tamoxifen Resistant Cell lines display distinct morphological changes

Exposure to tamoxifen for an extended period time resulted in distinct morphological changes when compared to the parental cell lines. Brightfield images taken using the Olympus IX50 microscope connected to the Zeiss AxioCam MRm (Figure 5) display the phenotypic differences between parental and TamR cells. Especially in MDA-MB-134 parental, which presents as round cells, the TamR cells are enlarged and take on a more cuboidal shape. Additionally, the MDA-MB-134 TamR cells do not present as an even monolayer of cells, rather a collection of cells layered on top of each other. 3D growth cultures will be important to further elucidate the unique morphological changes that occur after long term exposure to tamoxifen in ILC.

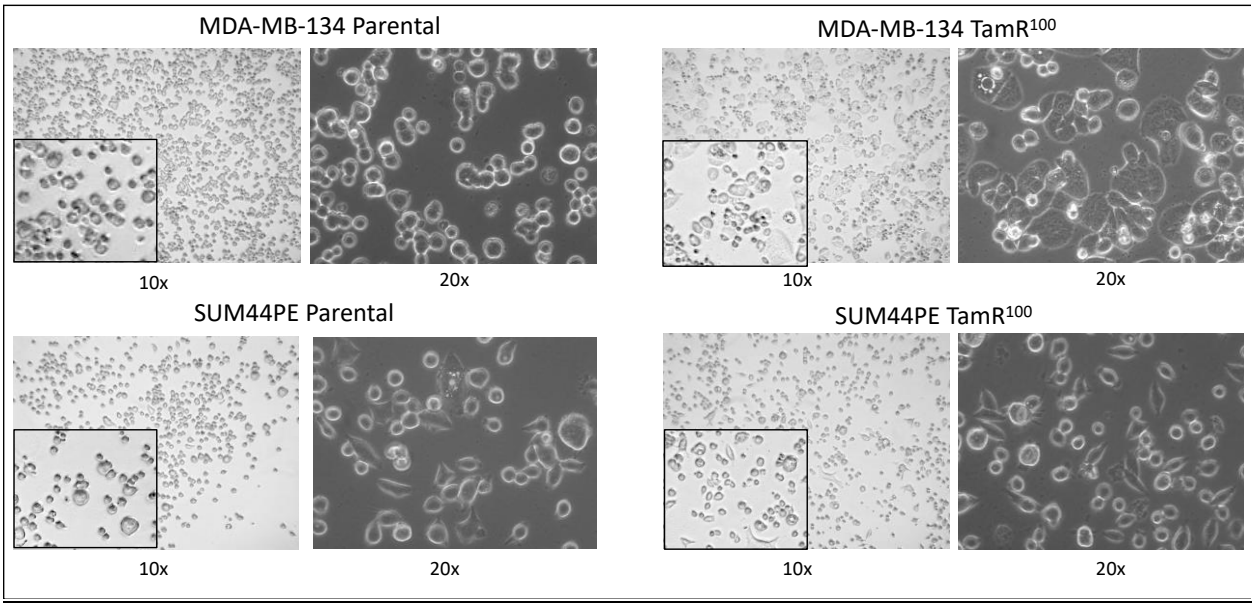

Figure 5: Representative brightfield microscope images taken of exponentially growing cells taken using Olympus IX-50 microscope attached the Zeiss AxioCam mRm. Images were taken at 10x magnification and 20x magnification.

Endocrine therapy resistant ILC Cell Lines show increases in HER2

The tamoxifen resistant derivatives of the parental ILC cell lines showed unique responses to tamoxifen monotherapy and were different morphologically. We were interested in seeing how both long-term exposure to tamoxifen or long-term estrogen deprivation would impact the HER2 expression levels in ILC. HER2 enrichment and mutations are known to occur in endocrine-resistant ILC[35]. Western blot analysis revealed that endocrine resistant cell lines express higher levels of HER2 than the parental lines (Figure 6). The MDA-MB-134-VI TamR^{100} line shows a 4-fold increase in HER2 expression, while the SUM44PE TamR^{100} has a 2-fold increase in HER2 expression. These increases in HER2 expression match the increases in the resistance to tamoxifen (Table 1). The LTED cell lines, which generally did not display significant resistance to tamoxifen, showed comparable HER2 level as the parental lines, with the exception of MM134 LTED A.

While there was discernable increase in HER2 expression in the derivatives of ILC lines, the level of HER2 is still significantly less than BT474, which is a HER2 overexpressing cell line and was used as a control in this study.

ERα protein level was also measured and compared among the ILC cell lines. Generally, the LTED cell lines (with the exception of MDA-MB-134-VI LTED E) showed decreases in ERα levels in comparison to the parental cells, suggesting that long term estrogen deprivation leads to the downregulation of ERα. ERα levels were unchanged in TamR cells.

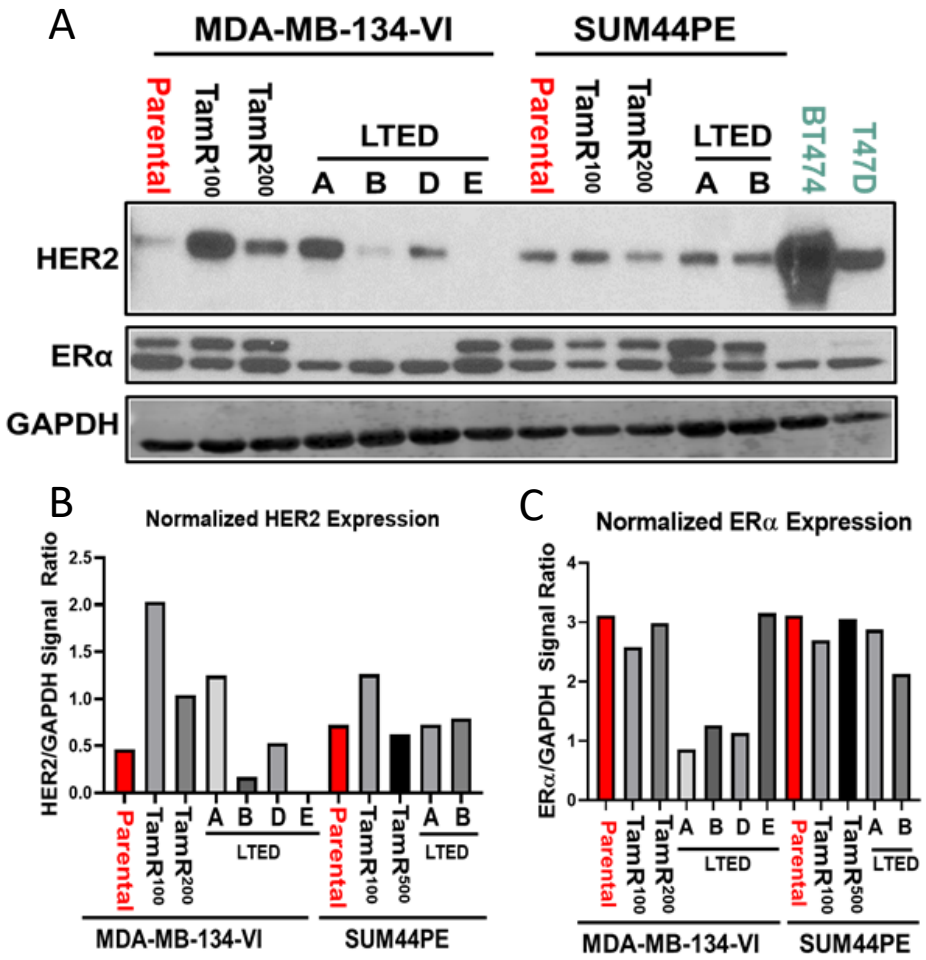

Figure 6: A. Western blot analysis using whole cell extracts of exponentially growing parental and endocrine resistant ILC cell lines. 30μg of protein was separated on SDS-PAGE and probed for Her2, ERα and GAPDH (loading control). BT474 and T47D are IDC cell lines used as a

positive control for HER2 expression. **B.** Quantified HER2 protein normalized to GAPDH and **C.** quantified ERα protein normalized to GAPDH.

Combination of Tamoxifen and Trastuzumab with Neratinib is most effective against SUM44PE cells

To determine whether anti-HER2 strategy is a therapeutic option in ILC, MTT assays were performed for cells treated with tamoxifen alone, combination of tamoxifen and TKI (neratinib or tucatinib), and a combination of tamoxifen, TKI, and trastuzumab. The triple combination of tamoxifen, trastuzumab, and neratinib (TTN) was most effective in reducing the viability of the SUM44PE cell lines after 5 days of treatment (Figure 7). Among the SUM44PE lines, TamR and LTED B showed significant reduction (p-value < 0.05) in cell viability with the TTN triple combination when compared to tamoxifen monotherapy, while parental and LTED A had a marginal, and non-significant change in viability. The triple combination of TTN was more effective than TTT in SUM44PE cells. The greater effectiveness of neratinib is potentially due to the role of neratinib as a HER2/EGFR dual inhibitor, where tucatinib only targets HER2. Combined treatment with either of the TKI and tamoxifen was not as effective as the triple combination.

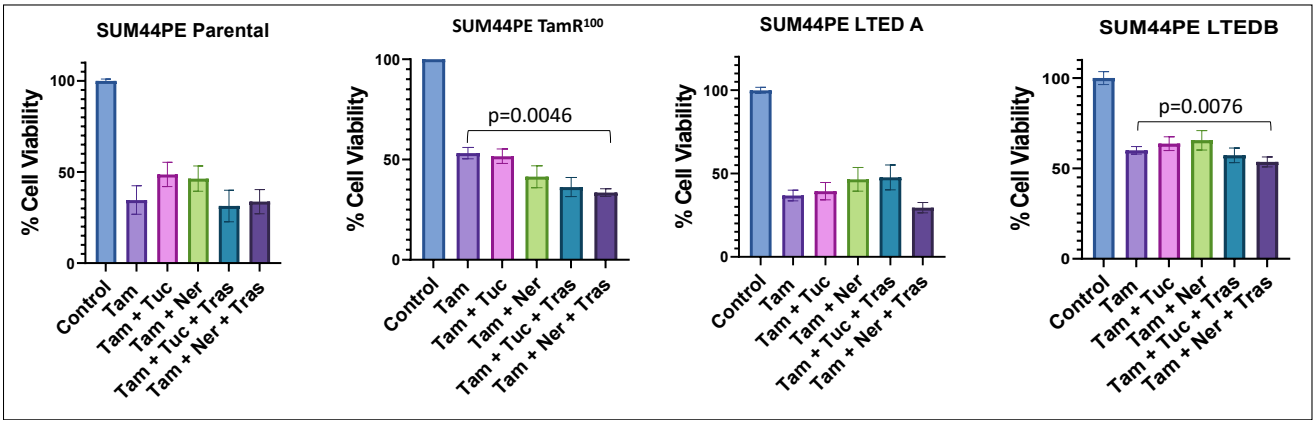

Figure 7: Freshly seeded cells (15,000) were estrogen deprived for 24 hours and treated with drugs as indicated. Cell viability, which is calculated as the ratio of treated to control cells, was measured 5 days after treatment using MTT reagent. Cells were treated with tamoxifen (10μM), neratinib (100nM), tucatinib (100nM), and trastuzumab (10μg/mL). Mixed-ratio one-way anova with multiple comparisons was used to calculated p-values. Figures and statistical analysis represent 3 separate experiments each conducted with triplicates.

Combining TKI with Tamoxifen and Trastuzumab reduces viability in MDA-MB-134 TamR, LTED A, LTED D, LTED E, but not Parental and LTED B cells

In MDA-MB-134-VI cells, combining tamoxifen, tucatinib, and trastuzumab (TTT) significantly reduced cell viability in TamR (p-value = 0.047), LTED A (p-value = 0.026), LTED D (p-value = 0.011), and LTED E (p-value = 0.007) when compared to tamoxifen monotherapy (Figure 8). In LTED A cell however, the effect of TTN on cell viability is more pronounced than TTT (p-value = 0.001). TamR and LTED B lines had marginal (3% cell viability reduction), but not a significant, reductions in viability with the TTN combination therapy. The parental cell lines did not show viability change in response to any combination treatment. The cell lines that displayed sensitivity to HER2 targeted treatments (TamR, LTED A, and LTED D) have higher HER2 expression levels compared to the parental lines, however LTED E, which does not have higher HER2 expression, still showed sensitivity to HER2 targeted treatments (Figure 6A, 6B).

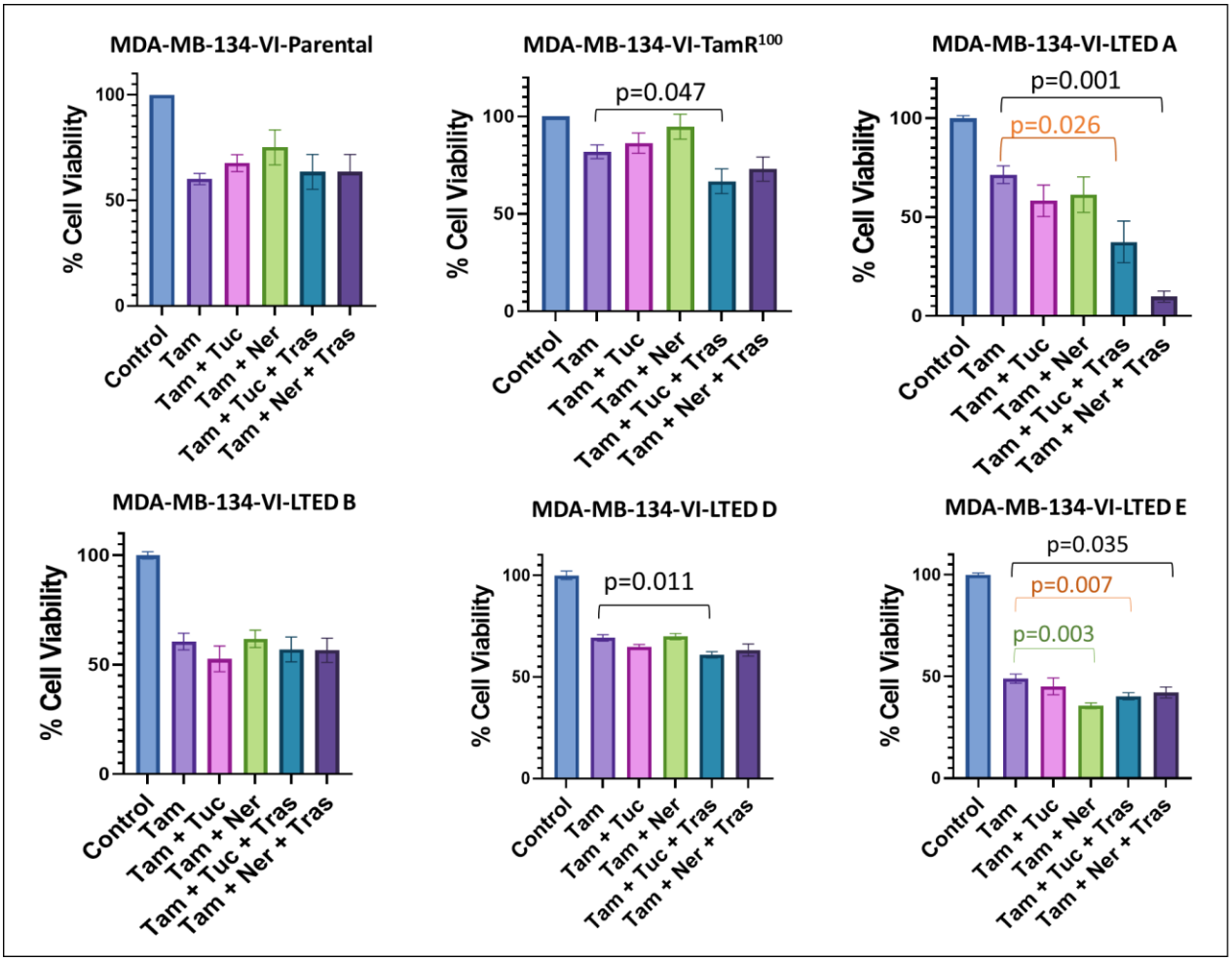

Figure 8: Freshly seeded MDA-MB-134 parental and endocrine therapy resistant cells (15,000) were estrogen deprived for 24 hours and treated with drugs as indicated. Cell viability was measured 5 days after treatment using MTT reagent. Cells were treated with tamoxifen (10μM), neratinib (100nM), tucatinib (100nM), and trastuzumab (10μg/mL). Mixed-ratio one-way anova with multiple comparisons used to calculated p-values. Figures and statistics represent 3 separate experiments each conducted in triplicates.

Combination of TKI and antiestrogen inhibits AKT signaling

The mechanisms by which targeting HER2 impacts cell signaling pathways and downstream signaling was analyzed using western blotting. Two main pathways that are downstream of HER2, the phosphoinositide 3-kinase/protein kinase b (PI3K/AKT) and mitogen-activated protein kinase (MAPK) pathways (Figure 3) were further studied. The PI3K/AKT and

MAPK pathways are essential signaling pathways that facilitate cell proliferation and survival[36]. SUM44PE parental cells were serum starved for 48 hours to reduce the level of pAKT and pMAPK to baseline by depriving of growth factors. Cells were subsequently stimulated with low dosage of EGF (0.1ug/ml) for 15 minutes to stimulate EGFR signaling that leads to downstream AKT and MAPK signaling. Higher dosage of EGF and prolonged treatment was not used to avoid saturation of phosphorylation of signaling molecules and proper assessment of the effects of different drugs. While EGF treatment showed a marked increase in pAKT and pMAPK, prior treatment with either tamoxifen, tucatinib, or a combination of the two resulted in marked reduction in pAKT level (Figure 9). The effect of the combined treatment was more pronounced and significant on AKT phosphorylation compared to single agent treatment (82% decrease vs 37% decrease). On the contrary, tucatinib alone showed maximum reduction in pMAPK level, compared to tamoxifen alone or combination of tamoxifen and tucatinib (70% decrease vs 41% decrease). Further investigation with more cell lines and additional downstream targets are important to fully elucidate mechanisms by which endocrine therapy resistance can be overcome.

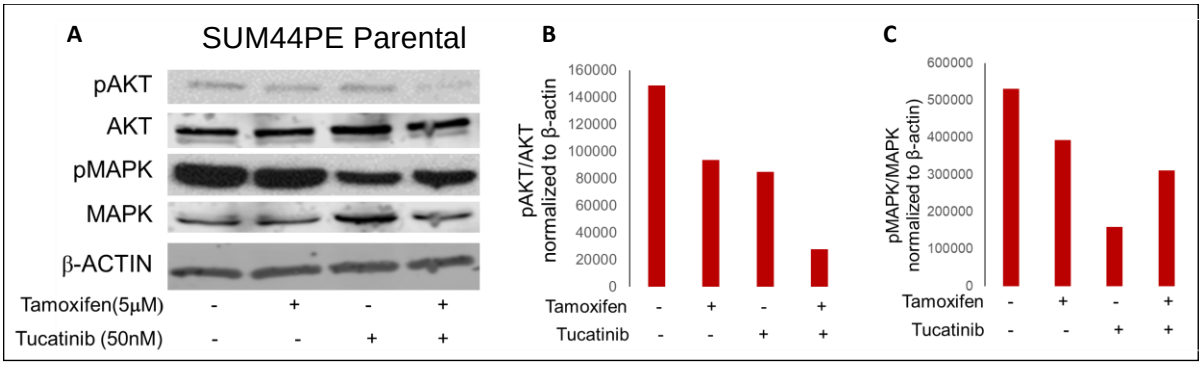

Figure 9: SUM44PE cells (500,000) were seeded in 60mm dishes, and serum starved for 48 hours. Cells were then treated with indicated drugs for 8 hours, then stimulated with 0.1μg/mL EGF for 15 minutes. Whole cell lysates were separated on SDS-PAGE gel and probed with antibodies against indicated proteins.

ACK1: A RTK target is phosphorylated in ILC cells

Further analysis of downstream signaling in parental ILC cells was undertaken using a receptor tyrosine kinase (RTK) array, containing 71 RTKs. Whole cell extracts from exponentially growing SUM44PE parental and MDA-MB-134-VI parental cells showed phosphorylation of several RTKs, namely, ACK1, FYN, and HCK1 (Figure 10). ACK1 is a non-receptor tyrosine kinase that is associated with cancer invasiveness and progression[37]. FYN and HCK1 are tyrosine kinases that belong to the Src family of kinases, and mediate cell development, survival, and proliferation. Dysregulation of these proteins is considered a driving force for cancer initiation[38]. Not much is known about the role of these three RTKs in ILC and needs further investigation.

We next analyzed if drug treatment alters phosphorylation of any of the three newly identified RTKs in ILC. We used whole cell extracts from serum starved SUM44PE either treated with TKI and antiestrogen or left untreated (control) and stimulated with EGF. Whole cell extracts were hybridized with the membranes. Treatment with tamoxifen, tucatinib alone or in combination displayed marked reduction in pACK1 levels (Figure 10D). ACK1 signaling is implicated in promoting breast cancer survival through PI3K independent AKT phosphorylation, inhibiting tumor suppression via wwox, and radioresistance through the androgen receptor (AR) (Figure 10C)[37, 39]. ACK1 signaling is involved in many aspects of cancer and understanding the specific role ACK1 plays in ILC may provide a new drug target for patients.

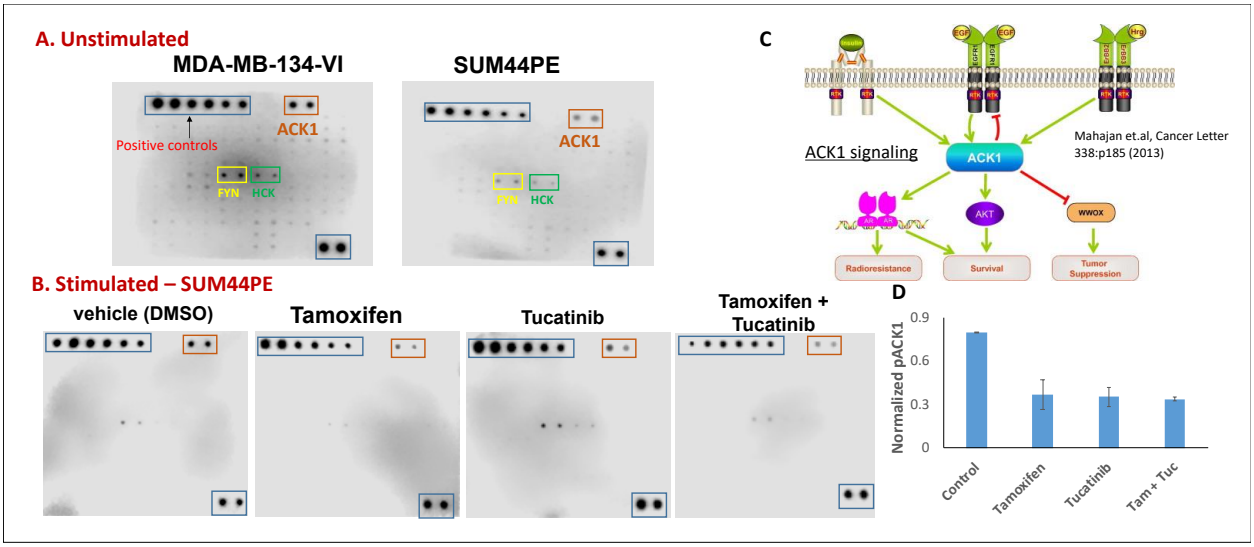

Figure 10: Human Receptor Tyrosine Kinase Array: whole cell lysates from A**.** exponentially growing ILC cell lines and B. EGF-treated (0.1 µg/mL for 15 minutes) SUM44PE cell lines exposed to either tamoxifen (5 µM), ONT-380 (100nM), or a combination of both for 24 hours, were hybridized to RTK array (Abcam). Membranes were imaged using LICOR imaging system and quantified using Image J. C. ACK1 signaling pathway and its implications in cancer[39]. D. Quantification the pACK1 status in treated cells.

Discussion

Invasive Lobular Cancer is an understudied subtype of breast cancer that impacts around 44,000 women each year[1]. The hallmark mutation of ILC is the loss of the cell-cell adhesion protein, E-cadherin, which results in a unique growth pattern[1, 8]. With the more "web-like" growth pattern, ILCs are a stealthy cancer more difficult to detect via mammogram and often diagnosed a later stage[40]. Since ILCs are generally ER+/PR+ and HER2 negative, the standard course of treatment involves endocrine therapy, including tamoxifen[8, 14]. Endocrine resistance is a major challenge in the treatment of ILC, as a third of patients fail to respond to treatment and develop resistance in the long run[23]. Endocrine resistance in ILC leads to the higher risk of recurrence, which leads to significant morbidity[8]. There are many contributors to endocrine resistance in ILC, including FOXA1 amplification, upregulation of orphan nuclear receptor ERRγ, increased WNT

signaling, and upregulation of MAPK/ERK pathway and glutamate receptor[8, 12, 23, 25]. The HER2 signaling pathway, which is routinely targeted in breast cancer, is also known to cross-talk with the hormone receptors and induce therapy resistance[2, 24, 26]. Our study set out to investigate whether targeting HER2 in endocrine resistant ILC is a viable option to overcome endocrine therapy resistance.

HER2 is an oncogene and a key biomarker in breast cancer, which when overexpressed, is an excellent therapeutic target as efficient targeting antibodies to these surface proteins have been developed over the years. Although ILC does not overexpress HER2, studies have revealed 27% of patients to harbor HER2 activating mutations, and these mutations combined with E-cadherin loss yield significantly worse prognosis[26, 31]. HER2/EGFR cross talk with ER are known to induce endocrine therapy resistance in breast cancers [31, 33, 34], **recurring cancers show increased HER2 mutations and HER2 enrichment**, **highlighting the importance of testing the efficacy of HER2 targeted therapies**[35]**.**

We used long-term estrogen deprived (LTED) ILC cells obtained from a lab at UPMC and the tamoxifen resistant ILC (TamR) cell lines generated in our lab as models of endocrine resistant ILC. These cells we generated showed significant resistance to tamoxifen and will be a unique tool to study endocrine resistant ILC both *in vitro* and *in vivo* in the future. The altered morphology and growth pattern of TamR MDA-MB-134-VI cells is interesting and could reveal major alteration in cell adhesion properties that are yet to be investigated. High-throughput gene expression analysis may reveal new pathways that mediate resistance to endocrine therapy which could be therapeutically targetable.

In our study, we utilized two different treatment strategies to target HER2. The TKIs we used, neratinib and tucatinib, differ slightly in their specificity for HER2, with neratinib being a

dual HER2/EGFR targeting molecule. One key importance of these drugs is that they are already being used in the clinic and approved by the FDA, indicating their safety for patient use. Combination therapy is an important anti-cancer strategy that can reduce drug resistance, decrease metastatic potential, and reduce tumor growth in a cost effective manner, especially when the drugs are already FDA approved[41]. In cancer therapy, a positive response to a therapy can lead to time added to overall survival, emphasizing the importance of providing physicians more tools for their patients[42]. The small, but significant decreases in cell viability seen with the combination of HER2 targeted therapies with tamoxifen may result in months added to overall survival in patients. Especially in ILC, which is currently treated no differently than IDC[1, 8], understanding how the loss of E-cadherin impacts responses to HER2 targeted therapies is critically important[43].

Our study also uncovered ACK1, which has not been previously studied as a target in ILC. ACK1, which is a non-receptor tyrosine kinase, is known to be frequently amplified or mutated in breast cancers, and its overexpression increases the invasive phenotype of cancer[37, 44]. One pathway through which ACK1 has been shown to increase the invasive phenotype of cancer is by escalating the EGFR signaling pathway[45]. Additionally, ACK1 expression is positively correlated with disease progression and negatively correlated to overall survival in breast cancer[46]. Studies have also indicated ER and estrogen binding regulates the ubiquitylation and degradation of ACK1, consequently, lack of ER increases the stability of ACK1[47]. In endocrine resistant ILC, especially in the LTED lines, where we saw decreases in ER protein levels (Figure 6), ACK1 signaling may be contributing to the poor patient outcomes. The interconnectedness of ACK1, ER, and the HER2/EGFR signaling pathway makes it an important area of study in ILC. Further studies utilizing AIM-100, a drug targeting ACK1, will yield important information on the role that ACK1 plays in ILC, and determine whether it is therapeutically targetable.

A challenge faced throughout this study revolves around the lack of cell lines available for ILC and the slow growing nature of these cells. With so few lines and high heterogeneity among breast cancers, a greater number of cell lines will provide more insight to the different pathways and mechanisms which are implicated in endocrine therapy resistance. The slow growing nature of the cancer cells makes it difficult to conduct both *in vitro* and *in vivo studies*. One potential limitation of the MTT assay in ILC cells revolves around its measurement of metabolic activity, which is inherently lower in the slow growing cells. A potential solution to address this limitation include supplementing the MTT assay with other growth measuring assays such as the colony formation assay. When planning for *in vivo* testing of HER2 targeted therapy for ILC, the difficulty of executing orthotopic tumor models becomes clear, with cell lines taking extended periods of time to form tumors[45]. While a few genetically engineered mouse models (GEMMs) for ILC have recently come to light, they do not mirror the high rate of ER positivity seen in ILC[8]. Innovations in modeling ILC in *in vivo* models will be critical to studying endocrine therapy resistance and validating drug targets.

This study has opened up many exciting new paths, in particular with novel triple combination therapy approaches with trastuzumab, TKIs and tamoxifen. In addition to further studying endocrine therapy mechanistically using our TamR cell lines, we hope to identify more drug targets in ILC. We speculate that gene-expression analysis and reverse phase protein arrays of the TamR lines will reveal upregulated pathways critical to imparting therapy resistance to ILC, which can then be targeted. Another approach we are currently studying involves lenvatinib, a multi-kinase inhibitor approved for use in thyroid cancers can be used to target RTKs on a broader scale. This study, ultimately, sets the foundation for HER2 targeted therapy to be used in

combination with antiestrogen therapy in patients with ILC, and future studies in this area will be critical to improving outcomes for patients.

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