8-2010

Understanding acquired resistance to Lapatinib in breast cancer cells

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UNDERSTANDING ACQUIRED RESISTANCE TO LAPATINIB
IN BREAST CANCER CELLS

by

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A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

August, 2010

Understanding acquired resistance to Lapatinib in breast cancer cells

Publication No._____

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Signaling through epidermal growth factor receptor (EGFR/ErbB) family members plays a very important role in regulating proliferation, development, and malignant transformation of mammary epithelial cells. ErbB family members are often over-expressed in human breast carcinomas. Lapatinib is an ErbB1 and ErbB2 tyrosine kinase inhibitor that has been shown to have anti-proliferative effects in breast and lung cancer cells. Cells treated with Lapatinib undergo G_1 phase arrest, followed by apoptosis. Lapatinib has been approved for clinical use, though patients have developed resistance to the drug, as seen previously with other EGFR inhibitors. Moreover, the therapeutic efficacy varies significantly within the patient population, and the mechanism of drug sensitivity is not fully understood. Expression levels of ErbB2 are used as a prognostic marker for Lapatinib response; however, even among breast tumor cell lines that express similar levels of ErbB2 there is marked difference in their proliferative responses to Lapatinib.

To understand the mechanisms of acquired resistance, we established a cell line SkBr3-R that is resistant to Lapatinib, from a Lapatinib-sensitive breast tumor cell line, SkBr3. We have characterized the cell lines and demonstrated that Lapatinib resistance in our system is not facilitated by receptor-level activity or by previously known mutations in the ErbB receptors. Significant changes were observed in cell proliferation,

cell migration, cell cycle and cell death between the Lapatinib resistant SkBr3-R and sensitive SkBr3 cell lines. Recent studies have suggested STAT3 is upregulated in Lapatinib resistant tumors in association with ErbB signaling. We investigated the role that STAT3 may play in Lapatinib resistance and discovered higher STAT3 activity in these resistant cells. In addition, transcriptional profiling indicated higher expression of STAT3 target genes, as well as of other genes that promote survival. The gene array data also revealed cell cycle regulators and cell adhesion/junction component genes as possible mediator of Lapatinib resistance. Altogether, this study has identified several possible mechanisms of Lapatinib resistance.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Understanding the acquired resistance of breast tumor cells to pharmacological agents remains an open and challenging area of research. This report investigates the acquired resistance from prolonged treatment of Lapatinib in a breast tumor cell line. The background provided here covers breast cancer, advances in the field, specifically in the area of targeted therapy, the drug Lapatinib and its targets, and previous studies in drug resistance. Additionally, a review of the literature on STAT3 and its link to ErbB signaling and drug resistance will be discussed. Finally, the specific aims of this project will be discussed.

BREAST CANCER

Worldwide, breast cancer is the leading cause of death from cancer in women (3). It is the second most common cause of cancer death among women in the US, following lung cancer. In 2009, the American Cancer Society estimated that 192,370 new cases of invasive breast cancer would be diagnosed among women in the United States, and over 40,170 deaths by year's end (4). Historically, incident rates increased in the 1980s and mid 1990s, afterwhich it reached a plateau, when the use of mammography screening was increased. Between 1999 and 2006 incident rates dropped 2.0% annually (4), likely due to decreased use of menopausal hormones as well as increase in mammography screening.

The breast anatomy includes the glands (lobules), the ducts (small tubes that connect the lobules to the nipple), fatty and connective tissue, blood vessels, and lymph

vessels. Milk is produced by the glands and carried to the nipple through the ducts. The connective tissue holds everything together. Breast cancer can be classified based on tissue of origin: lobular and ductal. Both type of carcinoma can be classified into two subgroup based whether cancer cells are in situ or invasive. Invasive carcinoma accounts for about 80 percent of breast cancer (5).

Breast cancer, like many other forms of cancer, is believed to be caused by both environmental and hereditary factors. Exposure to chemicals, viruses, or radiation can cause DNA damage that leads to genetic mutations (6). Another risk factor is the failure of early stage immune surveillance system (7). Abnormal growth factor signaling in the cells has also been linked to tumor expansion (2). Inherited defects in DNA repair genes, such as BRCA1, BRCA2, PTEN and TP53, can also contribute to breast cancer development (8).

ADVANCES AND THERAPIES

Over the past decades there have been many advancements in the areas of prevention, diagnosis, and treatment of breast cancer. Today, nearly 90% of patients who have been diagnosed with breast cancer will survive at least five years (9). Mastectomy alone is no longer the only accepted surgical option for treatment. Lumpectomy, followed by radiation therapy has replaced mastectomy as the preferred approach for women with early stage breast cancer (10). Mammographic screening has reduced mortality (11). It is now routinely utilized as an accepted standard for early detection. Combinational chemotherapy has become standard in the adjuvant treatment of patients in early stage of cancer. The use of chemotherapy is to help reduce metastatic tumors.

Clinical trials are currently underway using neoadjuvant chemotherapy, which is a treatment given before surgery to shrink tumor mass. Hormonal therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, and aromatase inhibitors is also standardized in the treatment of women with estrogen receptor (ER) positive breast cancer. SERMs prevents estrogen from binding to its receptor, thus preventing growth stimulation by estrogen (11). In contrast, aromatase inhibitors block estrogen production (11). Some Food and Drug Administration (FDA) approved aromatase inhibitors include anastrozole, exemestane, and letrozole. The use of hormonal therapy has been adapted for both early stage and advanced stage of cancer. Tamoxifen and another SERM, raloxifene, have been shown to prevent the development of invasive breast cancer. Hence, tamoxifen has been an effective treatment option as well as prevention strategy.

Her2 (human epidermal growth factor receptor 2), also known as ErbB2, is overexpressed in about 20% of breast cancer, and therapies targeting activity of this protein are being investigated (12-14). Monoclonal antibodies and small-molecule tyrosine kinase inhibitors are currently in clinical use for the advanced and metastatic ErbB2 positive breast cancer patients. For the next few sections of this chapter, we will focus on the role of ErbB2 protein in cancer.

ERBB RECEPTORS

ErbB2 is a member of the epidermal growth factor receptor family of tyrosine kinases [ErbB1/EGFR, ErbB2/Her2/Neu, ErbB3, and ErbB4] that regulate cell growth, survival, migration, adhesion, proliferation and differentiation (15-17). Members of this family have an extracellular ligand binding region, single membrane spanning region, and a cytoplasmic tyrosine kinase containing domain. The epidermal growth factor (EGF) family of growth factors serve as ligands for the ErbB receptors and is divided into three groups based on the receptors they bind. The first group includes EGF, transforming growth factor (TGFα), amphiregulin (AR), and epigen (EPG); this group binds to EGFR. The second group binds to EGFR and ErbB4, and includes beta-cellulin (BTC), heparin-binding EGF (HB-EGF) , and epiregulin (EPR). The third group is further divided into two subgroups of neuregulins (NRGs): 1) NRG1 and NRG2 which are specific for ErbB3 and ErbB4, and 2) NRG3 and NRG4 which are specific for ErbB4 only (18).

Following ligand binding, an ErbB receptor homodimerizes or heterodimerizes with another ErbB family member, followed by autophosphorylation of specific tyrosine residues in the intrinsic kinase domain (2). See Figure 1. The known combinations of dimerization are shown in Figure 1A where ErbB1, ErbB3, and ErbB4 can all form homodimers or heterodimers with ErbB2. ErbB2 lacks the extracellular binding domain and therefore does not bind to a ligand; it can however dimerize and activate itself and is a preferred partner for heterodimerization with the other ErbB receptors (Hynes and Lane 2005).

The phosphorylation sites serve as docking sites for recruitment of proteins that lead to activation of intracellular signaling pathways. Several different signaling pathways may be activated depending on which specific receptor residues phosphorylated and the proteins recruited, as shown in Figure 1B. For example, the mitogen-activated protein kinase (MAPK) cascade that affect growth, differentiation and apoptosis is activated by recruitment of growth-factor-receptor-bound protein 2 (GRB2) and Src homology 2 domain-containing (SHC) protein to the receptor. The recruitment of GRB2 is dependent on the phosphorylation on EGFR at residues 1068/1086 and 1173 and on ErbB2 at residues 1139 while SHC recruitment depends on ErbB2 phosphorylation at 121/122. Another important pathway, the phosphatidylinositol 3-kinase (PI3K)−AKT, which mediates anti-apoptotic activities, is stimulated through recruitment of the p85 adaptor subunit of PI3K to the ErbB3 receptor (2). Other pathways, such as those containing effectors like SRC tyrosine kinase, mammalian target of rapamycin (mTOR), are also activated as a result of EGFR activation (2). Alternative signaling cascades, such as those of the signal transducer and activator of transcription (STAT) family, leads to activation of transcription factors; for example STAT3, which translocates to the nucleus and activate pro-survival factors (2).

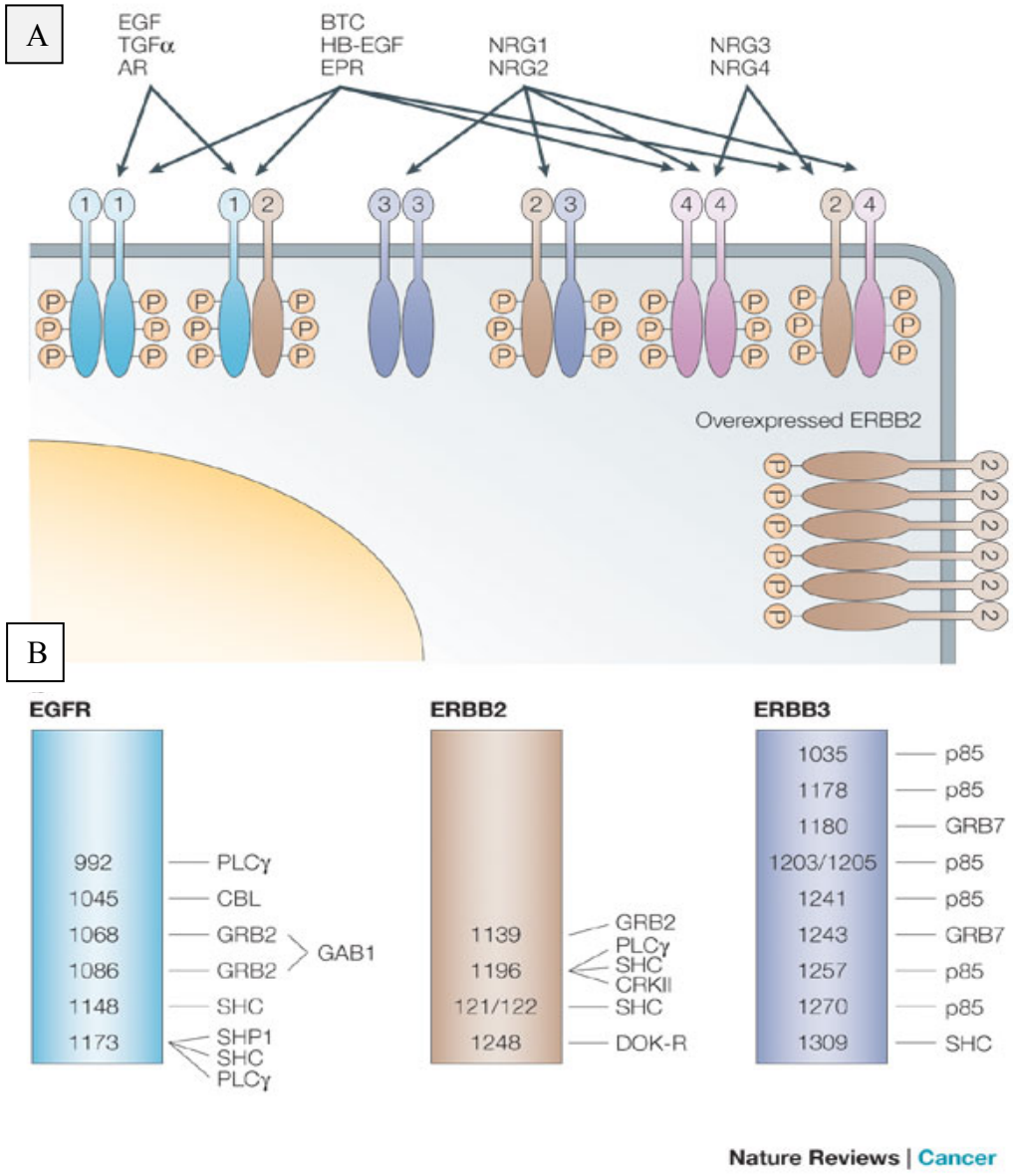

Figure 1. Ligands that bind known dimerizations and phosphorylation sites of the ErbB family of receptors.
A) Different homo and hetero dimers that can be formed by the ErbB receptors, along with ligands that can bind and activate the corresponding dimers. B) The phosphorylation sites in the kinase domain of the ErbB receptors and the intracellular signaling proteins that are recruited to the corresponding sites. Reprinted by permission from Macmillan Publishers Ltd: Nature Review Cancer (2), copyright 2005.

ERBB1 AND ERBB2 IN CANCER

Many studies have shown that EGFR and ErbB2 have been associated with a variety of characteristics of cancer, such as increased proliferation, decreased apoptosis, metastasis, and resistance to chemotherapy and radiotherapy (19, 20). In addition, EGFR and ErbB2 have been reported to be amplified, overexpressed, or mutated in numerous solid tumor types, including 20-30% of breast cancers where they are linked to aggressive cancer category and poor patient outcome (12-14). For example, in gliomas EGFR amplification was found concomitantly with mutations in the extracellular domain of type 3 variant of EGFR (21). Furthermore, many EGF-related growth factors are produced either by the tumor cells themselves or the surrounding stomal cells, causing constitutive activation of EGFR (22, 23).

The ErbB2 gene has also been reported to be amplified in human breast cancer (24), and its overexpression by transfection methods results in transformation of normal human fibroblasts (25). In nude mice, ErbB2 amplified breast cancer cells exhibit higher rates of DNA synthesis, proliferation, invasion and metastatic potential (26, 27). It has been demonstrated that ErbB2 transgenic mice develop breast cancer (28). ErbB2 overexpression has shown to increase the outgrowth of metastatic tumor cells in the brain.

ERBB TARGETED THERAPEUTICS

The established role of EGFR and ErbB2 in promoting growth and survival of various tumor types make them attractive therapeutic targets. Drugs that are in clinical use or advanced pre-clincal studies comprise of monoclonal antibodies and tyrosine kinase inhibitors (TKIs) (1). Several companies have developed monoclonal antibodies targeting the extracellular domains of these receptors and preventing activation. Other drugs include the TKIs that enter the cells and target the ATP binding sites, thus preventing receptors from phosphorylating target proteins. See Figure 2 for illustration of their mechanisms. Table 1 below shows current drugs used to target EGFR or ErbB2, or both.

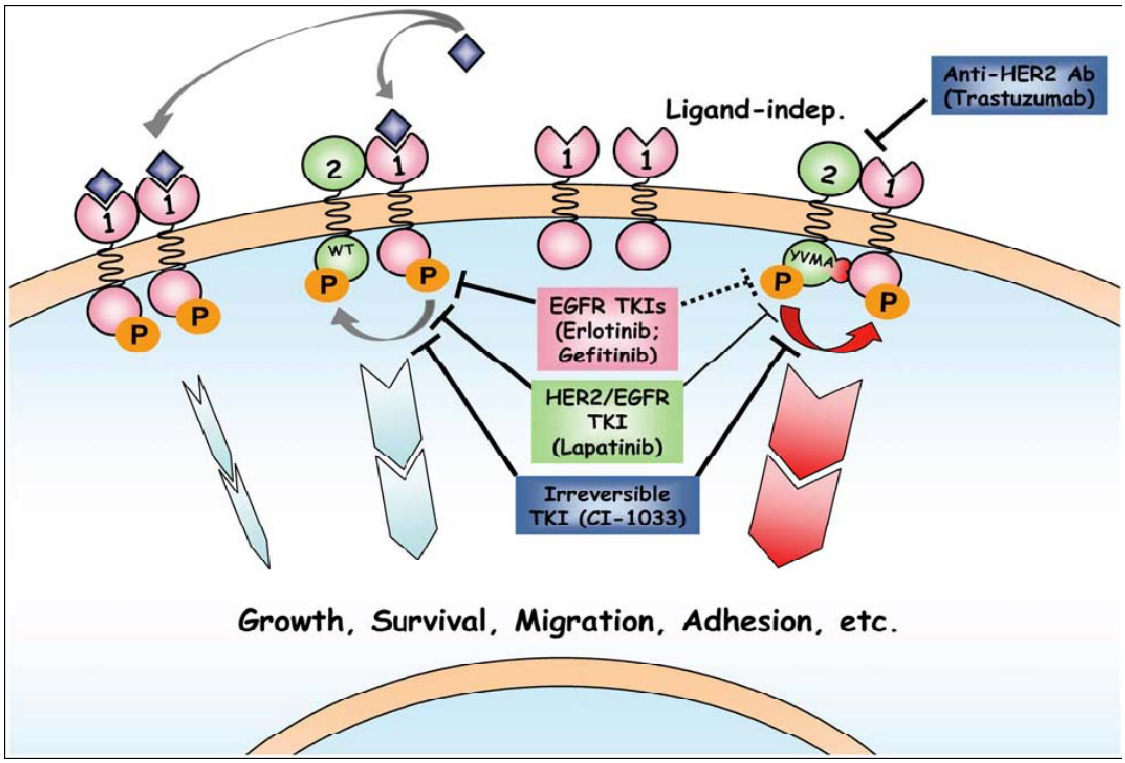

Figure 2. Current anti-EGFR drugs and the sites at which they target the receptor. Reprinted by permission from Elsevier: Cancer Cell (1), copyright 2006.

Table 1: Current ErbB targeting drugs.

Source: (2)

Monoclonal Antibodies	Target	Tyrosine Kinase Inhibitors	Target
Trastuzumab (Herceptin)	ErbB2	Gefitinib (Iressa)	EGFR
Pertuzumab (Omnitarg)	ErbB2	Erlotinib (Tarceva)	EGFR
Cetuximab (Erbitux)	EGFR	Lapatinib	EGFR /ErbB2
Matuzumab	EGFR	EKB-569	EGFR /ErbB2
Panitumumab	EGFR	AAE788	EGFR/ErbB2/VEGF
		CI-1033	EGFR /ErbB2

The drug trastuzumab (Herceptin) by Genetech has been the focus of many studies. Trastuzumab is a monoclonal antibody that binds to the extracellular domain of ErbB2. FDA approved the use of trastuzumab in clinics in 1998. Several publications have shown that in conjunction with adjuvant chemotherapy, trastuzumab lowers the risk of recurrence in ErbB2 positive breast cancer patients, compared to chemotherapy alone (29-31) and has significant effect on patient survivorship (32). However, trastuzumab, like the rest of the monoclonal antibody drugs, is controversial because of its cost in production.

The introduction of the tyrosine kinase inhibitors as therapeutics has been more recent. These drugs can target either EGFR or both EGFR and ErbB2 receptors. Among these inhibitors, gefitinib and erlotinib both target only one receptor, EGFR, while newer FDA approved drugs, such as Lapatinib target both EGFR and ErbB2. Increased expression of EGFR and ErbB2 occurs in about 30% of breast cancers and since these two receptors are heterodimer partners, strategies in which the use of drugs like Lapatinib or combination of drugs are being considered for clinical trials.

Several studies have shown that targeting both EGFR and ErbB2 may have synergistic effects on proliferation for the BT474 and SkBr3 breast tumor cell lines (33). Our work focuses on Lapatinib because it targets both EGFR and ErbB2 that are implicated in cancer. Among the receptor tyrosine kinase inhibitors, it has been shown to be most specific to these two receptors, which is important in our study of acquired resistance.

LAPATINIB

Lapatinib is an orally active small molecule tyrosine kinase inhibitor developed by GlaxoSmithKline. This compound is a potent ATP-competitive inhibitor that targets EGFR and ErbB2. In cell free biochemical kinase assays it has been shown to inhibit EGFR and ErbB2 tyrosine kinases by 50% (IC_{50}) at concentrations of 10.8 and 9.3 nM, respectively (Rusnak, Lackey et al. 2001). In a study where the binding affinity of 20 kinase inhibitors for 100 different kinases were screened, Lapatinib was found to be the most specific because it bound EGFR and ErbB2 almost exclusively (34). Compared to other ErbB receptor tyrosine kinase inhibitors, Lapatinib has slower dissociation from receptor, resulting in prolonged effect on receptor downregulation (35). In vitro, it has been shown that Lapatinib blocks EGFR and ErbB2 phosphorylation and decreases phosphorylation of downstream MAPK and Akt (36). Lapatinib has shown to have anti-proliferative effects on breast and lung cancer cells (37, 38). In cell lines across multiple tumor types, it has been observed that Lapatinib-treated cancer cells undergo apoptosis or G_1 cycle arrest (39, 40).

In phase I clinical studies, Lapatinib was tolerated up to 1800 mg once daily in breast cancer patients, with side effects of diarrhea, nausea, rash, fatigue, anorexia, and vomiting. Clinical activity was observed at a minimum of 650 mg/day (41). Pharmacokinetic data from these studies showed serum level of Lapatinib peaked 4 hours after dosing, accumulate two fold with daily dosing, with steady state achieved in 7 days. A phase II trial showed that Lapatinib was effective in approximately 20% of patients with ErbB2-positive metastatic breast cancer who had not received first-line chemotherapy (42). In a phase III trial, it was demonstrated that women with ErbB2-positive metastatic breast cancer benefit from Lapatinib, whereas ErbB2-negative breast cancer did not (43). In 2007, FDA approved Lapatinib for use in combination with capecitabine for patients (previously treated with anthracycline, taxane, or trastuzumab) who have metastatic breast cancer that overexpresses ErbB2 (44), after several phase III trials that demonstrated the synergistic effect compared to either alone (45-47).

Lapatinib offers improvements over trastuzumab. Aside from its specificity to EGFR and ErbB2, Lapatinib induces apoptosis in trastuzumab-resistant breast SkBr3 cancer cells (48). In 2009, Scaltriti et. al showed that Lapatinib enhances the effects of trastuzumab in MCF7 and SkBr3 breast cancer cell lines (49). Additionally, Lapatinib's anti-tumor activity was observed in Japanese patients with ErbB2-positive metastatic breast cancer that relapsed after trastuzumab-based therapy (50). Furthermore, several studies demonstrated synergistic effects for Lapatinib in combination with trastuzumab in xenograft tumor reduction (36, 51).

Therapeutic efficacy of Lapatinib in patient populations is limited by both primary and acquired resistance. Multiple phase II trials have revealed that only 20% to 35% of patients with ErbB2-positive metastatic breast cancer respond to Lapatinib (42, 52). Similar to trastuzumab, the medium duration of response to Lapatinib is less than one year (52, 53). Thus, Lapatinib resistance is a vital issue, especially considering ErbB2 is used as a biomarker to initiate Lapatinib treatment in patients. However, the mechanisms of drug sensitivity and acquired resistance are not fully understood at this time.

In an in vitro model, it was discovered that Lapatinib resistance in BT474 breast tumor cells was mediated in part by estrogen receptor (ER) and progesterone receptor (PR) signaling upregulations in response to Lapatinib, with evidence in increased activity in FOXO3a and caveolin-1, as well as Bcl-2 anti-apoptotic protein (38). Furthermore, ErbB2+/ER+ tumor biopsies after 14 days of Lapatinib treatment also reflect increased expression of FOXO3a, PR, and Bcl-2. Consequently combinational treatment with tamoxifen demonstrated resistance prevention, suggesting such therapeutic approach is appropriate for ErbB2+/ER+ patients (38).

Within the past decade, many studies have investigated EGFR/ErbB2 tyrosine kinase inhibitors and the development of subsequent resistance following treatment in lung and breast cancer patients. The major contributing factor was identified as mutations in the kinase domain of EGFR and/or ErbB2. Recent studies by Tam et. al identified mutations in EGFR which confer different degree of sensitivities to gefitinib in lung adenocarcinomas (54). Earlier work by Wang and collaborators discovered that

lung cancer NCI-1781 cells with mutated ErbB2 are insensitive to EGFR inhibitors while remain sensitive to ErbB2 inhibitors (1). These studies confirmed that mutations in EGFR and ErbB receptors may confer anti-ErbB drug resistance. However, there have been several studies in lung cancer patients harboring specific mutations in EGFR which have been linked to increased sensitivity to tyrosine kinase inhibitors, such as gefitinib and erlotinib, compared to patients that express wild type EGFR (55-57). Some groups proposed the activation of alternate pathways when EGFR and ErbB2 are inhibited as the sources of resistance. In 2007, Engelman et. al discovered that hepatocyte growth factor receptor (MET) amplification leads to gefitinib resistance in lung cancer cells by activating ErbB3 leading to increased Akt signaling (58). Besides the ErbB receptors, other molecules have been implicated in ErbB targeted drug resistance. Activated Src and Ras were also implicated as causes of gefitinib resistance by activating either or both Akt and MAPK signaling pathways in human gallbladder adenocarcinoma cells (59). Another study by Martin et al. reported that in the HCT116 colorectal carcinoma cell line, Lapatinib resistance was mediated by elevated induced myeloid leukemia cell differentiation protein (MCL-1) and decreased Bcl-2 homologous antagonist/killer (BAK) activation, and not by an ErbB mutation (60).

STAT3

Recent studies have suggested a role for signal transducer and activator of transcription 3 (STAT3) in anti-ErbB resistance. In 2005, Greulich et al. observed that cell lines harboring EGFR mutations have increased levels of phosphorylated STAT3 which correlated with gefitinib sensitivity (61, 62). Interleukin-6 (IL-6) and STAT3

signaling were also linked to cetuximab and radiation resistance in pharyngeal cancer (63). Thus, we hypothesize that STAT3 may play a role in the Lapatinib acquired resistance.

STAT3 is one of seven members of the STAT family: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. The proteins of this family have two roles: 1) transduce signal through cytoplasm and 2) initiate transcription of genes involved in cell cycle, apoptosis, proliferation, survival, differentiation and development (64-66). STATs were originally discovered as mediators of signaling from IL-6 and IFN receptors following ligand binding (67, 68). Each STAT family member responds to specific cytokines, and each regulates a specific set of genes. Following receptor activation, the Janus kinase (JAK) family kinases (JAK1, JAK2, JAK3, JYK2) phosphorylates STAT proteins. In response, STATs homodimerize or heterodimerize with other STAT members via phosphotyrosine Src homology 2 (SH2) domain interaction. The dimers then translocate to the nucleus where they function as transcription factors for target genes, many of which encode for cytokines and growth factors, thus providing a mechanism for autocrine and paracine STAT activation (69, 70).

Like other STATs, STAT3 is activated by tyrosine phosphorylation in response to stimulation by cytokines and growth factors. Its activation is specifically mediated by IL-6 cytokine family members, oncostatin M (OSM), and leukemia inhibitory factor, and by growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factors (VEGF) and EGF (71-74). STAT3 is phosphorylated at tyrosine residue 705 and at serine residue 727, which results in maximal activation of STAT3's transcriptional activity (75). In addition to JAK family

members, STAT3 is tyrosine phosphorylated by two other types of kinases: 1) receptor tyrosine kinases such as EGFR, FGFR, or PDGFR, and 2) nonreceptor-associated tyrosine kinases like Src, Ret, or Bcl-Abl protein (76).

Following tyrosine phosphorylation, STAT3 either homodimerizes or heterodimerizes with STAT1, then translocates to the nucleus to begin transcription of numerous genes, including survivin, bcl-2, bcl-xL, mcl-1, p21, c-Myc, VEGF, and cyclin D1 (74, 77-80). STAT3 is regulated by several different mechanisms. Suppressors of cytokine signaling (SOCS) proteins attenuate STAT3 activity by inhibiting upstream JAK activation (81). Other mechanisms include protein inhibitors of activated STATs (PIAS) proteins and protein tyrosine phosphatases that target STAT3 directly (81-83).

Studies have implicated STAT3 in oncogenesis, promoting abnormal apoptosis, cell cycle progression, angiogenesis, and tissue invasion (64). Bromberg et al. in 2002 demonstrated that STAT3 is required for many cancer cell lines to maintain a transformed phenotype (84). Numerous studies have shown that STAT3 is constitutively activated in a variety of cancer types including: breast (85), prostate (86), leukemia (87), lung (88), thyroid (89), and head and neck squamous cell carcinoma (90). Consequent studies show that small molecule tyrosine inhibitors targeting STAT3 activation result in growth suppression and apoptosis (87, 91, 92). Similar effects were observed using dominant-negative STAT3 and antisense oligonucleotides (92-94).

HYPOTHESIS

We wanted to show that for a particular breast cancer cell line that acquired resistance to Lapatinib is mediated by alterations in the gene regulatory and signaling networks. Specifically in this project, we aimed to characterize the resistant cell line and

identify changes in the ErbB signaling proteins and gene expressions that may be involved in Lapatinib resistance. This study consisted of three specific aims. Aim 1 was to develop and characterize a Lapatinib resistant cell line derived from SkBr3, a Lapatinib-sensitive breast cancer cell line. Aim 2 was to identify ErbB signaling network changes in the developed resistant cell line and to compare it to the parental line. Aim 3 was to determine gene expression changes in the resistant cell line in response to Lapatinib. The details of the study for Aim 1, 2, and 3 will be discussed in chapters 2, 3, and 4, respectively.

CHAPTER 2: DEVELOPMENT AND CHARACTERIZATION OF RESISTANT CELLS

INTRODUCTION

To understand how breast cancers may acquire resistance to Lapatinib, we developed a Lapatinib-resistant cell line. Studies performed by Xia et. al described the establishment of a Lapatinib-resistant clonal cell lines using the breast cancer BT474 cell line (38). We adapted their protocol to develop a Lapatinib-resistant SkBr3 breast cancer cell line, named SkBr3-R. The parental SkBr3 cell line, like BT474, has a GI_{50} value of 0.03 μM and is among the most sensitive cell lines to Lapatinib, as shown in Figure 3 (51, 95). It is interesting, though not surprising that the most sensitive cell lines all overexpress ErbB2.

In this chapter, the protocol used in establishing the SkBr3-R cell line will be discussed first. The remaining sections of the chapter will report our findings in the characterization of this Lapatinib resistant cell line, including cell viability assays that verify the increased in Lapatinib resistance.

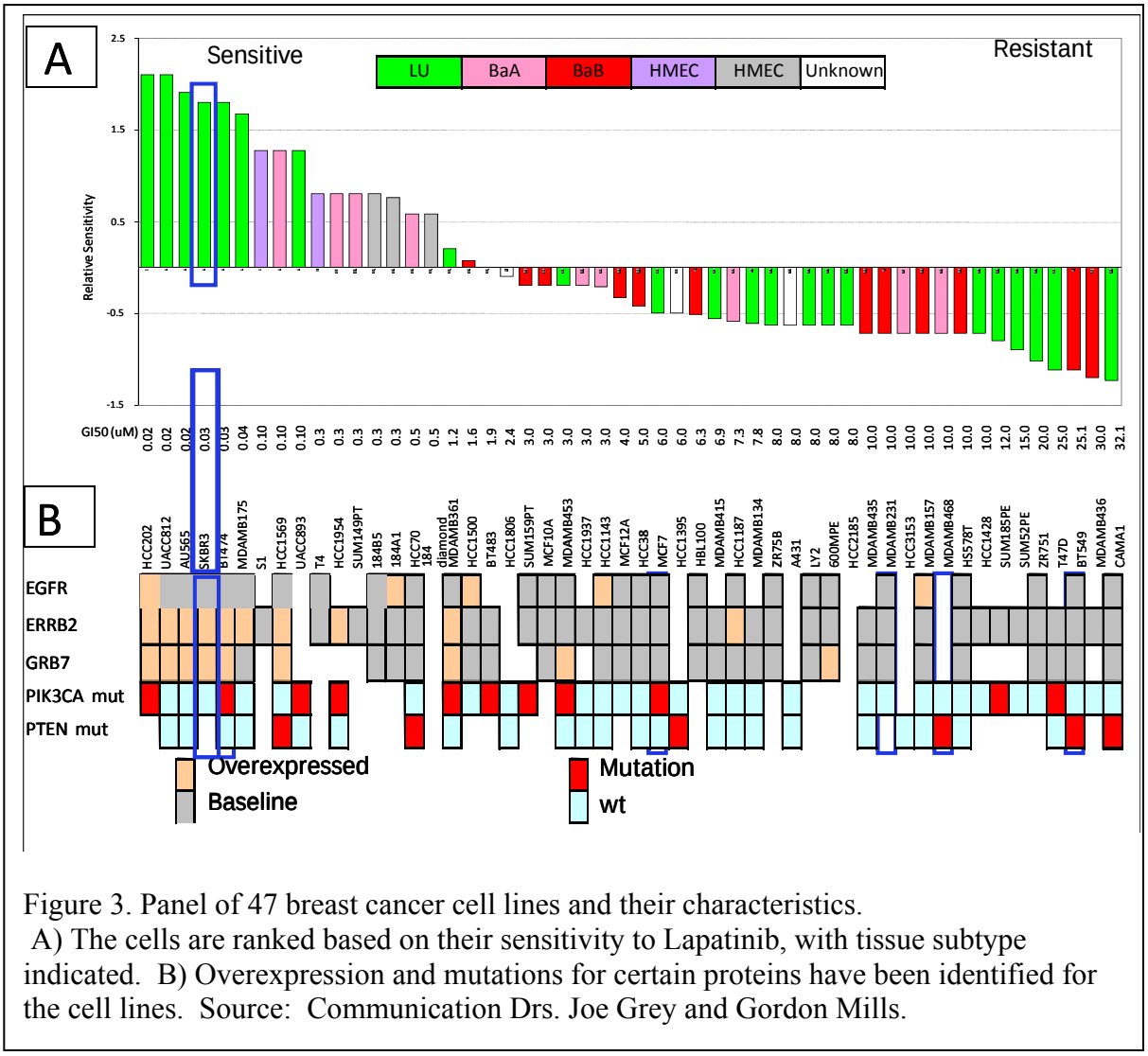

Figure 3. Panel of 47 breast cancer cell lines and their characteristics.
A) The cells are ranked based on their sensitivity to Lapatinib, with tissue subtype indicated. B) Overexpression and mutations for certain proteins have been identified for the cell lines. Source: Communication Drs. Joe Grey and Gordon Mills.

ESTABLISHMENT OF RESISTANT CELLS

Initially, the SkBr3 cells were grown in Lapatinib at concentration 0.01 μM, well below GI_{50} value of 0.03 μM. We adopted a set of rules that allowed us to increase Lapatinib concentration while maintain viable cells. First, following each passage (when confluency hit 90%), cells were allowed to attach overnight, after which Lapatinib was added. Second, Lapatinib was removed from the media for the remaining time of that

passage whenever confluency stalled for more than two weeks. Third, the media were refreshed every three to six days, depending on the confluency. Our prior protocol called for Lapatinib-containing media to be refreshed every day; however, that resulted in non-viable plates after two to three weeks. This modification of changing from continuous exposure to periodic acute exposure of Lapatinib allowed the concentration-increasing process to continue past the one month time point. Fourth, we increased Lapatinib concentration only after a minimum of four successful passages. Fifth, regarding the pace of concentration increase, we doubled the concentration until 0.2 μM was reached, at which point we increased at increments of 0.2 μM. After 12 months, the Lapatinib-insensitive SkBr3-R reached 1.5 μM and could not tolerate higher concentrations. We attempted single-cell cloning but were unsuccessful. Two techniques were tested: cloning cylinder isolation of cells and 96-well serial dilution cell isolation. Both resulted in non-viable wells of cells after three weeks. Subsequently, using the pooled SkBr3-R cells we performed Lapatinib dose response assays to determine the cells' GI_{50} value.

Cell viability assays confirmed a right shift in the dose response curve for the resistant cell line, which we named SkBr3-R, with GI_{50} value at 2.6 μM, over 100 fold decrease in sensitivity compared to the parental cell line. See Figure 4.

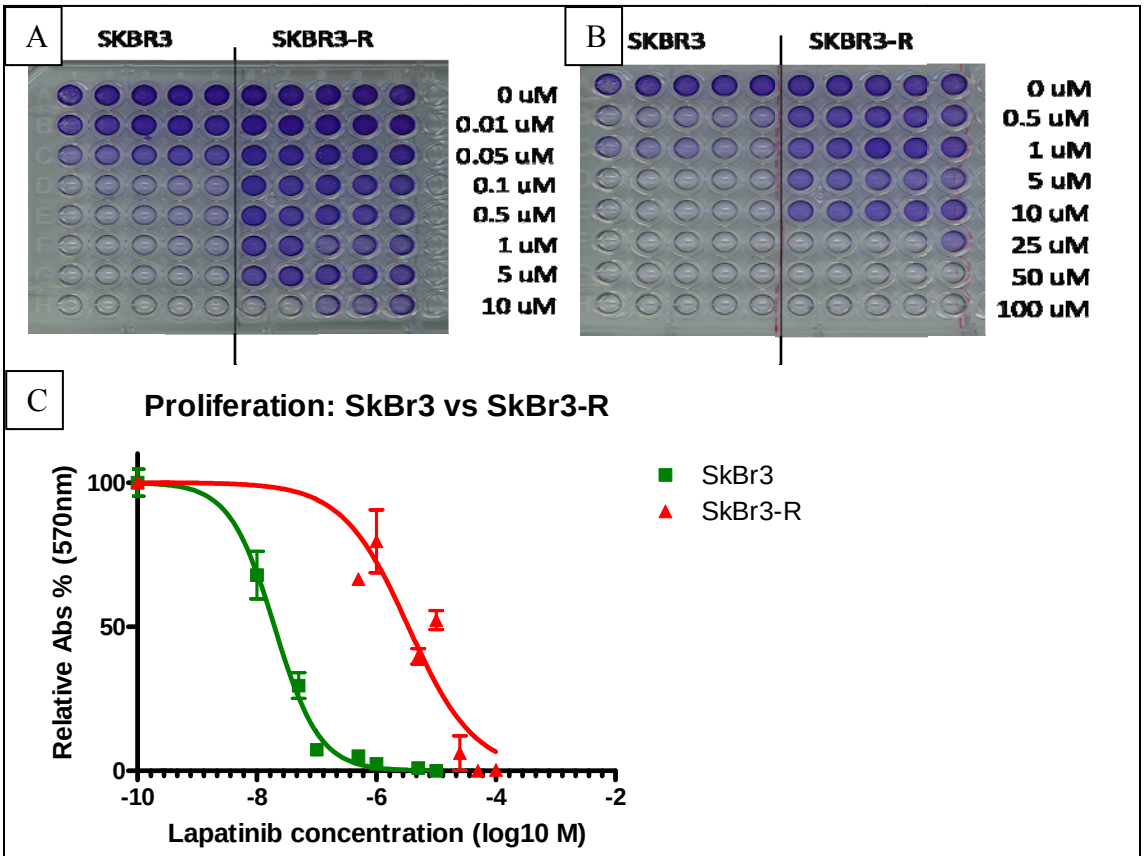

Figure 4. Crystal violet cell viability assays.
A) 96 well plate showing the crystal violet cell viability assay of parental SkBr3 and Lapatinib resistant SkBr3-R cells four days after treatment with different concentrations of Lapatinib as indicated on the figure. B) Dose response curves showing a right shift in the dose response curve for the resistant cells, and calculating the GI50 values shows the parental cells have a GI50 of 0.025 µM while the resistant SkBr3-R cells have a GI50 of 2.6 µM, exhibiting greater than 100X insensitivity to Lapatinib than the parental SkBr3 cells.

MATERIALS AND METHODS

Cell lines and reagents

SkBr3 breast cancer cell line was obtained from UT M.D. Anderson Cancer Center Characterized Cell Line Core Services. Both SkBr3 and SkBr3-R cell lines were characterized by Core Services to be related and known mutations verified. Cells were routinely maintained in RPMI 1640 (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS)(Sigma-Aldrich, St. Louis, Missouri) and penicillin/streptomycin (Mediatech, Manassas, Virginia). Other cell culture supplies include trypsin-EDTA (Invitrogen, Carlsbad, California), cloning discs (Fisher Scientific, Waltham, Massachusetts), phosphate buffered saline (PBS) (Mediatech, Manassas, Virginia). Lapatinib (LC Laboratories, Woburn, Massachusetts) was dissolved in dimethyl sulfoxide (DMSO) from EMD. Triton X-100 (EMD, Gibbstown, New Jersey), RNase A(Fisher Scientific, Waltham, Massachusetts), propidium iodide (MP Biomedicals, Solon, Ohio), and ethanol (Pharmaco-Aaper, Brookfield, Connecticut) were used to fix and stain cells in cell cycle analyses. Crystal violet (Sigma-Aldrich, St. Louis, Missouri), sodium citrate (Fisher Scientific, Waltham, Massachusetts), methanol (Ricca Chemical Co, Arlington, Texas) were used in crystal violet cell viability assays. Bovine serum albumin (BSA)(Fisher Scientific, Waltham, Massachusetts) was used for western blots. For DNA gel electrophoresis, we used ethidium bromide (EMD, Gibbstown, New Jersey) and agarose (EMD, Gibbstown, New Jersey).

Antibodies

The following antibodies were used for immunoblotting: anti-phospho-EGFR (Y1068); anti-phospho-EGFR(Y992); anti-phospho-EGFR (Y1045); anti-phospho-EGFR

(Y845); anti-phospho-HER3/ErbB3 (Y1289); anti-phospho-HER2/ErbB2 (Y1248); anti-EGFR; anti-ErbB2; anti-ErbB3; anti-ErbB4; (Cell Signaling Technology, Boston, Massachusetts); and anti-β-Actin (Sigma-Aldrich, St. Louis, Missouri).

Cell viability assay

Cells were seeded (5 x10^3 per well) in 96-well plates. Next day, cells were treated with Lapatinib for 8 concentrations (0, 0.01, 0.05, 0.10, 0.5, 1.0, 5.0, 10.0, 25.0 μM) and for another 8 concentrations (0, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 μM). At the 0, 48, 96, 120 hour time points, plates were collected for reading. Volume of 50μL of crystal violet solution (0.5% crystal violet w/v, 20% methanol v/v) was added to each well to allow staining for 10 minutes, followed by gentle rinse with water to remove excess stain. Once dried, the wells were filled with 100μL of sorensins buffer (0.1M sodium citrate [pH4.2], 50% v/v ethanol) to redissolve crystal. After one hour with the crystal violet uniformly dissolved, cell viability was determined by measuring the absorbance at 570nm using a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, California). Each sample was measured in quintuplicate.

SDS-PAGE and immunoblotting

Assays were performed as previously described (96). SkBr3 and SkBr3-R cells were seeded in 6-well plates (500,000 cells per well) in triplicates. The following day, cells were treated with Lapatinib at 1 μM. Controls were DMSO used in equal volumes. Cells were lysed by incubation on ice for 15 minutes in a sample lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EGTA, 10 mM sodium prophosphate, pH 7.4, 100 nM NaF, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100 plus protease inhibitors; aprotinin, bestatin, leupeptin, E-64, and pepstatin A). Cell lysates were centrifuged at 15,000 g for

20 minutes at 4°C. The supernatant was frozen and stored at −20°C. Protein concentrations were determined using a protein-assay system (BCA, Bio-Rad, Hercules, California), with BSA as a standard. For immunoblotting, proteins (25 μg) were separated by SDS-PAGE and transferred to Hybond-C membrane (GE Healthcare, Piscataway, New Jersey). Blots were blocked with 3% BSA TBS-T for 60 minutes and incubated with primary antibodies overnight, followed by goat anti-mouse IgG-HRP (1[ratio]30,000; Cell Signaling Technology, Boston, Massachusetts) or goat anti-rabbit IgG-HRP (1[ratio]10,000; Cell Signaling Technology) for 1 hour. Secondary antibodies were detected by enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, New Jersey). Quantification of bands were performed by ImageJ (National Institutes of Health).

Polymerase chain reaction

DNA templates were isolated from cells using FlexiGene DNA Isolation kit (QIAGEN). Primers were designed using Primer3 Online (See Appendix A for primer sequences). PCR master mix consisted of the following μL amount per sample: 10x Hif buffer 2.5, dNTPs 1.5, forward primer (0.05μg/μL) 1, reverse primer (0.05μg/μL) 1, Taq Hif 0.2, MgSO4 0.5, H2O 8.3, DNA template (1μg) 10. Total volume per PCR reaction was 25μL. PCR reactions were carried out in Bio-Rad MyCycler thermal cycler (SN#580BR). Two sets of PCR programs were used: 1) [94C, 2' (94C, 30"; 54C, 30"; 68", 1') $_{35\ cycles}$, 68C, 10'; hold 4C] and 2) [94C, 2' (94C, 30"; 60C, 30"; 68", 1') $_{35\ cycles}$, 68C, 10'; hold 4C].

Sequencing

PCR products were visualized using ethidium bromide on a 1% agarose gel and separated based on product size using electrophoresis. Desired DNA fragments were extracted from PCR products using QIAquick PCR Purification Kit (QIAGEN). DNA samples were submitted to UT M.D. Anderson Cancer Center DNA Core Services for sequencing.

Wound healing assay

SkBr3 and SkBr3R cell lines were plated to confluency in 6-well plates and treated with Lapatinib (1 μM) or DMSO for 24 hours prior to scratches with a sterile P200 pipette tip (SureOne: Fisher Scientific, Pittsburg, PA). The scratch was photographed with an inverted microscope (Eclipse TE2000E: Nikon, Melville, NY) over a 3 days period after medium was refreshed to observe any healing migration.

Matrigel invasion assay

BD BioCoat matrigel Invasion Chamber (BD Biosciences) was utilized. Following rehydration of Matrigel inserts and control inserts, cells were seeded ($5x10^4$ cells/mL) in a 24-well chamber plate in triplicates per condition. FBS was used as chemoattractant, Lapatinib was used at 1 μM. Inserts were transferred to wells containing FBS and cells were placed on inserts. The chamber plate was incubated for 22 hours in a humidified tissue culture incubator, at 37C, 5% CO_2 atmosphere. After staining, cells were counted per Matrigel. Analysis was performed by calculating % invasion using ratio of cells invading through Matrigel insert to cells migrating through control insert. Invasion Index = % Invasion Test Cell / % Invasion Control Cell.

Cell cycle analysis

Cells were seeded into 6-well plates in triplicates, with consideration of the 50-hour doubling rate, growth retardation by Lapatinib, and harvesting at confluency of 70-90%. Harvesting time points were day 0 (when 1 μM Lapatinib was added), day 2, day 4, and day 6. At each harvest, cells were trypsinized for 2-5 minutes, resuspended in medium, followed by centrifugation for 6 minutes at 200g at room temperature. After two centrifugations to remove supernatant using media containing serum, cells were counted and single-cell-resuspended in PBS ($1x10^6$ to 10^7 cells in 0.5mL). Cells mixture was added to 4.5mL of 70% ethanol in 12x75mm centrifuge tubes for fixation and kept in 4C for storage (2 hours minimum). After all time points collected, the samples were resuspended in 5mL PBS, centrifuged after which supernatant was decanted. The cells were incubated at room temperature for 30 minutes with 1mL propidium iodide staining solution (0.1%(v/v) Triton X-100 in PBS, 0.2mg/mL RNase A, 0.02mg/mL propidium iodide). Cell fluorescence was measured by flow cytometry. For each sample, 20000 cells were scanned. Analyses of data were done with DNA content histogram deconvolution software Cell Quest Pro.

Annexin V apoptosis assay

Cells were seeded into 6-well plates (500,000 per well) in triplicates. Following day, cells were treated with Lapatinib (1 μM). Controls were DMSO used in equal volume. At the 24 and 48 hour time points, cells were washed by cold PBS, trypsinized, and resuspended in 1X binding buffer (10X Binding Buffer: 0.1 M Hepes, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. 100μL of cell mixture was added to 5mL culture tube where it was stained with 2μL Annexin V-FITC solution (BD

Biosciences). After gentle mix and incubation for 15 min at room temperature in the dark, 400μL of 1X binding buffer was added to each tube. Addition of propidium iodide staining solution was followed immediately by analysis by flow cytometry, performed at the UT M.D. Anderson FACS Services department. Controls included unstained cells, Annexin V-FITC stained and propidium iodide (to account for sub G_1 cells) stained samples.

Statistical analysis

Microsoft Excel and GraphPad prism software (GraphPad Software) were utilized in the analyses of cell viability assays and generation of bar graphs and boxplots.

RESULTS

No differences in ErbB receptor levels or phosphorylations between sensitive and resistant cells

To assess whether change in sensitivity to Lapatinib is characterized by changes at the receptor level, we performed immunoblotting using antibodies against total EGFR and ErbB2 and found no significant differences between the two cell lines in their ErbB receptor protein expressions, with or without Lapatinib treatment (48 hours). See Figure 5. Concentration of Lapatinib used was 1 μM because it was the concentration at which resistance cells were maintained; above GI_{50} value of sensitive but below that of resistant cells. It has been previously shown that Lapatinib-resistant BT474 cells do not differ from its parental Lapatinib-sensitive cells in ErbB receptor expression (38). Thus, we find our result similar to that of Xia's group. We also examined the effect of Lapatinib on the phosphorylation of the ErbB receptors, after 48 hours of treatment. Again, similar

to Xia's reported results, Lapatinib decreased phosphorylated EGFR and ErbB2 in both the sensitive and resistant cells.

It was previously demonstrated in BT474 and SkBr3 cells that gefitinib (another anti-ErbB TKI drug) decreases both EGFR and ErbB2 phosphorylations. In another study, gefitinib was shown to initially inhibit the phosphorylation of ErbB3 but loses its suppression after 48 hours (97). In our cell lines we did not observe differential changes in EGFR or ErbB2 phosphorylation upon exposure to Lapatinib, that is, Lapatinib similarly inhibits receptor activity for both cell lines. Based on the data, we hypothesized that ErbB receptors do not contribute to Lapatinib resistance.

Since mutations in the ErbB receptors have been found in previous cases of other tyrosine kinase inhibitors' resistances, we sequenced exons (exons 18, 19, 20, 21, 22, and 25 in EGFR, exons 19, 20, 21, 22 in ErbB2, and exons 20 in ErbB3) in the ErbB receptors where mutations have been reported in breast and lung cancer (98-100). In our analyses, both cell lines did not have any mutations within these exons. Unless mutations occurred in the complement set of exons for these receptors, the mechanisms of resistance may lie downstream.

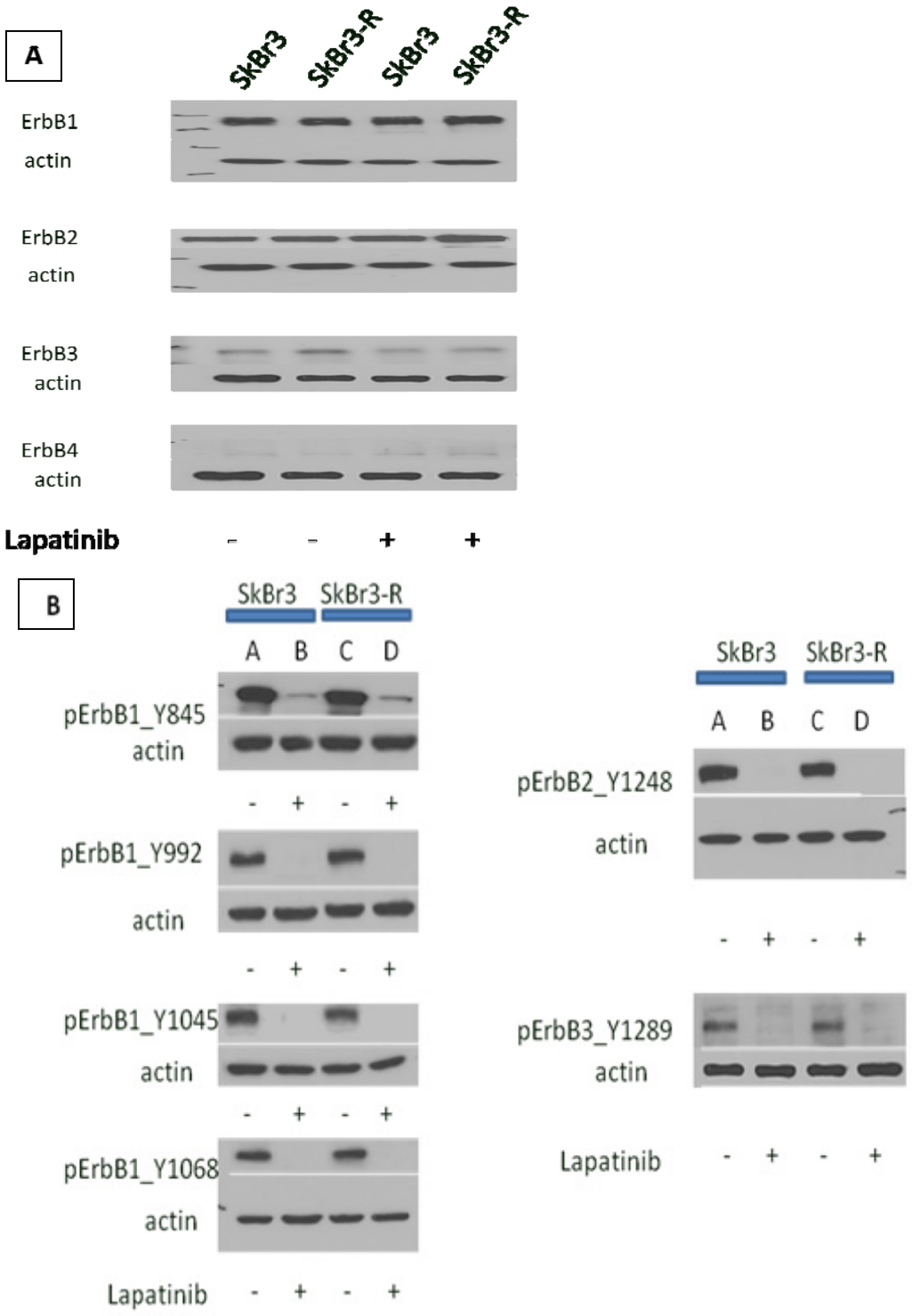

Figure 5. Western blots of ErbB receptors in sensitive and resistant cells.
A) Total proteins for ErbB1/2/3/4 were similar for both cell lines, with/without 1 μM Lapatinib after 48 hours. B) Western blot analysis using phospho specific antibodies shows no difference in the basal phosphorylation levels of the receptors (lanes A and C) and Lapatinib equally inhibits phosphorylation in both parental and resistant cells (lanes B and D).

Resistant cells have higher invasive index

Previous studies have found that in the acquisition of resistance to a drug, cell lines often significantly increase their invasive and mobile nature—like MCF7 breast carcinoma cells resistant to tamoxifen (101), HCT116 colorectal carcinoma cells resistant to Lapatinib (60), and A549 lung carcinoma cells resistant to gefitinib (102). We performed wound healing and Matrigel invasion assays to determine if the resistant cells have increased invasiveness and mobility. From the results of the Matrigel invasion assay, we determined the resistant cells have a higher invasive index value than the sensitive (1.42±0.07 vs. 1.00±0.10). However, in the presence of Lapatinib, their invasive indices are not significantly different. See Figure 6.

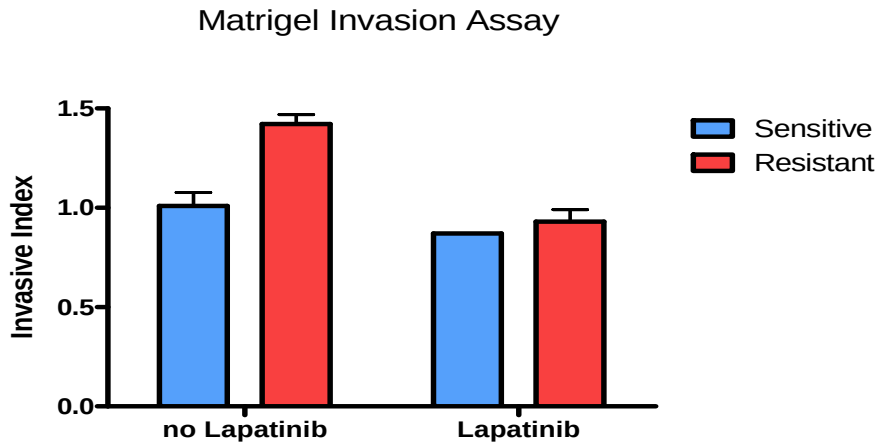

Figure 6. Matrigel invasion assay shows resistant cells have higher invasive index than the sensitive cells.
Matrigel assay shows that the resistant cells have a higher basal invasion index (1.4) as compared to the parental cells (1.0), significant with $p<0.05$. Lapatinib treatment reduced the invasion index of both parental and resistant cells.

The effect of Lapatinib on sensitive and resistant cells' mobility was observed using wound healing assay. See Figure 7. Both cell lines had similar profiles within the

first 24 hours (103), with no detectable wound healing. In the absence of Lapatinib, both sensitive and resistant cells slowly grew in the wound area over the period of three days. In the presence of Lapatinib, both cell lines reflected even slower wound healing over the three days period. Considering wound healing typically occurs within the first 24 hours, we conclude the resistant cells' migrative capacity is similar to the sensitive cells, which is minimal.

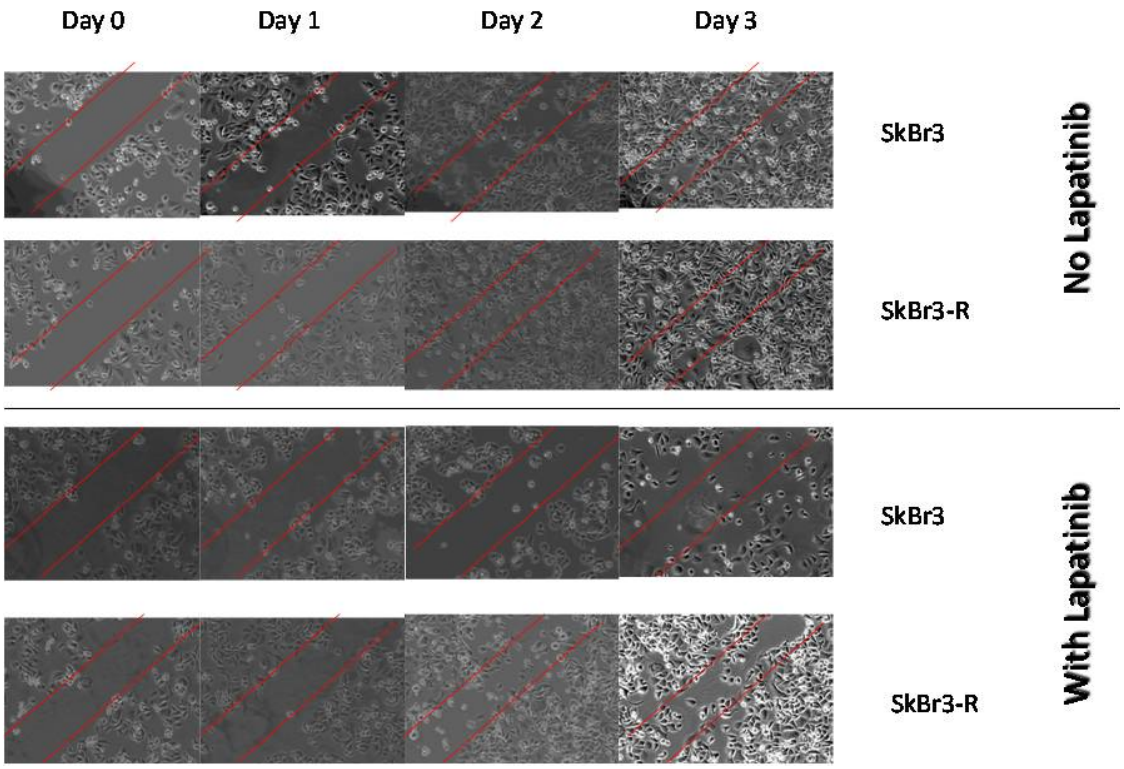

Figure 7. Wound healing scratch assay to measure cell migration.
Results from the scratch assay shows no difference between sensitive SkBr3 and Lapatinib resistant SkBr3-R cells, at Days 1-3. In the presence of Lapatinib, there was no difference on day 1 and the differences observed on day 2 and 3 are a function of decreased cell numbers in the Lapatinib treated sensitive cells.

Cell cycle distributions between resistant and sensitive

It was previously demonstrated that Lapatinib-treated cancer cells undergo apoptosis or G_1 cycle arrest (39, 40). From our dose response cell viability assays, we discovered sensitive cells start to die after two days of 1 μM Lapatinib treatment, with greater contrast between sensitive and resistant cells after six days. In order to stratify that contrast, we performed the cell cycle analysis to identify sub G_0/G_1, G_1, S, and G_2M subpopulations. Aside from cell cycle phase distributional changes, we wanted to know if the resistance cells evade cell cycle arrest; and if not, whether they evade apoptosis within this time frame (of three normal doubling cycles). Figure 8 shows the results from four time points collected after Lapatinib addition: days 0, 2, 4, and 6.

At day 0, prior to the addition of Lapatinib, both cell lines had similar cell cycle distribution: 49% cells in G_1 phase, 15% in S phase, 33% in G_2 and M phases, and 3% spontaneous deaths on average. Two days after the Lapatinib treatment, G_1 phase cell number increased in both cell lines, up to 70%, where as in the control groups, G_1 subpopulation remains closer to 50%. Though there were more cells in S phase in the resistant cell line, the sensitive cell line had significantly more dead cells, increased from 3% to 7%. Deaths in resistant cell lines remain at 3%, with or without Lapatinib conditions. On day 4, deaths jumped to 21% in sensitive cells treated to Lapatinib, compared to 5% in control. Resistant cells' deaths remain low for both control and Lapatinib condition, 5% and 7% respectively . Also to note at day 4 is that there was a higher percent of resistant cells in G_1 arrest than of sensitive cells, though that difference can be mirrored in the sensitive cells' dead population increase.

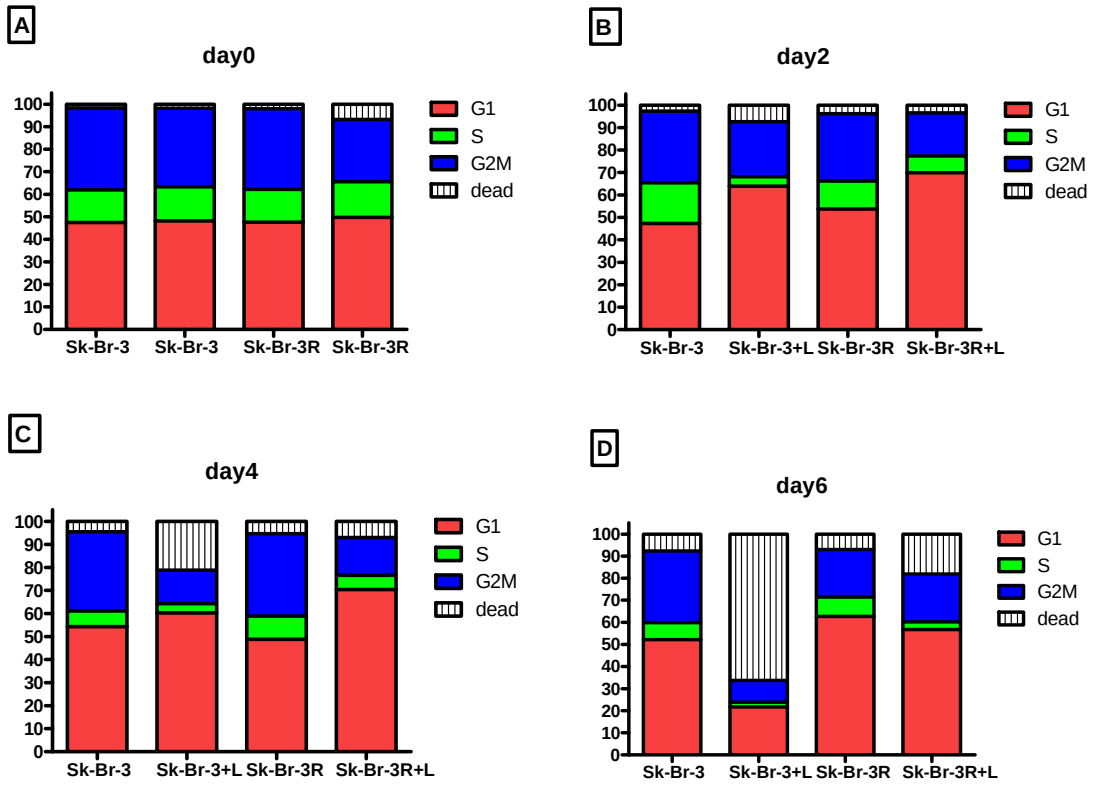

Figure 8. Cell cycle analysis.
Using FACS of 20,000 cells per condition, per day, collected at days 0, 2, 4, and 6 with and without Lapatinib treatment. A). Sensitive and resistant cells were treated with vehicle (columns 1 and 3) or 1 µM Lapatinib (columns 2 and 4) and cell cycle assayed by FACS analysis. B-D) Cell cycle analysis of sensitive and resistant cells on days 2, 4,6 without Lapatinib (columns 1 and 3) and with Lapatinib (columns 2 and 4). Lapatinib treatment had similar effect on the cell cycle for both sensitive and resistance cells leading to increase in cells with G_1 arrest. In the sensitive cells there was a significant increase in cell death and by day 6 up to 66% of the cells were dead, while in the resistant cells only 18% were dead cells.

Finally, after six days of Lapatinib treatment, deaths in sensitive cells skyrocketed to 66%, compared to accumulation of cell deaths at 8% in control condition. In the resistant cells, deaths increased to 18%, with control condition deaths at 7%.

At this point, cell deaths appear to be the main defining difference between the sensitive and resistant cells. A significant subpopulation of resistant cells do evade apoptosis, thus answering our first question. To answer whether resistant cells evade G_1 arrest, we re-examined the data by excluding the dead cells of our results and rescaled the population percentages of G_1, S, and G_2M such that the sum is 100%. See Figure 9. Two

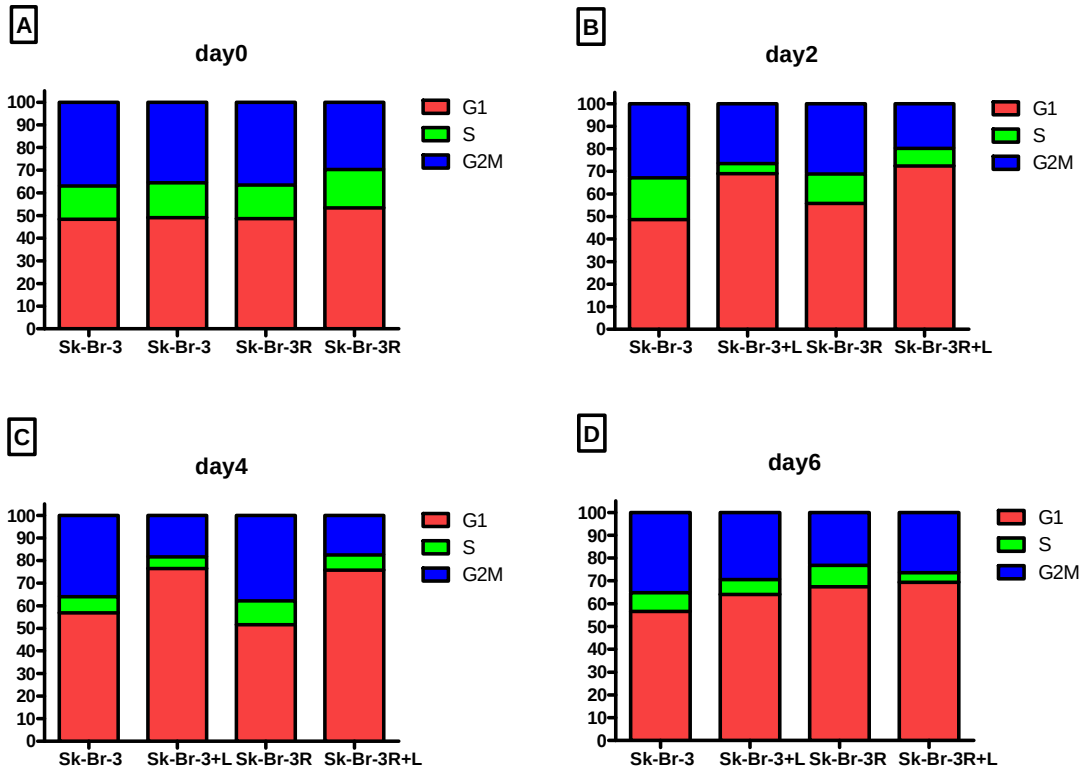

Figure 9. Cell cycle analysis of only live cells.
Days 0, 2, 4, and 6 after Lapatinib (1 μM) treatment. Deceased cells were excluded from the analysis as seen in Figure 9. Lapatinib treated cells entered G_1 arrest, evident at day2 and day4. Day6 indicated smaller percentages of G_1 arrested cells in both cell lines, compared to days 2 and 4.

days after Lapatinib treatment, both sensitive and resistant cells increased G_1 subpopulation to 70%. At day 4, G_1 subpopulation increased further to 75% in both cell lines. At day 6, G_1 subpopulation drops below 70% for both cell lines. Thus, for cells that evade Lapatinib-induced apoptosis, it appears they eventually escape G_1 arrest as

well. However, this hypothesis assumes no new cells proliferated, which was the case here since confluency increased over the days in the wells containing the resistant cells. Whether resistant cells escape G_1 arrest in addition to apoptosis remain uncertain at this point and may require studying extended time frame of more than three doubling generations.

Early apoptosis occurs in sensitive cells at 48hours

Since the major defining difference between the sensitive and resistant cells is cell deaths, we performed an apoptosis assay, Annexin V, to identify and separate cells in early apoptosis (when phosphatidylserine flips to extracellular membrane layer, marking the cell for phagocytosis), in late apoptosis/necrosis, and in necrosis after 48 hour treatment with Lapatinib at 1μM. See Figure 10. As before, the population of spontaneous dead cells was below 5% at the 48 hour time point (Figure 10A). For cells in late apoptosis and cells in necrosis, the populations were comparable across the conditions with exception of the resistant cells treated with Lapatinib, which was lower (Figure 10B). Finally, the subpopulations of cells undergoing early apoptosis provided greater contrast between the sensitive and resistant cell lines, showing almost three fold increase in cells in early apoptosis after 48 hour Lapatinib treatment, in the sensitive cells compared to the resistant cells (Figure 10C).

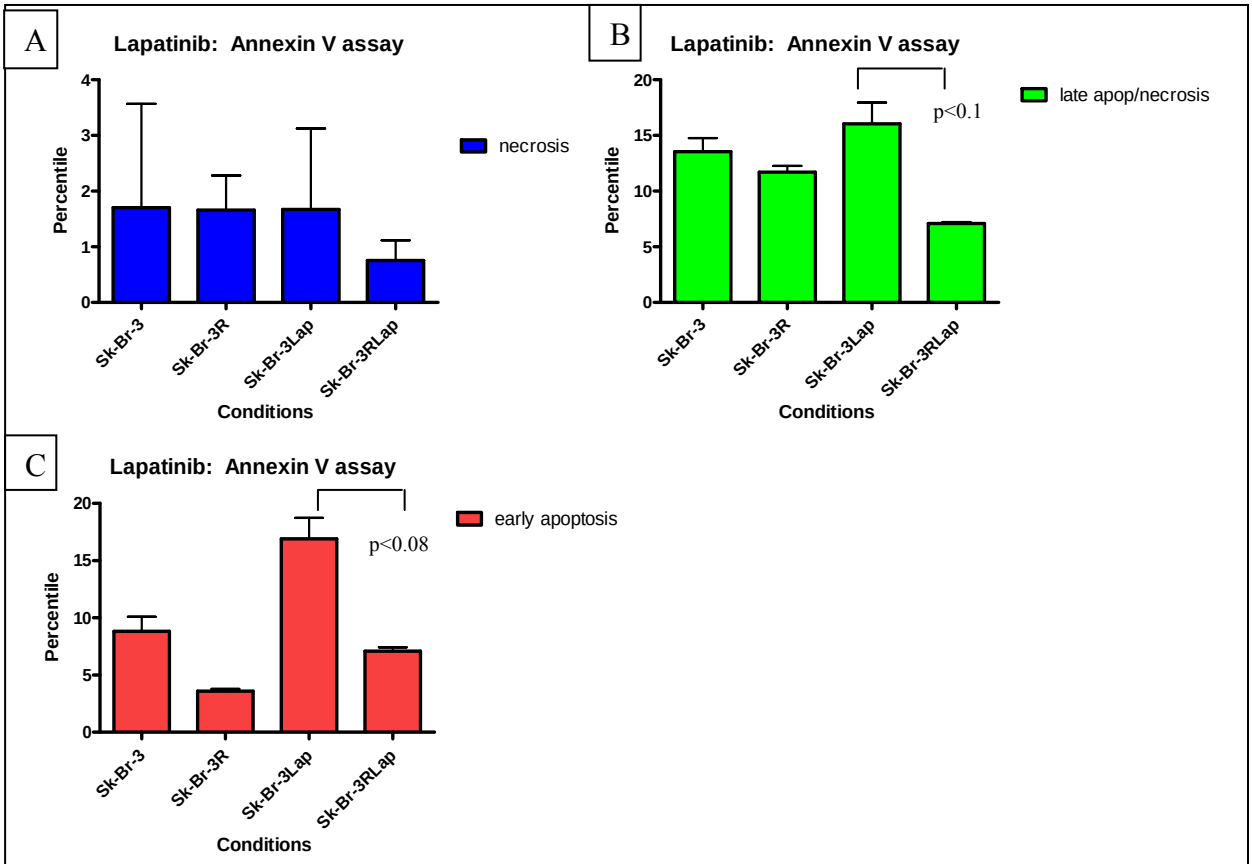

Figure 10. Identification of three subpopulations of dead and dying cells after 1 μM Lapatinib treatment for 48hours.
Fewer resistant cells underwent apoptosis. A) Necrotic cell populations were below 5% for all conditions. B) Lower population of late apoptotic and necrotic cells in the SkBr3-R cells treated with Lapatinib. C) Lower population of early apoptotic cell populations with/without Lapatinib for the resistant cells. Thus, sensitive cells have significantly higher population of cells in early apoptosis after Lapatinib exposure.

CONCLUSIONS

Mechanisms have been proposed for the development of acquired resistance to anti-ErbB therapies. One such proposal is that resistance is caused by mutations developed in the ErbB receptors. Numerous studies with other ErbB targeted drugs in various cancer types have discovered mutations in the EGFR (54-57) and ErbB2 (1, 104),

receptors that render cells to be insensitive to the drugs. Specific to Lapatinib, it was found in HCT116 cells that resistance is mediated by elevated MCL-1 and decreased BAK and not by ErbB mutation (60). However, in a recent study, randomly mutagenized ErbB2 expression library screen in murine bone marrow–derived Ba/F3 cells identified 16 mutations in ErbB2 (spanning from exons 11 to 27, many in the kinase domain) that affect sensitivity to Lapatinib (105). The exons that we sequenced for EGFR, ErbB2, and ErbB3 did not have mutations. At the time, our defined set of mutational hotspots only included exons 19-22 for ErbB2. Thus, since we did not sequence all exons of these receptors, we do not know fully if mutations did developed in these receptors in the resistant cell line during resistance acquisition. We do know that mutations did not develop for EGFR exons 18-22, 25, ErbB2 exons 19-22, and ErbB3 exon 20. However, together with our results where we examined phosphorylated and total ErbB receptors and discovered similar basal and response to Lapatinib in both sensitive and resistant cells, it is likely that no kinase domain mutations occurred in the exons for which we did not sequence. Nevertheless, further sequencing for all the ErbB receptor exons is needed to fully answer the question of whether mutations occurred in the ErbB receptors during acquisition of Lapatinib resistance.

We sought to determine if Lapatinib resistance was attributed by increase in migrative and invasive capacity. A previous study demonstrated adaptation in growth in A431-GR squamous cancer cells that had developed resistance to gefitinib from A431 cells; in 3D Matrigel, the resistant cells were able to form large colonies whereas the parental cells had impaired growth (106). Another gefitinib-resistant prostate cancer cell line PC3-GR was found by Boyden chamber assays to exhibit 2-fold greater migration

capability (107). Our wound healing assays indicated the resistant SkBr3-R cells have similar wound healing capacity compared to the sensitive cells. However, Matrigel assay demonstrated the resistant cells have higher invasive index than the sensitive cells. In this respect, Lapatinib resistance may contribute to an aggressive phenotype, similar to the case with gefitinib (106).

We made the observation during our proliferative assays that Lapatinib treatment at 1 μM was sufficient to demonstrate contrast between the sensitive and resistant cells. The cell cycle analysis performed using that concentration stratified the contrast and indicated difference in sub-G_0/G_1 populations. Lapatinib effectively induced G_0/G_1 arrest in both cell lines initially, but as the days passed more of the sensitive cells in cell cycle arrest underwent apoptosis. Since the cell cycle analysis does not identify live cells undergoing apoptosis, we performed Annexin V assay which differentiate live cells undergoing apoptosis from cells in late apoptosis and necrotic cells. From the apoptosis assay, we found significant difference in early apoptosis subpopulations between in the sensitive cells and resistant cells 48 hours after Lapatinib treatment. In the resistant cell line, the arrested cell population eventually decreased while sub-G_0/G_1 cells increased slowly (spontaneous deaths accumulated). When discarding the population of difference (sub-G_0/G_1) and considering only the live population, the cell cycle distributions of both cell lines appeared similar. This observation can be explained by either the result of either a mechanism that allows the resistant cells to continue cell cycle progression into the S phase or of the outgrowth of a subpopulation that does not undergo cell cycle arrest in response to Lapatinib. The latter case is possible since our SkBr3-R cell line is a pooled cell line.

CHAPTER 3 STAT3 SIGNALING ACTIVITY IN RESISTANT CELLS

INTRODUCTION

STAT3 is a signal transducer and transcription factor and has been found to be constitutively active in various tumor types (84-90). As research continues in elucidating the role of STAT3 in tumorigenesis, increasing evidence implicates STAT3's role in growth and survival dysregulation, angiogenesis promotion, immune suppression, and invasion and metastasis (64, 90, 108). Furthermore, recent studies have implicated STAT3 in cancer resistant to anti-ErbB drugs, such as gefitinib and cetuximab (61-63).

We discovered a correlation between STAT3 phosphorylation and Lapatinib sensitivity in a panel of breast cancer cell lines that were assayed using reverse phase protein array (RPPA). See Figure 11. Going from left to right are the cell lines in their order of increasing Lapatinib resistance. With the exception of SkBr3 cell line, the levels of phosphorylated STAT3 at tyrosine 705 increased with increased Lapatinib-resistance.

In our characterizations of the SkBr3 and SkBr3-R cell lines, we found the resistant SkBr3-R cells have higher invasive index than the sensitive cells. We also found that the resistant cells evade apoptosis after Lapatinib exposure whereas many of the sensitive cells did not. Considering STAT3's role in invasion, metastasis, survival, and its recent link in resistance to other ErbB inhibitors, we investigated STAT3's role in acquired Lapatinib resistance.

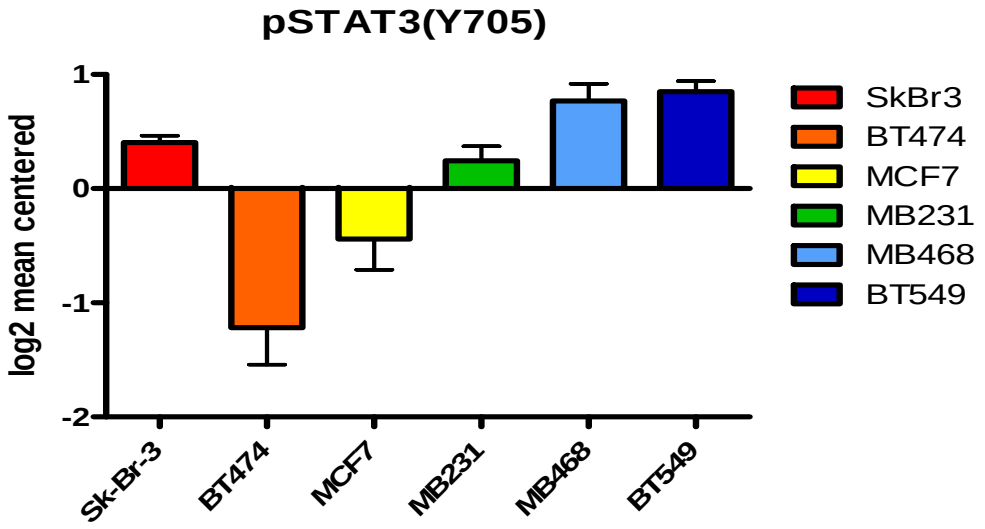

Figure 11. RPPA analysis of phospho STAT3 Y705 levels on a panel of breast cell lines.
Increasing levels of phospho Y705 STAT3 shows correlation with increasing Lapatinib resistance among breast tumor cell lines, with the exception of SkBr3 which show basal level of STAT3 phosphorylation.

MATERIALS AND METHODS

Cell lines and reagents

SkBr3 breast cancer cell line was obtained from UT M.D. Anderson Cancer Center Characterized Cell Line Core Services. Cells were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Lapatinib was purchased from LC Laboratories and dissolved in dimethyl sulfoxide (DMSO). Ly6E luciferase construct (1ug/mL) and beta-galactosidase construct (1ug/mL) were previously obtained from Dr. J Darnell Jr. (Rockefeller University, NY). Luciferase assay system kit (E1501) and reporter lysis buffer 5X (E397A) (Promega, Madison, Wisconsin) and beta-galactosidase assay reagents (Clonetech cat#631712, Mountain

View, California) were used for luciferase reporter assay. JetPRIME transfection kit was purchased from Polyplus Transfection, Inc (New York, New York) for the DNA transfection experiments. Reagents used in immunofluorescence assays include phalloidin (Oregon Green 488)(Invitrogen, Carlsbad, California), paraformaldehyde (USB Corp, Cleveland, Ohio), and DAPI (Sigma-Aldrich, St. Louis, Missouri). STATTIC (EMD, Gibbstown, New Jersey) stock solution was made at 50mM using DMSO.

Antibodies

The following antibodies were used for immunoblotting: anti-phospho-STAT3(Y705); anti-phospho-STAT3(S727); anti-STAT3; Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) (#4414)(Cell Signaling Technology, Boston, Massachusetts); and anti-β-Actin (Sigma-Aldrich, St. Louis, Missouri).

Reverse phase protein array

Assays were performed as previously described (109). Cells were seeded (300,000 per well) in 6-well plates in triplicates. Next day, cells were treated with Lapatinib at 1 μM for 4 hours, followed lysis using lysis buffer (as prepared for immunoblotting). Controls were DMSO used in equal volumes. In 1% SDS, cell lysates (1 μg/μl) were boiled and hybridized under stringent conditions. Using a GeneTac G3 DNA arrayer (Genomic Solutions, Ann Arbor, MI, USA), seven two-fold serial dilutions of cell lysates are arrayed on multiple nitrocellulose-coated glass slides (FAST Slides, Whatman Schleicher & Schuell, Keene, NH, USA). RPPA slides were produced in batches of 20. Printed slides were stored in desiccant at –20°C. Antibodies were screened for specificity by Western blotting with 25 μg of lysate protein per lane. An

antibody was accepted only if it produced a single predominant band at the expected molecular weight and if it behaved similarly between immunoblotting and RPPA across a dynamic range. Each slide was incubated with specific primary antibody, which was detected by using the catalyzed signal amplification (CSA) system (DAKO, Carpinteria, CA, USA). Briefly, each slide was washed in a mild stripping solution of Re-Blot Plus (Chemicon International, Temecula, CA, USA) then blocked with I-block (Tropix, Bedford, MA, USA) for 30 minutes. Following the DAKO universal staining system, slides were then incubated with hydrogen peroxide, followed by avidin for 5 minutes, and biotin for 5 minutes. Slides were incubated with primary and secondary antibodies then incubated with streptavidin-peroxidase for 15 minutes, biotinyl tyramide (for amplification) for 15 minutes, and 3,3-diaminobenzidine tetrahydrochloride chromogen for 5 minutes. Between steps, the slide was washed with TBS-T buffer. Loading is determined by comparing phosphorylated and nonphosphorylated antibodies. Multiple controls are placed on each slide to facilitate quantification and validation of the assay. Spot intensity was measured using MicroVigene by VigeneTech. Protein phosphorylation levels are expressed as a ratio to equivalent total proteins. Fold increases in spot intensities were calculated against nonstimulated control samples.

Transfection with reporter constructs

SkBr3 and SkBr3-R cells were seeded in 6-well plates (500,000 cells per well) in triplicates. The following day, the cells were co-transfected with Ly6E-luciferase and beta-galactosidase constructs [4:1 Ly6E:b-gal ratio (w/w)] using Polyplus transfection jetPRIME reagent at 1:2 DNA:jetPRIME ratio (w/v). One μg of DNA was diluted into 200μL jetPRIME buffer, followed by vortexing. Two μL jetPRIME was added. The

mixture was vortex for 10 seconds and spun down briefly. After incubation at room temperature for 10 minutes, 100µL of mixture was added dropwise to the cells, which were in 1mL medium. Transfection was allowed to occur 24 hours before replacement of transfection medium by complete medium. 48 hours after initial transfection, the cells were exposed to 1 µM Lapatinib. Controls were DMSO used in equal volumes.

Luciferase assay

Twenty-four hours after Lapatinib treatment, the cells were collected, washed with PBS and lysed with 100µL reporter lysis buffer. Lysates were freeze-thawed to lyse the cells completely, scraped and transferred to microcentrifuge tubes and kept on ice. Following 10 second vortex, the tubes were centrifuged at 12000g for 15 seconds. The supernatant was then used for the luciferase signal readout. With all reagents and samples at room temperature, 100µL of luciferase assay reagent was aliquot into each illuminometer tube. 20µL of sample lysate was added to each tube, followed by immediate reading on the Monolight 3010 illuminometer. The illuminometer was set to perform 2-sec measurement delay followed by 10-sec measurement read. The control used was 20µL of lysis buffer alone. To account for the number of cells successfully transfected, beta-galactosidase reporter system was used. For each sample, 100µL of beta-galactosidase assay reagent was added to an illuminometer tube prior to the addition of the sample, which was in the amount of 10µL. Sample lysates were added to the tubes. Samples were incubated for an hour at room temperature and the tubes were placed in the illuminometer for reading. The control was 10µL of lysis buffer alone. Luciferase readout of each sample required background subtraction from control readout, followed by division by the corresponding beta-galactosidase readout for that sample.

Immunofluorescence imaging

SkBr3 and SkBr3-R cells were seeded (10000, 5000, 2500 per well per row) in Packard Bell 96-well immunofluorescence plates. Each condition was done in triplicates. The following day, cells were treated with Lapatinib at 1 μM. Controls were DMSO used in equal volumes. After 48 hours, the cells were washed with PBS two times. The wells were then covered to a depth of 2-3mm with 4% formaldehyde in PBS. Cells were allowed to fix for 15 minutes at room temperature. Fixative was then aspirated and the wells were rinsed three times in PBS for five minutes each. The cells were then permeabilized with 0.3% TritonX-100/PBS for 10 minutes and rinsed again with PBS for 5 minutes. After rinse, the cells were blocked in 0.3% TritonX-100/PBS with 5% BSA for 60 minutes. The blocking solution was aspirated and cells were incubated with anti-phospho-STAT3 (Y705) diluted (1 to 100 ratio) in 200μL of 0.3% TritonX-100/PBS. Primary antibody incubation lasted 48 hours at 4C. After three rinses in PBS for five minutes each, the cells were incubated in fluorochrome-conjugated secondary antibody diluted (10ug/mL) in 200μL PBS/Triton for two hours at room temperature in the dark. At this point, cells were stained sequentially with fluorescent nuclear stain DAPI and actin stain phalloidin. Each stain was diluted 1:100 in 200μL PBS and incubation time was 20 minutes at room temperature. To avoid evaporation, the wells were covered. After the two staining, the cells were again washed with PBS twice. Finally, 200μL of PBS was added to each well prior to imaging. IN-Cell Analyzer 1000 was utilized in image acquisitions. Images were obtained using 20X objective. The quantifications of phospho-STAT3(Y705) were performed using IN-Cell Analyzer software.

Statistical analysis

Two-tailed t tests were used to assess the difference between two population's means. Differences were considered significant at $p < 0.05$. Microsoft Excel and GraphPad prism software (GraphPad Software) were utilized in the analyses of cell viability assays and generation of bar graphs and boxplots. The Student's t test was used to evaluate the statistical significance of the results. Statistical techniques were performed with the R statistical package (version 2.8) for Microsoft Windows (R Development Core Team).

RESULTS

The resistant cells have higher level of phosphorylated STAT3

Since STAT3 can be activated by EGFR, we wanted to determine if there were any changes in phosphorylation of STAT3 after treatment of Lapatinib in the sensitive and resistant cells. Our western blots show that STAT3 phosphorylation is decreased in the sensitive cells, but was not affected significantly by Lapatinib treatment in the resistant cells, even after 48 hours of exposure. See Figure 12.

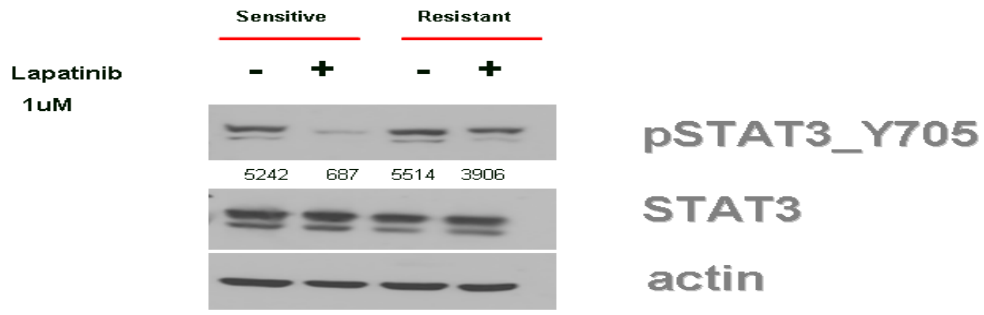

Figure 12. Analysis of STAT3 phosphorylation in sensitive and resistant cells. Sensitive and resistant cells were treated with l μM Lapatinib for 48 hours and probed for phospho Y705 STAT3. Lapatinib significantly decreased phosphorylation of STAT3 by 85% in the sensitive cells, while there was only a 30% inhibition of STAT3 phosphorylation in the resistant cells. Quantification of bands was performed with ImageJ.

The resistant cells have higher level of translocated activated STAT3

Transcriptional activation of STAT3 activity requires dimerization and translocation to the nucleus. To determine if there were changes in STAT3 nuclear translocation, we performed IN-Cell imaging assay to determine and visualize the localization of STAT3 from the cytoplasm to the nucleus in both sensitive and resistant cell lines. The cells were treated with Lapatinib at 1 μM for 48 hours, then imaged with IN-Cell Analyzer 1000. See Figure 13A. Quantification of phosphorylated STAT3 at tyrosine 705 using IN-Cell Analyzer software revealed higher level in the resistant cells, though levels did decrease after Lapatinib treatment. See Figure 13B. As illustrated by the box-plot, the ratio of nuclear to cytoplasmic phosphorylated STAT3 is higher in the resistant SkBr3-R cells than in the sensitive SkBr3 (median ratio), indicating higher level of STAT3 localized in the nucleus in the resistant cells, suggesting increased STAT3 activity. See Figure 13C.

The resistant cells have higher level of transcriptional activity of STAT3

To determine if the increased phosphorylation and nuclear localization of STAT3 resulted in an increase in STAT3 transcriptional activity, we performed a transcriptional activation assay in which cells were transfected with a STAT3-responsive luciferase reporter construct Ly6E. The results showed STAT3 transcriptional activity was significantly higher in the resistant cells and remained high even after treatment with 1 μM Lapatinib for 24 hours. See Figure 14. In the sensitive cells, basal STAT3 activity was four fold lower and decreased further upon Lapatinib treatment.

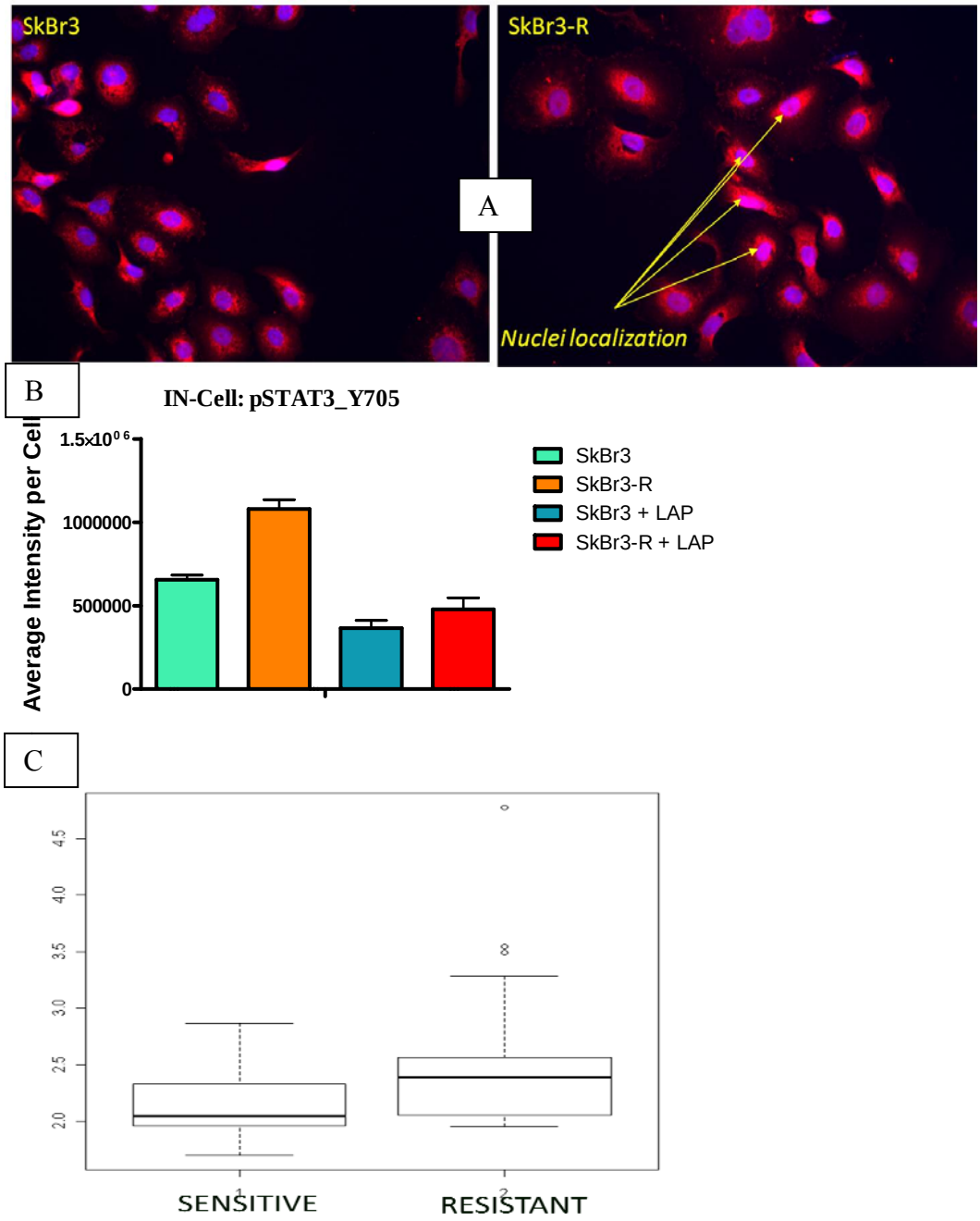

Figure 13. IN-Cell image and quantification of phosphorylated STAT3 at residue Y705.

Resistant cells have higher level of pSTAT3 than sensitive cells and higher ratio of translocated pSTAT3. A) In-Cell images of SkBr3 and SkBr3-R cells. Nuclei are stained blue while pSTAT3 are stained red. B) Quantification of pSTAT3 per cell. Resistant cells have more pSTAT detected per cell ($p<0.001$). With 1 µM Lapatinib treatment, pSTAT3 levels decreased in both cell lines. C) Boxplots of nuclei to cytoplasm ratio of sensitive and resistant populations. Sensitive cells have median ratio of 2.0 while resistant cells have ratio of 2.4 ($p<0.001$). Thus, more pSTAT3 localized in the nuclei in the resistant cells.

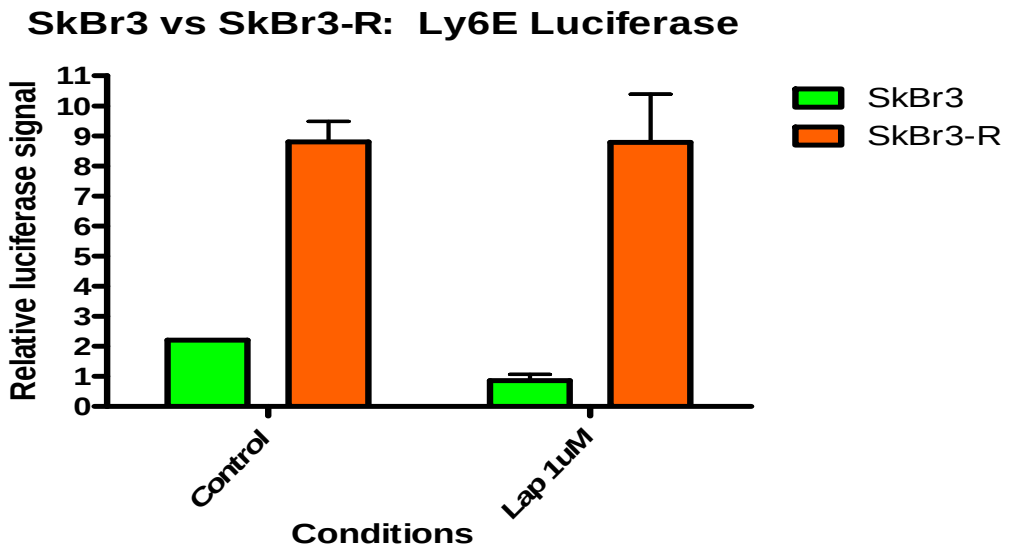

Figure 14. STAT3 transcriptional activity.
The transcriptional activity of STAT3 was determined using a luciferase based reporter assay using the Ly6E promoter STAT3 binding response element. STAT3 activity was higher in resistant cells under basal conditions. STAT3 activity in sensitive cells showed a 50% decrease in response to Lapatinib; however, STAT3 activity was not inhibited by Lapatinib in the resistant cells.

Targeting STAT3 with STATTIC

Since we observed an increase in STAT3 phosphorylation, nuclear translocation, and transcriptional activity, we wanted to determine if STAT3 interference or inhibition can reverse the Lapatinib resistance phenotype. We used STATTIC, which has been demonstrated to inhibit the function of the STAT3 SH2 domain and prevent the activation, dimerization, and translocation of STAT3 (110), and performed cell viability assays. Our observation indicated the GI_{50} value for STATTIC is 0.7 μM for both cell lines. See Figure 15 for dose response result. In combination with Lapatinib in gradient concentrations, we found that STATTIC at 0.3 μM significantly inhibited cell proliferation in the sensitive cells, whereas in the resistant cells, STATTIC 0.3 μM decreased Lapatinib GI_{50} value from 2.6 μM to 0.1 μM. At higher concentration of 10

μM, STATTIC plus Lapatinib inhibited cell proliferation in both cell lines. See Figure 16.

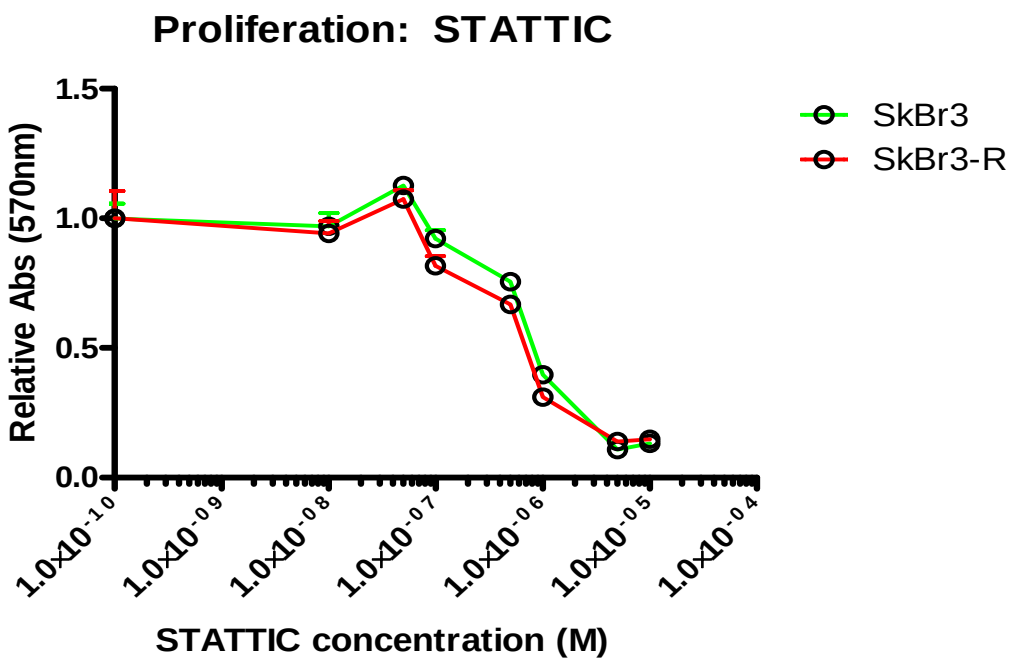

Figure 15. Dose response of sensitive and resistant cells to STAT3 inhibitor. Cell proliferation assay of sensitive and resistant cells treated with different concentrations of STATTIC shows both sensitive and resistant cells have GI_{50} 0.7 μM in response to STATTIC.

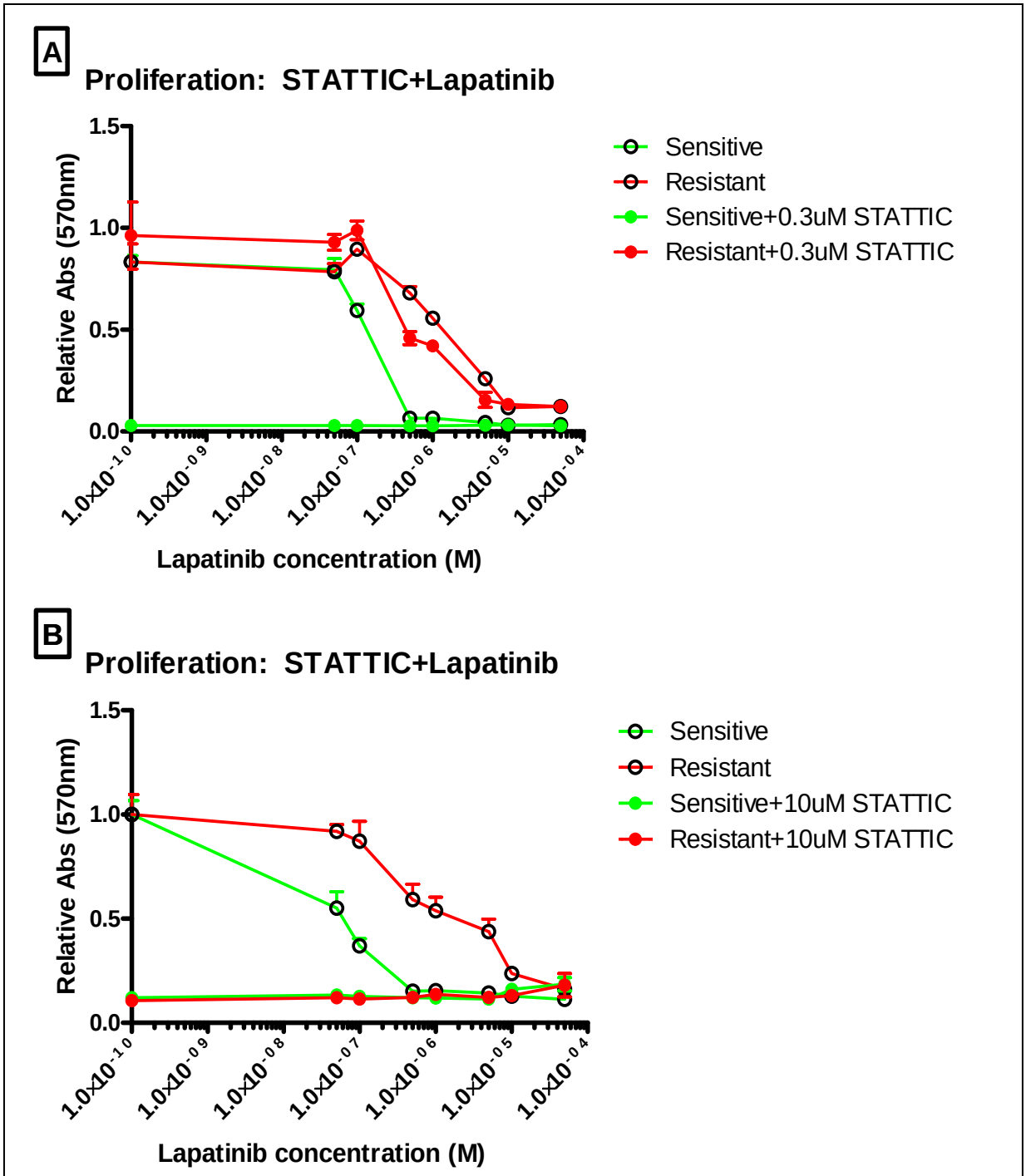

Figure 16. Cell viability assays using combination of STATTIC and Lapatinib.
A) STATTIC at 0.3 µM completely inhibited cell proliferation in combination with Lapatinib in the sensitive cells, but not in the resistant cells. B) STATTIC at 10 µM killed all cells in combination with Lapatinib.

CONCLUSIONS

From the RPPA we discovered a correlation between STAT3 phosphorylation and Lapatinib resistance across six breast tumor cell lines, with SkBr3 Lapatinib sensitive cell line being a special case. Here, the assays demonstrated 1) STAT3 phosphorylation is even higher in the SkBr3-R resistant cells, 2) more translocated STAT3 in the nuclei of the resistant cells than SkBr3, and 3) higher STAT3 activity reported by Ly6E-luciferase. Taken together, we have shown that STAT3 activity is further elevated in the resistant cells than in the sensitive cells. Since this project began, we have provided evidence that Lapatinib resistance has been linked growth acceleration and apoptosis evasion and increased invasive index. Interestingly, constitutive activation of STAT3 has been linked to those transformational phenotypes in breast carcinoma cells (94). Plus, in a study of lung cancers, gene array revealed that increased STAT3 activity increases expressions of genes involved in cell cycle progression, apoptosis suppression, angiogenesis and invasion (111).

Thus, the question to answer now is how STAT3 activity has increased in the SkBr3-R resistant cells and how this change may mediate Lapatinib resistance, or at least how it is attributed to it. Overactivation is thought to be either a consequence of dysregulation of upstream kinases or loss of endogenous inhibitors (112). Inhibition of STAT3 by SOCS3 (which inhibits JAK activation) or by PIAS3 (which inhibits STAT3 DNA binding in the nucleus) may be suppressed. Protein tyrosine phosphatases such as SHP1 and SHP2 which dephosphorylates active STAT3 complexes may also be suppressed. Further, in squamous cell carcinoma, breast cancer, and prostate cancer,

constitutive STAT3 activity is thought to be result from autocrine stimulation (Song and Grandis, 2000; Berclaz et al., 2001; Giri et al., 2001; Li and Shaw, 2002). STAT3 functions as a transcription factor for genes that encode for cytokines and growth factors, thus providing a mechanism for autocrine STAT activation (69, 70).

STAT3 overactivation may also be a result from dysregulation in other pathways that activate or inhibits STAT3. Besides JAK-STAT3 pathway, STAT3 activation is also regulated by the MAPK pathway. MEK kinase 1, in its inactive form inhibits STAT3 while its active form phosphorylates S727 and Y705 via Src and JAK (113). In another study, it was demonstrated that p38 MAPK activation by IL13 regulates STAT3 S727 phosphorylation (114).

It was previously demonstrated that constitutively activated STAT3 frequently coexpresses with EGFR in gliomas and targeting STAT3 sensitizes them to gefitinib (115). In our study, we targeted STAT3 using STATTIC that prevents activation and translocation of STAT3 to determine if the combination of STATTIC and Lapatinib will resensitize the resistant cells to Lapatinib. Our preliminary results indicated the resistant cells are more resistant to the combination of Lapatinib and STATTIC than the sensitive cells, though both cell lines are equally sensitive to STATTIC. It remains for us to explore further the combination of STATTIC and Lapatinib using different concentrations of STATTIC between 0 and 10 μM, at which toxicity is too great.

CHAPTER 4: GENE EXPRESSION CHANGES IN RESISTANT CELLS

INTRODUCTION

For the past decade, the use of DNA microarrays has accelerated research in the biomedical field. One of the most important applications for arrays is gene expression profiling where mRNA levels are measured that correspond to transcripts of tens of thousands of genes. The transcription of genes is the first step in the process of protein synthesis. Thus, gene expression changes can result in phenotypic differences or can be reflective of cellular responses to stimulation or perturbation. In order to get a global perspective of the transcriptional programming that may facilitate Lapatinib resistance, we next performed gene array on the pair of cell lines to identify patterns of gene expressions that may provide clues. Specifically, we sought to find differences in expression of genes involved in regulation of invasion, migration, cell cycle, survival and apoptosis.

MATERIALS AND METHODS

Transcriptional profiling

SkBr3 and SkBr3-R cells were seeded in 6-well plates (500,000 cells per well) in triplicates. Next day, the cells were treated with Lapatinib at 1 μM. Control was DMSO in equal volumes. After 48 hours of treatment, the cells were lyzed and total RNA was extracted using Ambion mirVana miRNA Isolation Kit (Applied Biosystems) and amplified using Illumina Totalprep RNA Amplification Kit (Applied Biosystems), according to the manufacturer's recommendations. RNA was quantified by measuring absorbance at 260 nm by spectrophotometric analysis (NanoDrop). RNA samples were

loaded onto HumanHT-12 Expression BeadChip (Illumina, San Diego, CA) that probes for 25,000 annotated genes with more than 48,000 probes. The chips were hybridized for 16 hours at 58Cand were scanned by UT Health Science Center Houston Microarray Services. Gene array data were analyzed using BeadStudio by Illumina.

Quantitative real-time polymerase chain reaction (RT-PCR)

Cells were lyzed and RNA was isolated by 1mL Trizol reagent (Invitrogen Life Technologies), following manufacturer's recommendations. RNA was quantified by measuring absorbance at 260 nm by spectrophotometric analysis (NanoDrop). RT-PCR was performed by reverse transcription-PCR, triplicate samples, using Applied Biosystems Taqman 1-step RTPCR Reagent kit and ABI Prism 7700 Sequence Detector. Samples were normalized to actin housekeeping gene.

Transfection of siRNA oligonucleotides

siRNA for PAGE2 was obtained from Dharmacon (Lafayette, CO). Control cells were transfected with non-targeting (N/T) siRNA (Dharmacon, Lafayette, Colorado). For siRNA validation via RP-PCR, cells were seeded (600,000 per well) in triplicates for each condition in 6-well plates. For cell viability assay, cells were seeded (3000 per well) in quintuplicates per condition per cell line. Next day, the cells were transfected with PAGE2 siRNA at 20nM, 50nM, and 100nM.

Statistical analysis

Two-tailed t tests were used to assess the difference between two population's means. Differences were considered significant at $p < 0.01$. GraphPad prism software (GraphPad Software) and Microsoft Excel were utilized in the analyses of cell viability

assays. The Student's t test was used to evaluate the statistical significance of the results. Statistical techniques were performed with the R statistical package (version 2.8) for Microsoft Windows (R Development Core Team). Pearson's correlation was utilized on the SkBr3/3R microarray data and the Gray's 47 breast tumor cell line microarray data.

RESULTS

Resistant cells: higher gene expressions in cell cycle regulators and DNA replication/repair genes

Transcriptional profiling microarray identified 394 genes (250 higher in resistant cells, 144 lower) in which their expressions were at least two-fold change between the sensitive and resistant cells when the cells were treated with Lapatinib (1 μM) for 48 hours. Figure 17 shows the connected subnetworks of the 250 higher expressed genes in response to Lapatinib. The graph was generated using Netwalk, created by Dr. Kakajan Komurov in Dr. Prahlad Ram's laboratory.

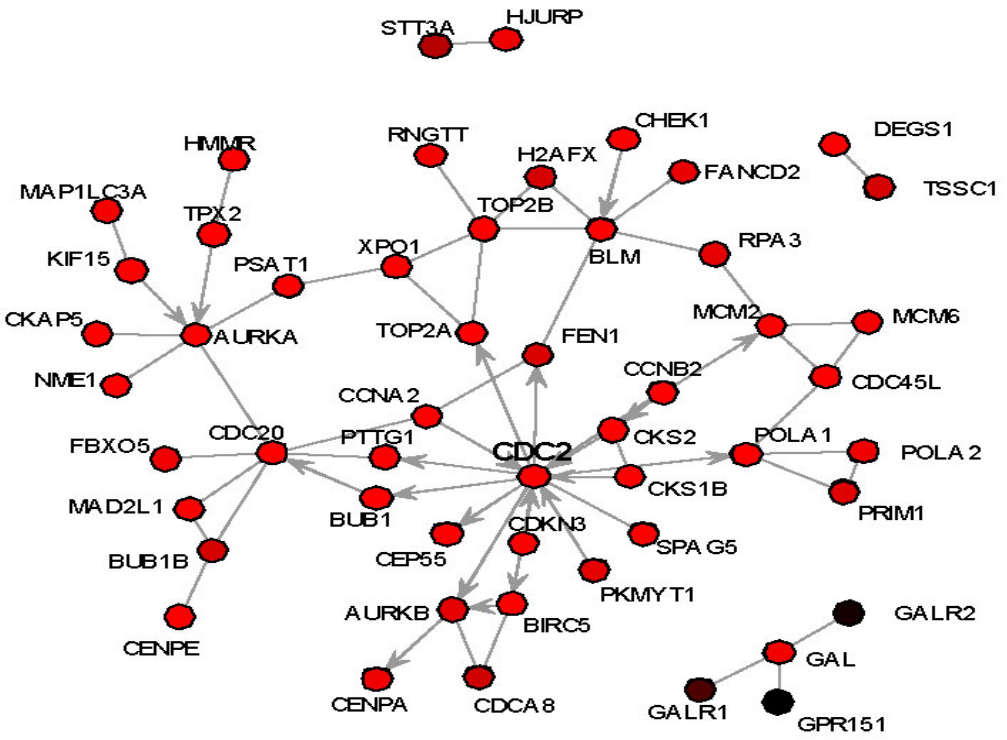

Figure 17. Gene array: higher expressed genes in resistant cells in response to 48 hour treatment of Lapatinib.

Two main subnetworks of genes populate this figure: cell cycle regulators and DNA replication and repair genes. Cell division control protein 2 homolog (CDC2), a central hub of this network, is required for S and M entry (116). Other cell cycle regulators include Aurora kinase B (AURKB)(M phase regulator), cyclins B2 and A2 (CCNB2, CCNA2)(CDC2 interactors) and cell division control protein 20 homolog (CDC20)(spindle checkpoint). On the other hands, genes involved in DNA replication and repair such as Aurora kinase A (AURKA)(centrosome separation), Aurora Kinase B (AURKB)(chromosome passenger complex), DNA topoisomerase 2-alpha (TOP2A) and TOP2B (breaks and rejoins DNA), and Bloom syndrome protein (BLM)(unwinds DNA

during DNA replication and repair), are also at least two times in magnitude in transcript expressions..

Resistant cells: lower gene expressions in cell adhesion genes and TGFb pathway genes

We also looked at two-fold minimal, transcriptional differences between the sensitive and resistant cells, in response to Lapatinib. Figure 18 shows the lower gene expressions in the resistant cells. Many of the claudin genes (CLDNs) were

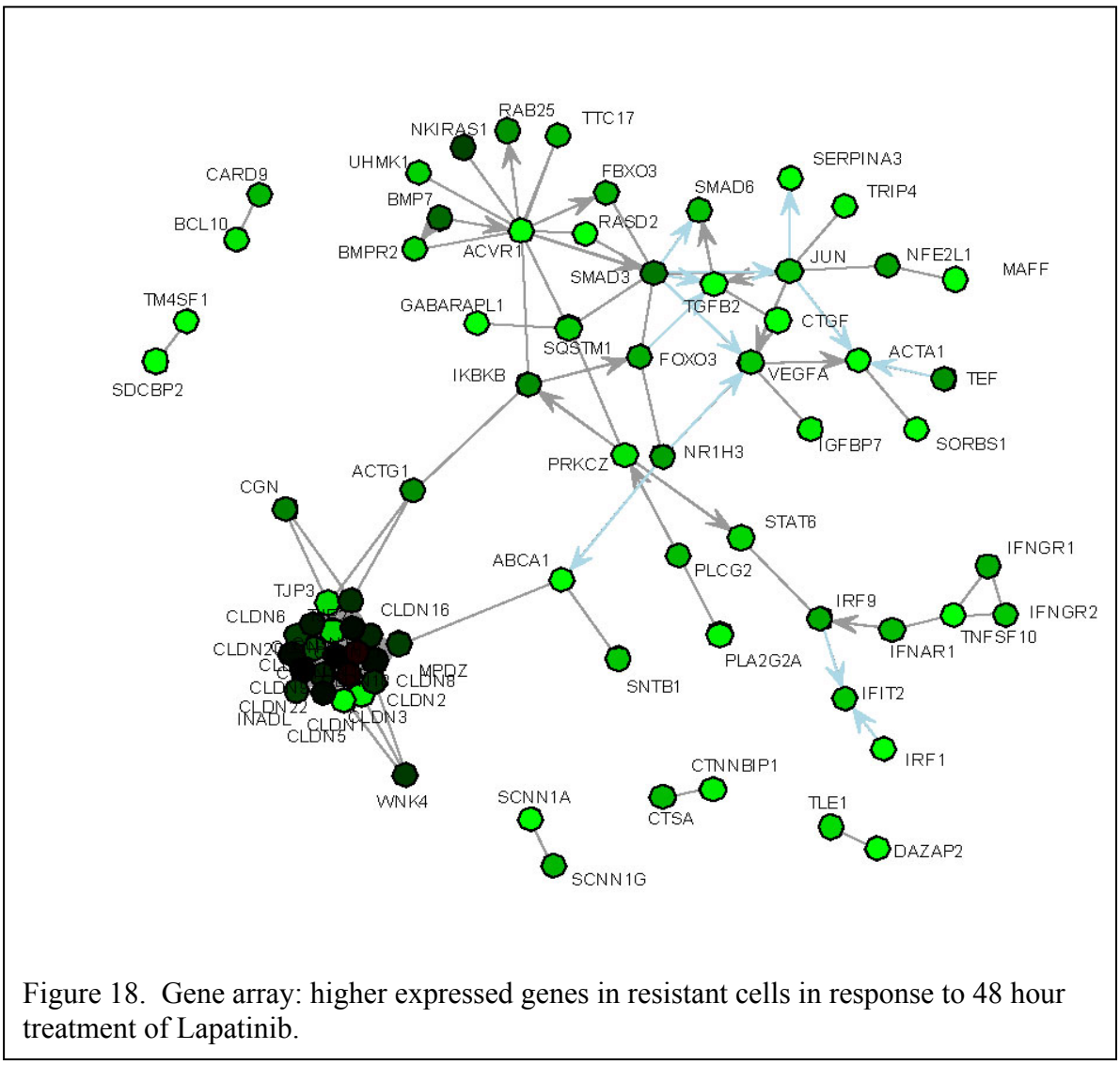

Figure 18. Gene array: higher expressed genes in resistant cells in response to 48 hour treatment of Lapatinib.

downregulated during Lapatinib treatment in the resistant cells, compared to the sensitive cells. These genes encode for proteins that are either components of or involved in cell junction and cell adhesion (117) and have been shown to be underexpressed in metaplastic breast cancers. Also a major part of the network are members of the tumor growth factor beta (TGFβ) pathway: activin receptor type-1 (ACVR1), TGFB2, Mothers against decapentaplegic homolog 3 (SMAD3), SMAD6, and FOXO3A, a proapoptotic gene.

Resistant cells: PAGE genes

Among the genes that were found to be most differentially expressed between the sensitive and resistant cells were members of the prostate-associated gene proteins (PAGEs): PAGE2, PAGE2B, and PAGE5, all located on chromosome X location p11.21. Alternatively known as putative G antigen family E members, the functions for their encoded proteins are unknown at the moment. See Figure 19 for gene array data on

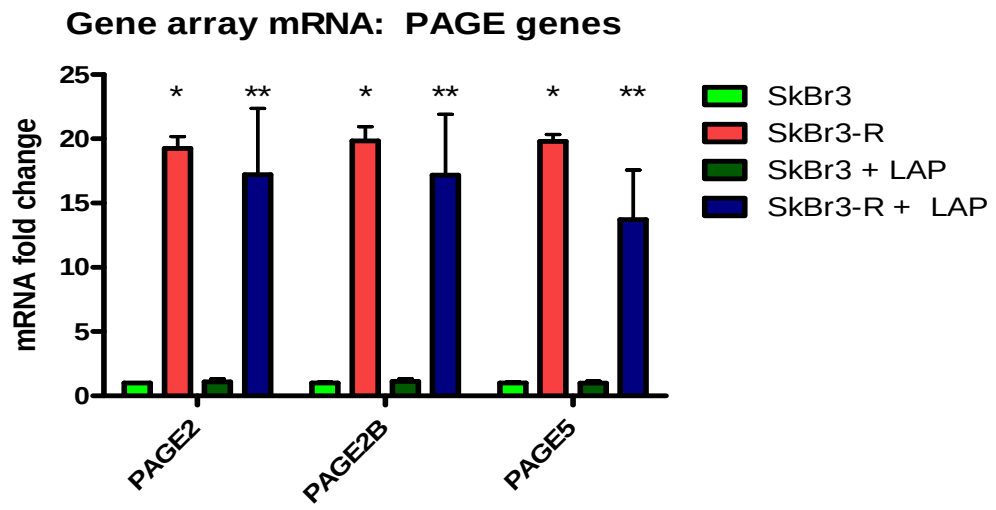

Figure 19. Levels of PAGE genes as measured from the gene array in sensitive and resistant cells.
*p<0.0001; **p<0.02

the PAGE genes. The mRNA levels for all three PAGE genes were found to be relatively

abundant (up to 20X) in the resistant cell line whereas in the sensitive cells, they were low.

To determine whether a PAGE gene is essential for the resistant cells to proliferate in the presence of Lapatinib treatment, we first validated the gene array mRNA data for one of the members, PAGE2. Using a customized set of primers purchased from AB Biosystems for PAGE2, we performed RT-PCR and verified the contrast in mRNA levels for PAGE2 between the two cell lines. See Figure 20. In the resistant cells, PAGE2 transcripts were abundant, even in the presence of Lapatinib.

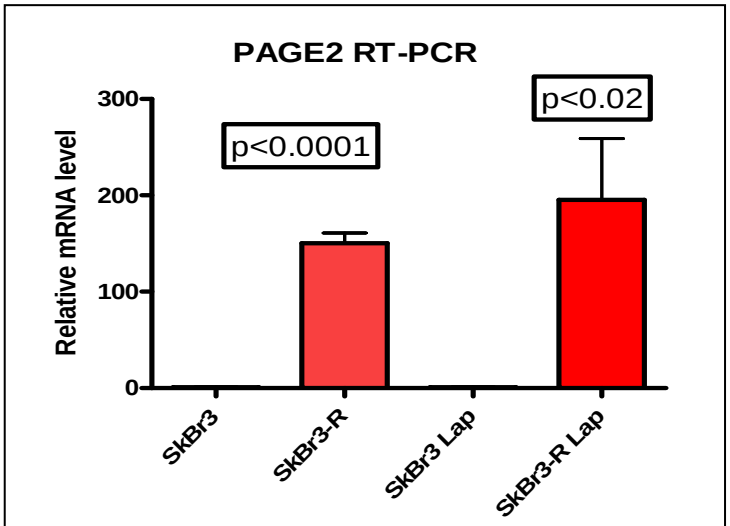

Figure 20. RT-PCR for PAGE2 mRNA levels. Resistant cells have abundant PAGE2 transcripts, compared to sensitive cells.

Next, we purchased PAGE2 siRNA oligonucleotides from Dharmacon and transfected the pair of cell lines to determine optimal siRNA concentration for knockdown of PAGE2 transcript. Concentrations used for testing were 20nM, 50nM and 100nM of PAGE 2 siRNA. From the RT-PCR result, we determined 50nM PAGE2 siRNA concentration to be optimal for an approximate 80% knockdown. See Figure 21. Again note, in the sensitive cells, mRNA for PAGE2 was low. Finally, using the 50nM siRNA concentration, we performed cell viability assays using Lapatinib gradient concentrations. See Figure 22. Comparing the Lapatinib dose response curves, there is no significant difference between N/T siRNA

and the 50nM PAGE2 siRNA conditions. Thus, our data indicated that PAGE2 knockdown does not increase nor decrease Lapatinib sensitivity in the resistant cells.

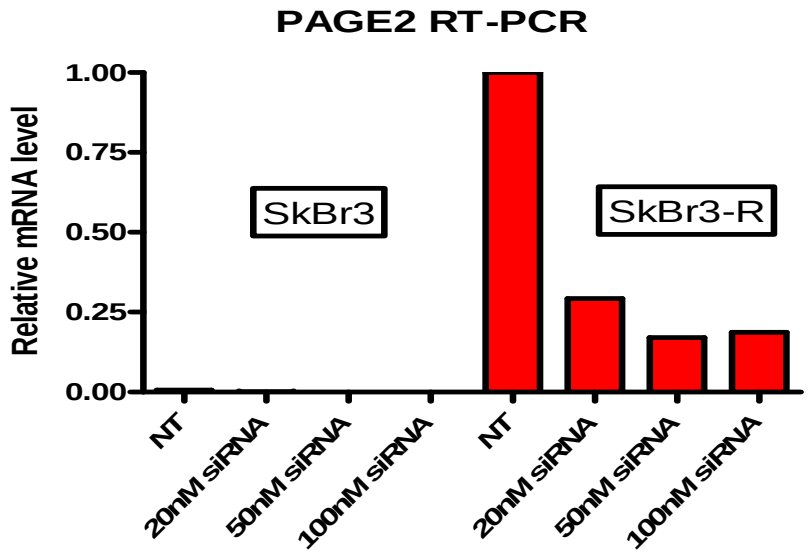

Figure 21. PAGE2 siRNA validation using RT-PCR. Different concentrations of PAG2 siRNA 20, 50, 100nM were transfected and PAG2 transcript measured using qPCR. 50nM was sufficient for ~80% knockdown in the resistant cells.

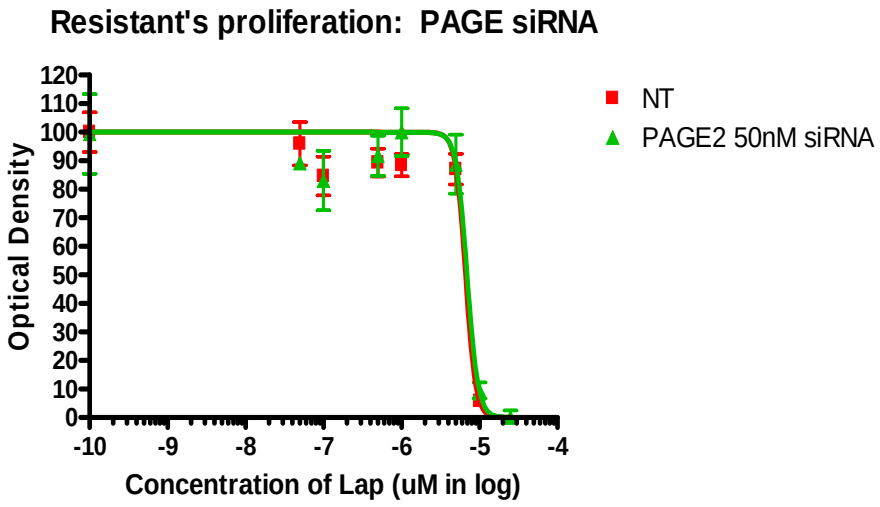

Figure 22. Cell viability assay using PAGE siRNA. Cells were transfected with PAGE2 siRNA and treated with Lapatinib. Proliferation assay showed no difference in the sensitivity to Lapatinib when PAGE2 is knocked down.

Resistant cells: STAT3 target genes

In the gene array, we examined genes downstream of STAT3 and found several target genes' expressions higher in the resistant cells after 48 hours Lapatinib treatment: Ly6E which promotes proliferation, BIRC5 which codes for an inhibitor of caspase 3 and 7, HBG1 which is involved in oxygen transporter activity, TIMELESS which is required for S-phase progression and involved in cell survival upon DNA damage and replication stress, CCND1 which controls cell cycle, and CBX5 which functions as a component of heterochromatin during mitosis. IRF1, which is a negative regulator of proliferation, was expressed lower in the resistant cells. See Table 2 below for gene array data values. Fold change (of mRNA levels) corresponds to ratio of resistant value over sensitive values for the Lapatinib condition.

Table 2: Gene array data on STAT3 target genes.

GENE	FOLD CHANGE	p-value
LY6E	1.39	0.1135
BIRC5	2.58	0.0003
HBG1	2.20	0.0719
TIMELESS	1.92	0.0219
CCND1	1.74	0.1369
CBX5	1.62	0.0548
IRF1	0.46	0.0028

DISCUSSION

Utilizing transcriptional profiling, we wanted to determine what genes are upregulated and downregulated in response to Lapatinib in the resistant cells compared to the sensitive cells. From the array data, we found many cell cycle regulating genes expressed higher in the resistant cells, most of which determine mitosis phase entry.

Additionally, genes involved with DNA replication and repair were higher expressed in the resistant cells. These two networks of genes involved have overlapping genes, which is not surprising considering how intertwined these two cellular processes are. Considering Lapatinib puts cells into G_1 arrest in sensitive cells, the data suggest that a significant subpopulation of the resistant cells were not in G_1 arrest but were progressing through the S and G_2M phases.

On the other hand, genes whose expressions were significantly lower belong in networks of genes that are involved in cell junction or cell adhesion, particularly genes of the claudin family. Low-claudin tumors has been identified to be aggressive, metastatic, chemoresistant and ”stem-cell like” (117). We have demonstrated in our characterization assays that the resistant cells have higher invasive index. Thus, the metastatic potential may be linked to the downregulations of the claudin genes. Claudin-low cancers have been shown to have elevated CD44/CD24 ratio, which has been proposed as a breast cancer stem cell-like marker (118). In our gene array, we did notice similar correlation between claudin-low and elevated CD44/CD24 ratio in the resistant cells, however, the correlation has low statistical significance in our dataset.

Several target genes of STAT3 were discovered to be expressed higher in the resistant cells compared to the sensitive cells: Ly6E, BIRC5, HBG1, TIMELESS, and CCND1, and CBX5. With the exception of HBG1, these genes code for proteins that promotes proliferation or survival or cell cycle progression. CBX5 was recently discovered by Gray’s lab to be upgregulated in Lapatinib resistant cells in vivo. IRF1 is also a target gene of STAT3 that is a negative regulator of proliferation, and in the array it is expressed significantly lower in the resistant cells.

Our gene array identified a family of genes that was abundantly present in the resistant cells but not in the sensitive cells. These PAGE genes were interesting in that their products and functions are unknown at this time. However, their sequences are similar to the G antigen family members (GAGEs) that are expressed abundantly in germline cells, associated with high proliferation, and may have antiapoptotic functions (119). Nevertheless, our cell viability assays using PAGE2 siRNA showed that knockdown of the gene did not reverse the Lapatinib resistance phenotype. It remains for us to test RNA interference for the other PAGE family members. However, given our preliminary results from PAGE2 experiments, it is likely that the PAGE2 is a passenger rather than driver of Lapatinib resistance.

So how did our array compared to array done by others in similar conditions? In another study, gene array was performed on the pair of Lapatinib-sensitive cell lines BT474 and SkBr3 (120). Consistent with their array with 12 hour Lapatinib treatment, we discovered in our array decreased transcripts for AKT1, IRAK1, and CCND1 and increased FOXO3A in the sensitive SkBr3 cells in response to 48 hours treatment of Lapatinib at the same concentration (1 μM). Interestingly, in our resistant SkBr3-R cells, AKT and IRAK1 did not decrease, CCND1 actually increased and FOXO3A only increased slightly. Previously, in a BT474 and a Lapatinib resistant clonal cell line treated with Lapatinib, FOXO3A was showed to be upregulated which resulted in a switch to ER signaling, survival factors regulation, and the cell line's ability to evade apoptosis (38). In our SkBr3-R Lapatinib resistant cell line, FOXO3A is one of the tumor suppressor genes shown to be expressed lower in the resistant cells than in the sensitive cells, see Figure 18 (previous Netwalk graph). Thus, ER signaling does not

appear to be an alternative pathway for pro-survival for the SkBr3-R cells, though BIRC5 (which codes for survivin) is significantly expressed, similar to Xia's observation. Another argument for the hypothesis that ER signaling not active in the SkBr3-R cells is that BT474 is ER+ while SkBr3 is ER-. The resistant SkBr3-R cells is also ER- based on the gene array data, though we have not determine ER protein expression experimentally.

CHAPTER 5 SUMMARY AND FUTURE DIRECTION

In human breast carcinomas, EGFR and ErbB2 are often overexpressed and associated with poor patient outcome. Thus, pharmacological agents that target these receptors provide attractive therapeutics. Lapatinib is one of the latest drugs approved by FDA for the treatment of breast cancer patients. However, acquired Lapatinib resistance has been recognized as a major concern today. Since mechanisms of drug sensitivity and acquired drug resistance were not fully elucidated, the purpose of this study was to understand acquired resistance to Lapatinib in breast cancer cells.

To understand acquired Lapatinib resistance, we established a system of isogenic cell lines in which one cell line is sensitive to Lapatinib (SkBr3) and the other resistant to it (SkBr3-R). The resistant cell line was established by gradual increase of concentrations of the drug over a span of 12 months. Once its Lapatinib resistance was verified, we began experimentation to determine mechanisms that may contribute resistance.

In our characterizations of the Lapatinib sensitive SkBr3 and resistant SkBr3-R cell lines, we first examined the usual suspects for drug resistance. Western blots indicated no significant difference in receptor expression or phosphorylation for ErbB1, ErbB2, ErbB3, and ErbB4. Since mutations have been identified to confer resistance in previous studies of other anti-ErbB drugs, we sequenced ErbB receptors of the resistant SkBr3-R cells for exons that have been reported to contain mutations in different tumor types. However, we did not find any mutations occurred in either cell line for those exons. It remains for us to sequence the remaining exons for these ErbB receptors, particularly those exons in the kinase domains. Other characterization assays performed

included wound healing and Matrigel; they provided evidence that the resistant cells have similar wound healing capacity, but higher invasive potential.

Cell cycle and apoptosis assays identified apoptosis evasion and cell cycle progression as attributes of Lapatinib acquired resistance. From the cell cycle analyses, we demonstrated that both sensitive and resistant cells initially underwent G_1 arrest upon Lapatinib treatment. However, as the days passed, the resistant cell population eventually decreased its G_1 phase population, indicating progression of cell cycle. That observation was further supported in our gene array where expressions of many cell cycle dependent genes that assist in cell cycle progression were higher in the resistant cells. In addition, several pro-apoptotic genes were expressed higher in the sensitive cells whereas anti-apoptotic genes were expressed higher in the resistant cells.

Because of STAT3 overactivation has been linked to abnormal growth and survival, invasion and metastasis in cancer and its involvement in other anti-ErbB drug resistance, we investigated into its role in Lapatinib resistance. From our western blots, IN-Cell images, and luciferase reporter assay, we showed that the resistant cells have elevated STAT3 activity. Complementary, the gene array showed increased STAT3 transcripts upon Lapatinib treatment, though for the sensitive cells it is not clear why transcript level increased despite inhibition of STAT3 phosphorylation in the western blots. Nevertheless, many STAT3 target genes' transcript levels were higher in the resistant cells, and their expression may contribute to Lapatinib resistance through promotion of cell cycle progression and cell survival.

We targeted STAT3 activity using STATTIC, an inhibitor of its activation. Preliminary results showed the resistant cell line to be more resistant to the combination

of STATTIC and Lapatinib despite same STATTIC GI_{50} value for both sensitive and resistant cell lines. It remains to determine if STAT3 inhibition in combination with Lapatinib will inhibit proliferation in the resistant cells.

In the gene array, we discovered a family of genes that provided the highest level of contrast in terms of mRNA levels between the resistant and sensitive cell lines. Little is known about this PAGE family, though information on a related family GAGE suggests PAGE proteins may have antiapoptotic functions and may be exclusive to germline cells. We performed cell viability assay using siRNA against one of the members (PAGE2) to determine its role in Lapatinib resistance, but did not confirm it as a driving force in resistance.

From the gene array, we also identified a family of genes, the claudins, that were lower expressed in the resistant cells in response to Lapatinib treatment. These genes code for proteins that are essential for cell junction and cell adhesion. The results suggest that the increase in metastatic potential may be contributed by the downregulation of these claudin genes. The clinical significance of these genes is that they are lowly expressed in metaplastic breast cancers with metastatic and chemoresistant characteristics and are stem-cell like.

Altogether, this study has identified genes and proteins implicated in several cellular processes that are involved in Lapatinib resistance, some of which may be contributors to the resistance: STAT3 and its target genes, PAGE genes, claudin genes, cell cycle regulatory genes, TGFb pathway genes. Further research is required in determining if targeting them will resensitize resistant cells to Lapatinib.

APPENDIX A

Receptor	Exon	Primer type	Tm	2ndary	Sequence	product size
EGFR	18	forward	59.4	weak	TGTAGAGAAGGCGTACATTTG	457
EGFR	18	reverse	58.2	none	TTTCCTCTCAATAACTTGGG	
EGFR	19	forward	72.6	strong	GCAATATCAGCCTTAGGTGCGGCTC	357
EGFR	19	reverse	61.4	weak	CATAGAAAGTGAACATTTAGGATGTG	
EGFR	20	forward	66.7	none	ATTCATGCGTCTTCACCTGGA	367
EGFR	20	reverse	64.0	moderate	ATGGCAAACTCTTGCTATCCC	
EGFR	21	forward	63.1	none	ACATGACCCTGAATCGGAT	308
EGFR	21	reverse	55.9	none	ACAATACAGCTAGTGGGAAG	
EGFR	22	forward	56.7	none	CACTCGTAATTAGGTCCAGA	255
EGFR	22	reverse	57.6	very weak	TGCATGTCAGAGGATATAATG	
EGFR	25	forward	66.2	weak	GACCCCTGCTCCTATAGCCAA	331
EGFR	25	reverse	55.1	none	CACTAGATGGTTATTTTCCC	
ErbB2	19	forward	60.4	weak	GGATGTTTGGAGGACAAGTAA	275
ErbB2	19	reverse	64.6	none	AACCCCAATGAAGAGAGACCA	
ErbB2	20	forward	65.7	none	TGGTTTGTGATGGTTGGGAG	346
ErbB2	20	reverse	64.1	none	CAGCAAGAGTCCCCATCCTA	
ErbB2	21	forward	71.2	weak	GGACTCTTGCTGGGCATGTGG	298
ErbB2	21	reverse	65.9	weak	CCACTCAGAGTTCTCCCATGG	
ErbB2	22	forward	65.2	weak	GTGGAGTGGTGTCTAGCCCAT	244
ErbB2	22	reverse	64.5	none	TAATTCTCCCCATCCCAGCT	
ErbB3	20	forward	63.8	none	TATGCCGCTAGGAGAGAGGA	534
ErbB3	20	reverse	68.5	very weak	TGCCGCTCACATGCTCTGT	
EGFR	18	forward	64.1	moderate	GGCACTGCTTTCCAGCAT	249
EGFR	18	reverse	67.5	none	TCCCCACCAGACCATGAGAG	
EGFR	19	forward	64.6	moderate	CATGTGGCACCATCTCACA	230
EGFR	19	reverse	64.2	none	CAGCTGCCAGACATGAGAAA	
ErbB3	20	forward	63.5	weak	TGTGCACATGCTGAGTGTATG	299
ErbB3	20	reverse	64.2	very weak	CCCCCAGACAAGCAGTTCT	

Primers used to amplified exons in EGFR, ErbB2, and ErbB3. Sequences used for primer designing program *Primer3* were extracted from genomic intron sequences 100 base pairs before and after each exon sequence.

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