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Role of Jagged1 in the Enrichment of Cancer Stem Cells in HER2+ Breast Cancer

Deep Shah
Loyola University Chicago, deep_pharma2010@yahoo.in

LOYOLA UNIVERSITY CHICAGO

ROLE OF JAGGED1 IN THE ENRICHMENT OF CANCER STEM CELLS IN HER2+ BREAST CANCER

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DEEP S. SHAH

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ADAM	A Disintegrin And Metalloprotease
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
APH1	Anterior pharynx-defective 1
ASCO	American Society of Clinical Oncology
ATM	Ataxia Telangiectasia Mutated
BCA	Bicinchoninic acid
BCL2	B cell lymphoma 2
BRCA1	Breast Cancer Associated1
BRCA2	Breast Cancer Associated2
BSA	Bovine Serum Albumin
CDH1	Cadherin-1
CDK2	Cyclin-Dependent Kinase 2
CHEK2	Cell cycle checkpoint kinase 2
CIR	CBF-1-interacting repressor
c-MET	cellular Mesenchymal to Epithelial Transition
CoAs	Co-activators

CREB	Cyclic AMP (adenosine monophosphate) response element binding
CSCs	Cancer Stem Cells
CSL	C promoter binding factor-1 [CBF-1], suppressor of hairless, Lag-1
Cyc: CDK8	cyclin C Cyclin-Dependent Kinase-8
DCIS	Ductal Carcinoma In Situ
DEAB	N, N-diethylaminobenzaldehyde
DLL1	Delta-like ligand-1
DLL3	Delta-like ligand-3
DLL4	Delta-like ligand-4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EHS	Engelbreth-Holm-Swarm
ELDA	Extreme Limiting Dilution Analysis
EMT	Epithelial to Mesenchymal Transition
EphA2	Ephrin A2
ER	Estrogen Receptor
ESA	Epithelial Specific Antigen
F.D.A	Food and Drug Administration
FGFR1	Fibroblast growth factor receptor 1

FISH	Fluorescence In-Situ Hybridization
FOXA1	Forkhead box A1
FOXO3	Forkhead box O3
GATA3	GATA binding protein 3
GCN5	General Control Non-depressible 5
GGH	Gamma Glutamyl Hydrolase
GSI	Gamma-Secretase Inhibitor
GSK-3β	Glycogen Synthase Kinase 3β
HBEGF	Heparin-Binding EGF-like Growth Factor
HER2	Human Epidermal Growth factor Receptor 2
HER3	Human Epidermal Growth factor Receptor 3
HIF	Hypoxia Inducible Factor
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IAP	Inhibitor of apoptosis
ICD	Intracellular domain
IGF-1R	Insulin-like Growth Factor 1 Receptor
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL8-CXCR	Interleukin 8- CXC motif chemokine receptor
Jak	Janus kinase
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
LAPTMB4	Lysosome-associated transmembrane protein 4-beta
MAP	Mitogen-Activated Protein

MET	Mesenchymal to Epithelial Transition
MFE	Mammosphere Forming Efficiency
MMTV	Mouse Mammary Tumor Virus
mTOR	mammalian Target Of Rapamycin
MUC4	Mucin-4
NECD-NTMIC	Notch extracellular domain-Notch transmembrane and intracellular domain
NEXT	Notch extracellular truncation
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NK	Natural Killer
NLS	Nuclear Localization Signal
NRR	Negative Regulatory Region
NSEBP1	Nuclease Sensitive Element Binding Protein 1
PALB2	Partner and localizer of BRCA2
PARP	Poly ADP-Ribose Polymerase
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDZ	postsynaptic density protein [PSD95], *Drosophila* disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]
PEA3	Polyoma Enhancer Activator 3
PEN2	Presenilin enhancer 2
PI3-K	Phosphoinositide3-kinase
PKB	Protein Kinase B

PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone Receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
RAM	RBPjκ Association Module
RANK	Receptor Activator of Nuclear factor Kappa-B
Rb	Retinoblastoma
RQ	Relative Quantitation
RTK	Receptor Tyrosine Kinase
SERD	Selective Estrogen Receptor Downregulator
SERM	Selective Estrogen Receptor Modulator
S.D.	Standard Deviation
SFK	Src family kinases
SHARP	SMRT/HDAC (histone deacetylase)-1-associated repressor protein
siRNA	small interfering RNA
SKIP	SKI-interacting protein
SLC39A6	Solute carrier family 39 zinc transporter, member 6
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
STK11	Serine-Threonine Kinase 11
TACE	Tumor necrosis factor-Alpha Converting Enzyme
TAD	Transcriptional Activator Domain
TBS	Tris-buffered saline
T-ALL	T cell Acute Lymphoblastic Leukemia

TGF	Transforming Growth Factor
TICs	Tumor Initiating Cells
TMA	Tissue microarray
U.S.	United States
VEGF	Vascular Endothelial Growth Factor
WAP	Whey Acidic Promoter
XBP1	X-box binding protein 1

CHAPTER I

INTRODUCTION

Anatomy of the Human Breast

The breast organs are endocrine glands that comprise mammary epithelial cells, adipose tissue and connective tissue. The mammary glands are immature during childhood and mature during puberty. The mammary gland is a complex network of lobes and ducts. There are about 15-20 lobes in each mammary gland and each lobe is made up of 20-40 lobules. Lobules produce milk after birth and the ducts help to carry the milk to the nipple (PP. 2001, SK. 2014)

Mammary stem cells are vital for the development of the mammary gland. A common progenitor cell is believed to give rise to two different types of epithelial cell lineages – luminal (ductal and alveolar subtypes) and myoepithelial (or basal). The regenerative capacity of the mammary epithelium during the reproductive cycles, as well its expansion during puberty and then pregnancy suggests a critical role of stem-like cells (Shackleton, Vaillant et al. 2006). Also, serial transplantation of epithelial regions of mouse mammary glands suggests that a single cell can reform the entire mammary gland (Kordon and Smith 1998). Moreover, a mouse mammary gland is progenitor cell-enriched (Welm, Tepera et al. 2002, Alvi, Clayton et al. 2003) and human mammary glands contain cells that can form myoepithelial as well as luminal cells *in vitro* (Stingl, Eaves et al. 2001, Gudjonsson, Villadsen et al. 2002, Dontu, Abdallah et al.

2003). It is hypothesized that transformation of the stem cell or luminal or basal progenitor cell compartment due to environmental insult or mutations leads to breast cancer (Petersen and Polyak 2010).

Breast Cancer

According to World Health Organization, breast cancer is the most common form of cancer among women worldwide. In the United States (U.S.), breast cancer is the second leading cause of cancer-related deaths among women, followed by lung cancer. It is estimated that about one in eight women in the U.S. would be diagnosed with breast cancer in her lifetime. The American Cancer Society estimates that 252,710 new cases of invasive breast cancer and 63,410 new cases of ductal carcinoma in situ (DCIS) will be diagnosed in the U.S. in 2017. Approximately, 40,610 women in the U.S. are estimated to die due to breast cancer in 2017 (Siegel, Miller et al. 2017).

Risk Factors for Breast Cancer

Various risk factors for breast cancer have been reported. Like most other forms of cancer, age is a primary risk factor for breast cancer. The American Cancer Society reported that, invasive breast cancer is diagnosed in women over the age of 55 (Howlader N). Listed below are the other risk factors for breast cancer:

Breast Cancer Risk Factors That Are not Under Personal Control.

Menstrual History: Women who have had more menstrual cycles because they started menstruating early (before age 12) (Kelsey and Bernstein 1996) or because they went through menopause later (after age 55) (1997) have slightly higher risk of breast

cancer. The increased risk may be due to longer lifetime exposure to the hormones estrogen and progesterone.

Race and Ethnicity: Rates of breast cancer vary in the U.S. by race and ethnicity. White women have the highest incidence (rate of new breast cancer cases) overall, while Asian-American, Native Hawaiian and Pacific Islander women have the lowest (Howlader N).

Breast Density: Women with higher breast density are 4-5 times more likely to get breast cancer than women with low breast density (Boyd, Guo et al. 2007, Yaghjyan, Colditz et al. 2011).

Family History: Having a first-degree relative (male or female) with breast cancer or a first-degree female relative with ovarian cancer increases the risk of breast cancer (Pharoah, Day et al. 1997, Kharazmi, Chen et al. 2014).

Radiation Exposure: Women who as children or young adults were treated with radiation therapy to the chest for another cancer have higher risk of breast cancer (Guibout, Adjadj et al. 2005, Ibrahim, Abouelkhair et al. 2012).

Genetic risk factors. Although age and many other factors combined are risk factors that contributes to development of breast cancers, there are clear genetic factors that increase risk of developing breast cancer. Mutations or loss of expression of Breast Cancer Associated1 and 2 (*BRCA1* and *BRCA2*) genes are highly penetrant mutations that increase the risk of breast cancer by 50-90% (Taucher, Gnant et al. 2003). Under normal conditions, BRCA1/2 acts as a critical tumor suppressor by contributing to DNA repair during cell cycle progression and transcription. However, when *BRAC1/2* is

mutated or lost, this loss of function leads to inefficient DNA repair during proliferation and accumulation of subsequent mutations. Eventually this leads to evasion of cell death signals and hyper-proliferation, ultimately leading to malignant transformation and cancer. Women with the *BRCA1* mutation have 55-65% chance of developing breast cancer, whereas women with the *BRCA2* mutation are at about 45% risk of breast cancer (Antoniou, Pharoah et al. 2003, Chen and Parmigiani 2007). Mutations in genes such as *ataxia telangiectasia mutated (ATM), phosphatase and tensin homolog (PTEN), TP53, cell cycle checkpoint kinase 2 (CHEK2), cadherin-1 (CDH1), serine-threonine kinase 11 (STK11) and partner and localizer of BRCA2 (PALB2)* have been detected but are not associated with increased risk of breast cancer (van der Groep, van der Wall et al. 2011).

Lifestyle-Related Breast Cancer Risk Factors.

Lifestyle also plays a critical role and unhealthy lifestyle certainly contributes to breast cancer.

Alcohol Consumption: Excessive alcohol consumption increases the risk of breast cancer, and other cancers as well. Having 2-5 drinks daily increases the chances of breast cancer occurrence by 1.5 times (Hamajima, Hirose et al. 2002).

Obesity and Physical Activity: Being obese or overweight after menopause enhances breast cancer risk, as more fat tissue after menopause can raise the estrogen levels (Huang, Hankinson et al. 1997, van den Brandt, Spiegelman et al. 2000). Physical activity reduces the risk of breast cancer. Brisk walking for 1.25-2.5

hours/week has been shown to reduce the risk of breast cancer by 18% (Eliassen, Hankinson et al. 2010, Hildebrand, Gapstur et al. 2013, Wu, Zhang et al. 2013).

Hormone Therapy after Menopause: Estrogen plus progestin increases the risk of developing and dying from breast cancer (Rossouw, Anderson et al. 2002, Manson, Chlebowski et al. 2013). Estrogen alone was found to slightly decrease the risk of breast cancer after short-term use(Chen, Manson et al. 2006). However, long-term use of estrogen (for more than 10 years) has been found to increase the risk of breast cancer(Brinton, Richesson et al. 2008).

Having Children: Women who have not had children or who had their first child after age 30 have a slightly higher breast cancer risk overall (Bernstein 2002). Having more children lowers the risk of breast cancer (Ewertz, Duffy et al. 1990, Lambe, Hsieh et al. 1996, Nelson, Zakher et al. 2012).

Subtypes of Breast Cancer

Breast cancer is a heterogeneous disease and is categorized into various subtypes, each one with distinct biological features that determine the course of treatment and clinical outcome. There are five molecular subtypes of breast cancer:

i. Luminal A

ii. Luminal B

iii. Human Epidermal Growth Factor Receptor 2 (HER2)

iv. Triple negative/basal like

v. Normal like breast cancer

Approximately 75% of breast cancers express steroid receptors, estrogen receptor (ER) and/or progesterone receptor (PR). ER positive tumors express ER responsive genes which include, -PR and the genes that code for proteins found in luminal epithelial cells. For this reason, ER positive tumors comprise the luminal subgroup (Yersal and Barutca 2014). There have been numerous classifications of the luminal subtype based on gene expression analysis. Hu et al. created a data set based on the gene cluster analysis of three independent studies and established two subtypes: Luminal A and Luminal B (Hu, Fan et al. 2006), which was further validated by subsequent studies (Sotiriou, Neo et al. 2003, Abd El-Rehim, Ball et al. 2005, Habashy, Powe et al. 2012).

Luminal A.

Luminal A is the most common subtype of breast cancer and it accounts for 50-60% of all breast cancer cases. It is characterized by expression of ER and/or PR, lack of *ERBB2* gene amplification and/or protein overexpression and lower levels of Ki-67 (a marker for cell proliferation) (Carey, Perou et al. 2006, Yersal and Barutca 2014). Moreover, they express cytokeratins 8 and 18, other markers including ER1, ER function associated genes like *LIV1* (zinc transporter *ZIP6* or *SLC39A6*; solute carrier family 39 zinc transporter, member 6), *Forkhead Box A1 (FOXA1), X-box binding protein 1* (XBP1), *GATA binding protein 3 (GATA3), B cell lymphoma 2 (BCL2), ERBB3* and *ERBB4* (Carey 2010). This subtype has the best prognosis and has higher survival rates and lower recurrence rates than other subtypes (Parise and Caggiano 2014, Shim, Kim et al. 2014).

Anti-hormonal therapy is currently the standard treatment for women with Luminal A breast cancer. Pre-menopausal women (18-49 years of age) are treated with tamoxifen, a selective estrogen receptor modulator (SERM). Post-menopausal women (50-85 years of age) are treated with aromatase inhibitors (anastrozole, letrozole or exemestane) or tamoxifen (Swaby and Jordan 2008). Tamoxifen is a competitive inhibitor of 17β-estradiol, the active form of estrogen. It binds directly to the ligand binding domain of the ER and prevents binding of 17β-estradiol. Aromatase inhibitors act by blocking aromatase-mediated synthesis of 17β-estradiol from androstenedione or testosterone. The U.S. Food and Drug Administration (F.D.A.) has approved the use of a selective estrogen receptor downregulator (SERD), fulvestrant for the treatment of breast cancer in post-menopausal women that are diagnosed with advanced (metastatic) hormone receptor positive breast cancer refractory to tamoxifen or aromatase inhibitors.

Luminal B.

The luminal B subtype of breast cancer accounts for 15-20% of breast cancer cases. This subtype has a poorer prognosis as compared to luminal- A due to higher levels of Ki-67 and higher histological grade (Creighton 2012). Also, the luminal B subtype has a higher recurrence rate and lower survival rates (Ellis, Tao et al. 2008). Higher recurrence rates of luminal B breast cancers as compared to luminal A is limited to first 5 years after the diagnosis (M. Ignatiadis 2009).

Luminal B breast cancer is characterized by expression of ER and/or PR, and gene amplification and/or protein overexpression of HER2 in about 30% of cases (Loi,

Sotiriou et al. 2009). The major contrast to the luminal A subtype is the increased expression of proliferation-associated genes such as *avian myeloblastosis viral oncogene homolog (v-MYB), lysosome-associated transmembrane protein 4-beta (LAPTMB4), gamma glutamyl hydrolase (GGH), nuclease sensitive element binding protein 1 (NSEP1)* and *cyclin E1 (CCNE1)* (Reis-Filho, Weigelt et al. 2010).

Advances in recent research have attributed the higher proliferation rate of luminal B breast cancer to activation of growth factor pathways including fibroblast growth factor receptor 1 (FGFR1), HER1, sarcoma proto-oncogene (Src) and phosphoinositide 3 kinase (PI3K) catalytic alpha polypeptide (Tran and Bedard 2011).

If the tumor is non-metastatic, patients are treated with endocrine therapy along with anti-HER2 therapy, depending on the HER2 status. The addition of chemotherapy was found to be beneficial in patients with lower ER expression and a higher recurrence score (as assessed by a 21-gene score referred to as OncotypeDX) (Albain, Barlow et al. 2010)

Triple Negative.

The triple negative subtype of breast cancer accounts for nearly 15%-20% of all breast cancer cases (Dawson, Provenzano et al. 2009). They do not express ER, or PR, and lack gene amplification of ERBB2, hence, they are termed as triple negative. Within triple negative breast cancer, there are numerous subtypes (Lehmann, Bauer et al. 2011). The major subtype, basal-like breast cancer, express high levels of myoepithelial basal markers including laminin and cytokeratins 5,14, and 17 (Nielsen, Hsu et al. 2004, Cheang, Voduc et al. 2008). Also, basal-like breast cancer overexpress

P-cadherin, fascin, caveolins 1 and 2, alpha-B crystallin and epidermal growth factor receptor (EGFR)(Schneider, Winer et al. 2008). Basal-like cancer is associated with frequent mutations of *TP53* gene, inactivation of the *retinoblastoma (Rb)* pathway and genomic instability. These tumors have a higher proliferative index, higher metastasis to lung and brain, and demonstrate aggressive clinical behavior (Heitz, Harter et al. 2009). It is important to note that although most triple negative breast cancers are basal-like, they are other sub-categories including luminal, mesenchymal, stem-like, and androgen receptor positive.(Lehmann, Bauer et al. 2011, Toft and Cryns 2011). Triple negative breast cancer has the worst prognosis due to the lack of targeted therapy. Chemotherapeutic agents have been traditionally used by oncologists for this subtype of breast cancer. Since chemotherapeutic agents also affect rapidly dividing non-cancerous cells, the patient experiences adverse effects such as alopecia, bone marrow suppression and gastrointestinal disturbances(Chan and Giaccia 2008).

Based on immunohistochemistry and micro-array data, the basal-like subtype of breast cancer accounts for about 75% of all *BRCA1* mutant breast cancers. *BRCA1* is located on the long arm of chromosome 17(q21) and is a critical gene involved in the repair of DNA damage. *BRCA1* cancers are associated with a triple negative phenotype, presence of high Ki-67, basal cytokeratins, *TP53* mutations, EGFR, P cadherin and X chromosome abnormalities. Just like women with basal-like cancer, metastatic disease pattern and early relapse could be observed in cases of women with BRCA1 cancers (Foulkes, Brunet et al. 2004). Clinical trials are underway for the treatment of BRCA1 mutant breast cancer with poly ADP-ribose polymerase (PARP)

inhibitor, which acts by completely shutting down the ability of the tumor cell to repair the damaged DNA, thus resulting in accumulated damaged DNA, cell arrest in the G2/M phase of the cell cycle, and apoptosis (Livraghi and Garber 2015).

Normal Breast-Like.

They comprise nearly 5-10% of all breast cancer cases. They are characterized by the lack of ER/PR and HER2 expression. Because of this, they could be considered as triple negative. However, they are not basal-like as they lack the expression of EGFR and CK5. They show gene characteristics of adipose tissue and have an intermediate prognosis between luminal and basal subtype of breast cancers. This is not a widely-studied subtype of breast cancer and its clinical significance remains unclear. No cases of normal breast-like subtype were observed when microdissection was performed to isolate tumor cells, supporting the notion of some researchers that this subtype might be an artifact from contamination with normal tissue during microarrays (Weigelt, Mackay et al. 2010, Yersal and Barutca 2014).

Human Epidermal Growth Factor Receptor 2 (HER2).

The HER2 positive subtype of breast cancer accounts for about 20% of all breast cancer cases and is characterized by the gene amplification of *ERBB2* located on chromosome 17q12 and /or overexpression of its protein. The gene amplification is detected by using fluorescence in-situ hybridization (FISH) and the protein overexpression is detected by using immunohistochemistry (IHC) (Press, Finn et al. 2008). These tumors are highly proliferative (Peppercorn, Perou et al. 2008), more aggressive (Slamon, Clark et al. 1987, Slamon, Godolphin et al. 1989), have early

relapse and higher recurrence rates and harbor *TP53* mutations in about 71% of the cases (Peppercorn, Perou et al. 2008, Weigelt, Kreike et al. 2009). This subtype has improved prognosis due to the availability of HER2 targeting agents (Slamon, Leyland-Jones et al. 2001).

HER2 or ErbB2 is a proto-oncogene which belongs to HER family of receptor tyrosine kinase and was initially identified in a rat glioblastoma model. Other members of the family include HER1/ErbB1 (EGFR), human epidermal growth factor receptor 3 (HER3)/ErbB3, and human epidermal growth factor receptor 4 (HER4)/ErbB4. HER family of proteins are Type I growth factor receptor tyrosine kinases (RTKs) which respond to extracellular growth factors to activate multiple signaling cascades. Their structure consists of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Citri, Skaria et al. 2003). Subdomains I (L1) and III (L2) of the extracellular domain mediate ligand binding (Lax, Bellot et al. 1989, Summerfield, Hudnall et al. 1996), whereas the cysteine-rich subdomains II (S1) and IV (S2) are critical for receptor dimerization. There are 11 known ligands for the four HER receptors. All the ligands possess an EGF-like domain and intramolecular loops containing three disulfide bonds. This receptor-binding domain is a part of a large precursor which also contains immunoglobulin-like domains, glycosylated linkers, and heparin binding sites (Yarden and Sliwkowski 2001). The transmembrane region's expression and processing is tightly regulated. Some ligands that bind multiple receptors have narrow specificity, whereas others bind to distinct receptors (Jones, Akita et al. 1999). Table 1 depicts the ligands that bind to each receptor. HER3 receptor

has a weak tyrosine kinase activity (Guy, Platko et al. 1994) and HER2 receptor has no known ligand (Klapper, Glathe et al. 1999). Thus, by dimerizing with HER2, HER3 can mediate distinct signaling pathways.

HER Receptor	Ligands
HER1	Amphiregulin, Epidermal Growth Factor (EGF), Epigen, Epiregulin, β-Cellulin, Heparin-Binding EGF-like Growth Factor (HBEGF) and Transforming Growth Factor – α
HER3	Neuregulin1 and Neuregulin2
HER4	Neuregulin1, Neureulin2, Neuregulin3, Neuregulin4, β-Cellulin, HBEGF and Epiregulin

Table 1: Ligands for HER Family Receptors. (Shah and Osipo 2016).

The extracellular domain of the HER family of receptors can be in an open confirmation or a closed confirmation. In the closed confirmation, the S1 and S2 subdomains of HER1, HER3 and HER4 interact with each other. Upon ligand binding in the L1 and L2 subdomains of the receptor, there occurs a structural change which protrudes the S1 dimerization loop and favors the open confirmation. HER2 exists in a constitutively active confirmation and does not cycle between active and inactive states. The HER2 receptor either homodimerizes or heterodimerizes with other HER receptor (Citri, Skaria et al. 2003). Selection of the dimerizing HER partner is critical for the signaling activity and a hierarchical pattern is followed, favoring heterodimer formation over homodimer formation. Overexpression of a certain receptor can skew the hierarchy. For example, *ERBB2* gene amplification and thus HER2 protein overexpression leads to more HER2 homodimer and heterodimer formation, depending on expression of other HERs and their growth factors. HER2 has a strong catalytic

kinase activity and hence HER2 heterodimers have strongest signaling functions. This conclusion is based on the strong receptor transphosphorylation and mitogenesis observed due to extensive interaction between HER2 and the ligand occupied EGFR, HER3 and HER4 (Tzahar, Waterman et al. 1996). HER2-HER3 is the most active signaling dimer, and is known to be critical for various developmental processes and biological functions (Citri, Skaria et al. 2003, Moasser 2007).

Depending on the dimerization partners, both cis- and trans-phosphorylation of tyrosine residues within their intracellular domains can lead to activation of the receptor tyrosine kinase domain. Once activated, the receptor tyrosine kinase can lead to docking of intracellular signaling molecules and downstream activation of PI3K/protein kinase B (PKB)/Akt or mitogen-activated protein (MAP) kinase signaling cascade. When HER2 dimerizes with HER3, HER2 trans-phosphorylates many tyrosine residues on HER3. The result is recruitment of adaptor proteins that initiate PI3K or MAP kinase signaling. Specifically, the p85 subunit of PI3K interacts with phosphorylated tyrosine residues on HER3 at 6 consensus sites (Hellyer, Cheng et al. 1998). Upon docking with HER3, the regulatory subunit, p85 interacts with the p110 catalytic subunit and activates PI3-Kinase. Thus, HER3 drives HER2-mediated PI3K signaling. The signaling pathways have been shown to promote cell survival, proliferation, motility, invasiveness, resistance to apoptosis and angiogenesis. The output depends upon the cell context, the ligand involved and the dimer formed (Yarden and Sliwkowski 2001, Citri, Skaria et al. 2003, Linggi and Carpenter 2006, Moasser 2007, Dey, Williams et al. 2015). Figure 1 summarizes the HER signaling pathway.

Drugs that are used to target HER2 include humanized, therapeutic monoclonal antibodies, trastuzumab and pertuzumab, a dual EGFR-HER2 tyrosine kinase inhibitor, lapatinib, trastuzumab coupled with a cytotoxic agent, emtansine (T-DM1) and taxane based chemotherapeutic agents (Drakaki and Hurvitz 2015).

The two antigen specific sites of trastuzumab binds to the subdomain IV of the HER2 transmembrane protein. This prevents the activation of the intracellular receptor tyrosine kinase. There are several mechanisms by which trastuzumab inhibits HER2 signaling. These include – inhibition of HER2 dimerization, G1 cell cycle arrest associated with induction of $p27^{KIP1}$, inhibition of shedding of HER2 extracellular domain, increased endocytic downregulation of HER2 receptor, inhibition of angiogenesis, and antibody-dependent cell-mediated cytotoxicity (ADCC). Based on previous clinical trials, trastuzumab was not effective as a monotherapy in metastatic, HER2+ breast cancer, and the response rate ranged from 11-26% (Hudis 2007). Pertuzumab is another therapeutic monoclonal antibody that prevents the dimerization of HER2 with other HER receptors, primarily the formation of HER2-HER3 dimer. It acts by binding the extracellular domain of HER2 at a different epitope than trastuzumab (subdomain II). In the metastatic, HER2+ breast cancer setting, the combination of trastuzumab plus pertuzumab in combination with docetaxel increased overall survival by 15.7 months as compared to trastuzumab plus chemotherapy without pertuzumab in the CLEOPATRA clinical trial, (Swain, Baselga et al. 2015), suggesting that the combination therapy provides a more comprehensive blockade of HER2. As per the American Society of Clinical Oncology (ASCO) guidelines, this remains the first line of

treatment for patients with HER2+ breast cancer, unless the patient has severe heart problems. It is used in neoadjuvant and metastatic settings. Along with HER2+, if the tumor is hormone receptor positive, then hormonal therapy is included in the regimen along with anti-HER2 therapy. Only in HER2+/ER+ indolent metastatic breast cancer, hormonal therapy is prescribed along with anti-HER2 treatment.

T-DM1 is an antibody drug conjugate consisting of therapeutic humanized monoclonal antibody trastuzumab linked to cytotoxic agent DM1 through a non-cleavable linker. In the case of intrinsic resistance, or if tumor recurrence is observed within 12 months, T-DM1 is used as a second line of treatment. The EMILIA clinical study that was conducted in patients with locally advanced or metastatic HER2+ breast cancer found that the progression free survival of patients that received T-DM1 was significantly higher than the ones that received lapatinib plus capecitabine (Verma, Miles et al. 2012). Lapatinib is approved along with capecitabine or other chemotherapy and anti-HER2 combinations as the third line of treatment for advanced or metastatic breast cancer patients that have previously received anthracycline, taxane or trastuzumab (Geyer, Forster et al. 2006).

Despite the numerous advancement and efforts, the clinical challenge is that a significant number of patients develop either intrinsic or acquired resistance to the treatment. The tumor relapses in a more aggressive fashion, undergoes epithelial to mesenchymal transition (EMT) that facilitates metastasis and evades HER2 targeting. Hence, it is critical to understand the mechanisms of drug resistance and tumor

recurrence for anti-HER2 agents, and develop novel therapeutic strategies to prevent resistance.

HER2+ Breast Cancer: Drug Resistance

As mentioned previously, drug resistance to HER2 targeted agents is a major challenge. Numerous pre-clinical and clinical research studies have been undertaken to understand the mechanisms of intrinsic and acquired drug resistance. These mechanisms include:

i. HER2 mutations.

ii. Co-expression of other receptors or crosstalk with other signaling pathways.

iii. Defects in apoptosis and cell cycle regulation.

iv. Involvement of host factors that modulate drug response.

Emerging clinical evidence supports the use of combination therapy targeting these resistance mechanisms along with anti-HER2 therapy for more effective treatment of patients suffering from HER2+ breast cancer (Rexer and Arteaga 2012).

HER2 Mutations.

Mutations in HER2 cause alterations in the binding of the drug to the target. A truncated form of HER2 called p95-HER2 lacks the N-terminal extracellular region including the antibody binding region. This form arises through alternate transcription initiation sites in HER2 (Anido, Scaltriti et al. 2006), retains the kinase activity and hence lapatinib is effective under these conditions, but not trastuzumab (Scaltriti, Rojo et al. 2007). Cell lines expressing the splice variant of HER2 – D16 HER2 lacks exon16 in the extracellular domain and expression results in resistance to trastuzumab (Castiglioni,

Tagliabue et al. 2006, Mitra, Brumlik et al. 2009). The variant acts by stabilizing the homodimer and preventing its disruption by the antibody (Castiglioni, Tagliabue et al. 2006). Co-expresssion of HER2 with membrane associated glycoprotein mucin-4 (MUC4) can block the binding sites of trastuzumab (Price-Schiavi, Jepson et al. 2002, Nagy, Friedlander et al. 2005). A cleaved form of MUC1 was overexpressed in a cell line with trastuzumab resistance (Fessler, Wotkowicz et al. 2009). Moreover studies have shown that the cleaved form can homodimerize and initiate the signaling cascade for cell survival and proliferation (Hikita, Kosik et al. 2008, Mahanta, Fessler et al. 2008).

Activation of Compensatory Signal Transduction Pathways.

Receptor tyrosine kinases. HER2 can heterodimerize with other members of the HER family and signaling initiated by the ligands of EGFR, HER3 and HER4 would bypass the ligand independent effect of trastuzumab (Moulder, Yakes et al. 2001, Motoyama, Hynes et al. 2002). Transactivation of HER2 by other RTKs could amplify the signal transduction, and thus lapatinib and trastuzumab won't be effective. An acquired resistance model of trastuzumab in breast cancer xenografts has shown increased expression of ligands for HER3 and EGFR, resulting in HER3 and EGFR activation (Ritter, Perez-Torres et al. 2007). Increased availability of HER ligands is also related with activation of transforming growth factor (TGF)-β receptors, which activate a disintegrin and metalloprotease (ADAM) / tumor necrosis factor-alpha converting enzyme (TACE) to release amphirelgulin, heregulin and TGF-α. These ligands activate HER3. Expression of a constitutively active mutant form of TGF-β type I receptor results in a gene signature that correlates with poor clinical outcome and trastuzumab

resistance (Wang, Xiang et al. 2008). An increase in the insulin-like growth factor 1 receptor (IGF-1R) or signaling through IGF-1R/HER2 can lead to strong activation of PI3K-Akt signaling and confer resistance to trastuzumab (Lu, Zi et al. 2001, Nahta, Yuan et al. 2005). Lapatinib or a neutralizing antibody against IGF-1R was found to overcome IGF-1R mediated trastuzumab resistance (Nahta, Yuan et al. 2005, Nahta, Yuan et al. 2007, Browne, Crown et al. 2011).

Trastuzumab treatment has been shown to upregulate levels of cellular mesenchymal to epithelial transition factor (c-MET), the hepatocyte growth factor receptor. Overexpression of c-MET and its ligand, HGF was shown to confer tratuzumab resistance in breast tumors from subset of patients that did not respond to chemotherapy plus trastuzumab (Shattuck, Miller et al. 2008). Overexpression of ephrin A2 receptor (EphA2) was associated with reduced disease free survival in patients and conferred trastuzumab resistance in cell lines (Zhuang, Brantley-Sieders et al. 2010). Erythropoietin receptor was found to be overexpressed in the cell lines that overexpress HER2, and concomitant treatment of the cell lines with trastuzumab and erythropoietin has been shown to confer resistance through janus kinase (Jak) and Src signaling. In the metastatic setting, patients with HER2+ breast cancer treated with trastuzumab and erythropoietin showed reduced progression free survival and overall survival (Liang, Esteva et al. 2010).

The RTK, AXL was found to be upregulated in an acquired resistance model of lapatinib. AXL was found to activate PI3K by engaging p85, and hence lapatinib and

trastuzumab were not effective. Use of AXL inhibitor was found to restore the sensitivity to anti-HER2 agents (Liu, Greger et al. 2009).

Intracellular kinases. Mutations in the PI3K/AKT signaling pathway have been reported in about 30% of the invasive tumors. The *PIK3CA* gene encodes for the p110 catalytic subunit of PI3K, while the *PIK3R1* gene encodes for the p85 regulatory subunit. Gain of function mutations were identified in *PIK3CA, AKT1* or *PIK3R1* genes which promote constitutive activation of PI3K. Also, loss of *PTEN,* a gene that encodes a lipid phosphatase that reverses the PIP_2 to PIP_3 reaction mediated by PI3K has been detected in many breast tumors. (Bellacosa, de Feo et al. 1995, Li, Yen et al. 1997, Bachman, Argani et al. 2004, Carpten, Faber et al. 2007, Gewinner, Wang et al. 2009). Since anti-HER2 agents act by inhibiting the PI3K signaling cascade, this gain of PI3K mutations or loss of PTEN would lead to unregulated PI3K and resistance to anti-HER2 agents.

Loss of PTEN or presence of *PIK3CA/PIK3R1* mutations conferred trastuzumab resistance. Low levels of PTEN and presence of “hotspot” PIK3CA mutations were associated with poor outcome observed in patients after trastuzumab plus chemotherapy treatment (Berns, Horlings et al. 2007). The combination of trastuzumab and a PI3K inhibitor, or trastuzumab or lapatinib with dual PI3K- mammalian target of rapamycin (mTOR) inhibitor inhibited the growth of PIK3CA mutant-harboring breast xenografts that were resistant to anti-HER2 therapy (Eichhorn, Gili et al. 2008, Serra, Markman et al. 2008, Chakrabarty, Rexer et al. 2010) . Under conditions of PTEN loss, the trastuzumab resistance was partially reversed by targeting AKT or mTOR (Lu,

Wyszomierski et al. 2007). The strategy of targeting PI3K signaling cascade along with HER2 has been tried clinically. BOLERO-I clinical trial evaluated the use of trastuzumab along with the mTOR inhibitor, everolimus and paclitaxel as the first line of therapy for patients with HER2 positive advanced breast cancer. However, no significant difference was observed in progression free survival, even though there was prolongation of 7.2 months in the combination arm.

In case of acquired resistance to lapatinib, upregulation of Src family kinases (SFK) was identified through mass spectrometry. Using an SFK inhibitor along with lapatinib restored lapatinib sensitivity in a xenograft model and partially blocked PI3K activation in resistant cells (Rexer, Ham et al. 2011). The combination of trastuzumab and the Src inhibitor was able to overcome tarstuzumab resistance caused by D16 HER2 isoform and by PTEN's inability to dephosphorylate Src (Mitra, Brumlik et al. 2009, Zhang, Huang et al. 2011). Trastuzumab resistance conferred by erythropoietin receptor is mediated partly by Src, as the receptor activates Src thorugh Jak2 and Src associates with HER2, phosphorylates PTEN and inactivates it (Liang, Esteva et al. 2010). Moreover, the EphA2 activation observed after chronic trastuzumab treatment and during trastuzumab resistance is also mediated by Src (Zhuang, Brantley-Sieders et al. 2010).

Defects in Apoptosis and Cell Cycle Control.

HER2 targeted therapies are known to arrest cell proliferation and induce apoptosis. The levels of pro-apoptotic BH3-only Bcl2 family member BIM were reported to be predictive of therapeutic response in HER2+ breast cancer. In that study, even

though lapatinib inhibited HER2 and downstream signaling, apoptosis was only observed in the cell lines that expressed higher levels of BIM, suggesting that BIM might be a predictive biomarker for lapatinib response in an oncogene-addicted cancer (Faber, Corcoran et al. 2011).

Survivin belongs to the inhibitor of apoptosis (IAP) protein family. It acts by inhibiting caspases whose activity is critical for programmed cell death. Lapatinib resistant HER2+ breast cancer cells show upregulation of ERα, which in turn causes transcription of survivin through forkhead box O3 (FOXO3)a. Higher survivin levels resulted in mediating lapatinib resistance (Xia, Bacus et al. 2006). Enhanced levels of survivin and anti-apoptotic protein Mcl-1 were observed in trastuzumab resistant cells (Valabrega, Capellero et al. 2011). Survivin levels are regulated in HER2+ amplified breast cancer cell line by PI3K signaling, and inhibiting HER2-PI3K was shown to lower the levels of survivin and cause apoptosis in HER2 overexpressing breast cancer cells (Faber, Li et al. 2009, Tanizaki, Okamoto et al. 2011).

Alteration in the genes that are involved in the progression of cell cycle are also known to play a role in mediating resistance to anti-HER2 therapy. Amplification of cyclin E was associated with lower response to trastuzumab in HER2+ breast cancer, and cyclin-dependent kinase 2 (CDK2) inhibitors were found to be effective against trastuzumab-resistant xenografts (Scaltriti, Eichhorn et al. 2011). Downregulation of Cdk inhibitor $p27^{KIP1}$ is associated with trastuzumab resistance (Nahta, Takahashi et al. 2004) and the modulation of $p27^{KIP1}$ level is the common endpoint for several resistance

related pathways including signaling from IGF-1R and MET (Lu, Zi et al. 2001, Nahta, Yuan et al. 2005, Shattuck, Miller et al. 2008).

Involvement of Host Factors that Modulate Drug Response.

ADCC is the killing of an antibody-coated target cell by cytotoxic effector cells through a nonphagocytic process, characterized by the release of the content of cytotoxic granules or by the expression of cell death-inducing molecules. Receptors for the Fc region of Ig (FcR) on the plasma membrane of the effector cell are necessary for ADCC activity (Teillaud 2012). Trastuzumab is known to cause ADCC. Natural killer (NK) cells can target trastuzumab bound HER2 overexpressing cells through CD16 (FcγRIII)-mediated ADCC (Clynes, Towers et al. 2000). Host factors that affect the immunomodulatory function can have an impact on trastuzumab resistance. Mice lacking FcγRIII, and thus deficient in NK cells and macrophages binding to Fc region showed reduced sensitivity to trastuzumab (Clynes, Towers et al. 2000). Polymorphism in the human gene encoding FcgRIII is associated with trastuzumab response. Leukocytes from patients who showed a better response to trastuzumab had higher ADCC activity *in-vitro,* as compared to those who did not (Gennari, Menard et al. 2004). A later study found that the quantity and activity of CD16(+) lymphocytes affect ADCC induction by trastuzumab treatment, and thus the tumor response (Varchetta, Gibelli et al. 2007).

Conclusion

Numerous mechanisms of *de novo* and acquired resistance to anti-HER2 agents have been proposed in the pre-clinical and clinical settings. Emerging data suggest that

targeting the HER2 signaling cascade at various points would lead to a more effective therapy, rather than the use of single agents. An important challenge is trying to identify the resistance mechanism in the individual patient to provide personalized therapy. In order to achieve this, it would be important to perform biopsies of the primary and metastasized tumors at regular intervals for pathological examination and molecular analysis. Pre-operative therapy using the combination of anti-HER2 agents should guide the treatment based on pathological response rate, and extensive molecular profiling should be performed on the residual drug resistant tumors to identify better treatment strategies. Furthermore, the crosstalk of HER2 signaling pathway with other signaling pathways should be studied and novel biomarkers to predict drug response and resistance should be identified.

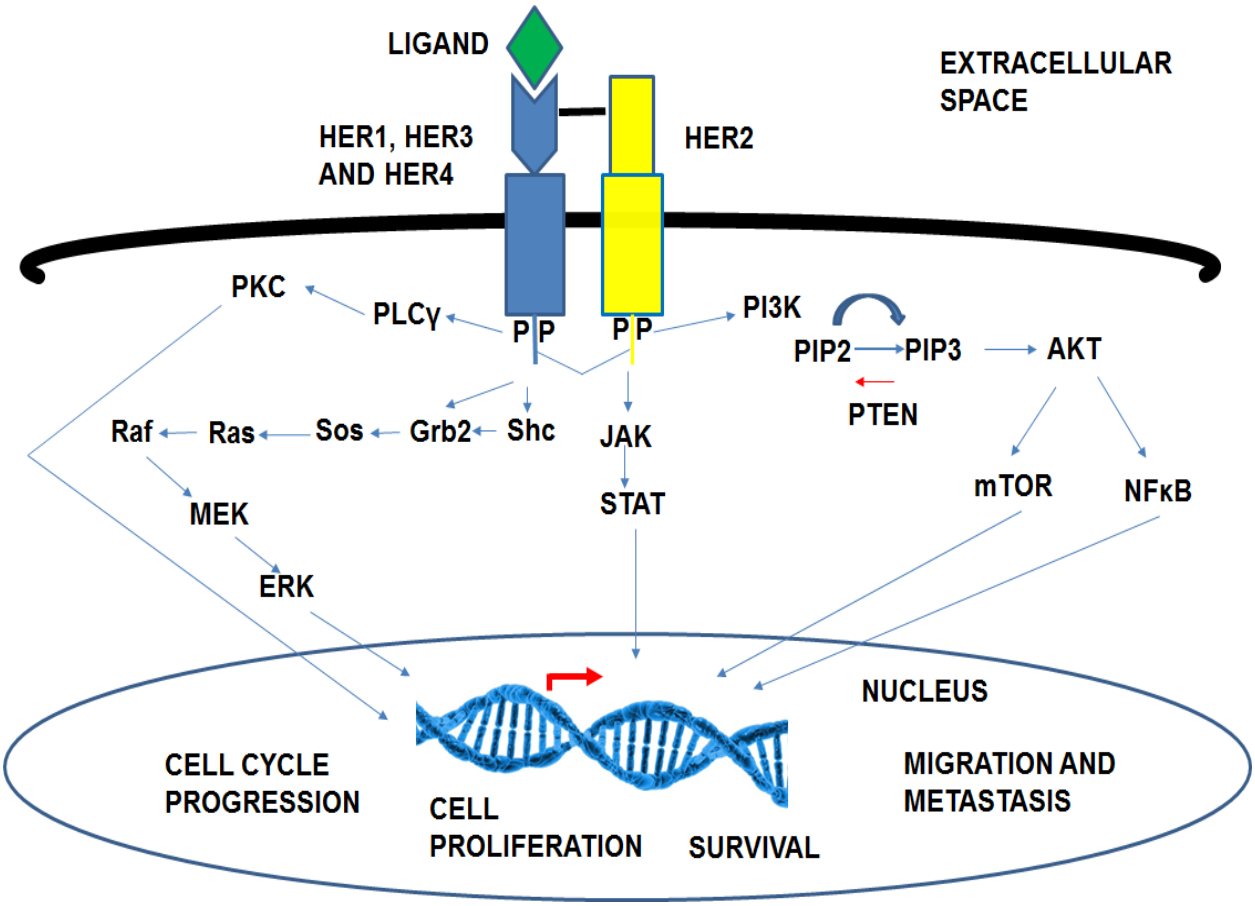


Figure 1: The HER Signaling Pathway.

CHAPTER II

CANCER STEM CELLS (CSCs) AND NOTCH SIGNALING: A LITERATURE REVIEW

The CSC Hypothesis

The drug resistance observed with the anti-HER2 agents could be explained by the presence of CSCs or tumor initiating cells (TICs). Since their identification in 1994, CSCs have received major attention in the field of cancer research, due to their ability to initiate and drive tumor growth, cause radiation and chemotherapy resistance, and promote tumor recurrence. The neoplastic subpopulation of CSCs is believed to comprise a subpopulation of the heterogeneous tumor mass. CSCs are at the apex of the hierarchy and possess the characteristics of self-renewal (yielding daughters that remain as CSCs), slow proliferation, and the ability to differentiate into multiple lineages of neoplastic cells that comprise the bulk of the tumor.

There are multiple theories regarding the origin of breast CSCs. According to some, dysregulation or mutations within normal cells leads to their transformation into CSCs. As per others, genetic modifications within the progenitor cells or normal stem cells can give rise to CSCs (Pattabiraman and Weinberg 2014, Bozorgi, Khazaei et al. 2015, Shah and Osipo 2016). Because of this discrepancy regarding the origin of CSCs, there is controversy in the field about the use of the term “CSCs”. Since the CSC fulfills the criteria of a true stem cell – i.e. it gives rise to a metastatic lesion by undergoing

self-renewal and recapitulating the entire tumor population, the nomenclature should hold valid (Jordan 2009). Figure 2 summarizes the CSC hypothesis.

Proposed Markers to Identify Breast CSCs.

Breast CSCs have been isolated from human tumors as well as from pleural effusions using flow cytometry. The cells were isolated based on expression of certain surface proteins such as $CD44^{+}/CD24^{-/low}/ESA^{+}$ (epithelial specific antigen). However, they lacked the expression of specific lineage markers (Lin^{-}). These cells showed enhanced tumorigenic potential, as compared to bulk tumor cells when injected into immune compromised mice. (Al-Hajj, Wicha et al. 2003). Since then, $CD44^{+}/CD24^{-/low}$ has been reliably used as a marker to isolate breast CSCs, and studies have reported the enrichment of these markers in basal like cell lines and primary breast carcinomas. CD44 is associated with stem cell characteristics and CD24 is related to differentiated epithelial features. CD44 is a specific receptor to hyaluronan and a cell surface glycoprotein. It is important for breast cancer adhesion, migration, and invasion. It is also involved in angiogenesis and cell proliferation. CD24 is another glycoprotein and when expressed at low levels, increases the ability of the tumor to grow and metastasize (Ricardo, Vieira et al. 2011, Bozorgi, Khazaei et al. 2015).

The other widely used marker for the isolation of breast CSCs is aldehyde dehydrogenase (ALDH), which is an enzyme involved in the oxidation of the aldehydes to carboxylic acids, i.e. acetaldehyde to acetic acid or formaldehyde to formic acid using NAD+ as a proton receptor. ALDH1A1 is the dominant form of the enzyme found in mammals and is involved in the conversion of retinaldehyde to retinoic acid. Through *in*

vitro and *in vivo* studies, Ginestier *et al.* showed that normal and cancer mammary epithelial cells that have enhanced ALDH activity show stem/progenitor properties. ALDH1A1+ CSCs are more resistant to platinum analogs, are more aggressive and predict poor patient prognosis (Ginestier, Hur et al. 2007, Ricardo, Vieira et al. 2011, Bozorgi, Khazaei et al. 2015).

Recently, cell lineage markers are used in tumor models to identify the hierarchy of CSCs and various studies have demonstrated that heterogeneity exists between CSC populations and that the cells have the capacity to interconvert between the stem and non-stem states (Owens and Naylor 2013). Whether different markers identify the same subpopulation or different remains unclear. Since $CD44^{+}/CD24^{-/low}$ and ALDH are the most widely studied markers for breast CSCs, a study was performed to study their relative distribution within the intrinsic molecular subtypes. Four hundred and sixty-six invasive breast carcinomas and eight breast cancer cell lines were used for the study. Basal like tumors contained a higher percentage of cells with $CD44^{+}CD24^{-/low}$ expression. About 40% of ALDH positive cases were also basal-like. The study of breast cancer cell lines showed that luminal cells were mainly $CD44^{-/low}CD24^{+}$, whereas basal/mesenchymal cell lines were enriched for the $CD44^{+}CD24^{-/low}$ phenotype. The remaining basal/epithelial cell lines were positive for both markers. ALDH activity was mainly observed in HER2 overexpressing basal/epithelial cell (Ricardo, Vieira et al. 2011). Recent work by Liu *et al.* suggests that ALDH is a better indicator for epithelial like breast CSCs and $CD44^{+}CD24^{-/low}$ is a better predictor of mesenchymal like CSCs.

Epithelial like CSCs are more localized and they proliferate rapidly, whereas mesenchymal like CSCs are more invasive and generally quiescent (Liu, Cong et al. 2014).

Besides these, there are other markers that have been used to identify breast CSCs. BRCA1 mutant tumors contain $CD44^{+}/CD24^{-}$/CD133+ cells (Wright, Calcagno et al. 2008) and ER negative breast CSCs possess CD44+ CD49fhi CD133/2hi phenotype (Meyer, Fleming et al. 2010). CD49f was identified as a breast CSC marker in MCF7 cell line (Cariati, Naderi et al. 2008), and CD61 was recognized as a breast CSC marker in certain mouse models of mammary tumorigenesis (Vaillant, Asselin-Labat et al. 2008). In a study performed using multiple breast cancer cell lines and primary tumors, different markers were expressed by different cell lines, and the cells obtained from primary tumors expressed varying amounts of different CSC markers. This suggests the presence of multiple lineages of human breast cancer stem/progenitor cells present within the heterogeneous breast tumor mass (Hwang-Verslues, Kuo et al. 2009).

In vitro propagation of human mammary stem/progenitor cells is studied by culturing the cells as mammospheres in a 3D culture system. The assay could be used for the quantification of stem cell activity and self-renewal of normal as well as cancer cells. Each mammosphere is believed to arise from individual stem cell and the quantification of the mammospheres gives an idea about the stem cell/ CSC frequency. It is calculated as % mammosphere forming efficiency (%MFE) -
%MFE = (Number of mammospheres formed/Number of cells seeded)*100. To assess the true self-renewal capacity, the primary mammospheres are disaggregated and

replated into subsequent medium conditions. Subsequent formation of secondary and tertiary mammosphere is evaluated and the result is indicative of the self-renewal capacity of the original CSC. (Shaw, Harrison et al. 2012).

The *In vivo* study is performed by using a limiting dilution assay. It remains the gold standard for the assessment of CSC frequency. The assay involves injecting SCID/NOD mice with different log fold dilution of cells (For example: 10 cells,100 cells, 1000 cells, 10k cells) and assessing tumor take. Based on the tumor take observed at each dilution, CSC frequency can be calculated using the extreme limiting dilution analysis (ELDA) software. The self-renewal is measured *in vivo* by re-implanting the tumor and assessing the tumor take (Hu and Smyth 2009).

HER2+ Breast CSCs.

The current work focuses primarily with breast CSCs from the HER2+ subtype of breast cancer. “However, it is important to note that HER2 is selectively expressed in HER2-, ER+ luminal breast CSCs and the HER2+ CSCs of HER2- breast cancer could be targeted by employing anti-HER2 agents as listed before (Ithimakin, Day et al. 2013). The HER2+ CD44 High/CD24 Low breast CSCs isolated from the HER2 negative breast cancer cells showed enhanced ALDH activity, invasiveness and *in vivo* tumorigenesis, as compared to HER2- breast CSCs (Duru, Fan et al. 2012).

HER2+ CSCs display a distinct genotype as compared to non-HER2+ CSCs through altered epigenetic regulation. HER2 strongly regulates genes related to stem cell and progenitor cell control (Rahmatpanah, Jia et al. 2012). Moreover, HER2+ CSCs display elevated levels of genes that promote S to G2/M transition and reduced

expression of genes that promote differentiation and immune response (Liu, Voisin et al. 2012, Shah and Osipo 2016).” The table below summarizes the distinctive features of HER2+ CSCs versus non-HER2+ CSCs-

Parameter	HER2+ CSC	Non-HER2+ CSC
HER2 overexpression	Observed	Not observed
Subtypes	Luminal A, Luminal B and HER2+	All subtypes
Anti-HER2 agents	Effective for treatment	Not effective for treatment
Phenotype	High ALDH activity, mammosphere formation, invasiveness, and tumorigenesis, as compared to Non-HER2+ CSC. Higher self-renewal and replicative potential.	Low ALDH activity, mammosphere formation, invasiveness, and tumorigenesis, as compared to HER2+ CSC. Lower self-renewal and replicative potential, as compared to HER2+ CSC.
Genotype	Increased regulation of genes related to stem cells and progenitor cell control. Increased expression of genes related to S/G2/M transition. Reduced expression of genes involved in differentiation and immune response.	Decreased regulation of genes related to stem cells and progenitor cell control, decreased expression of genes related to S/G2/M transition. Increased expression of genes involved in differentiation and immune response, as compared to HER2+ CSC.

Table 2: HER2+ CSCs Versus Non-HER2+ CSCs. (Shah and Osipo 2016)

Numerous signaling pathways have been implicated in the formation of CSCs in HER2+ breast cancer. These include developmentally conserved pathways like Notch, Wnt, and Sonic Hedgehog signaling. Besides these, other signaling pathways such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), IL-8-CXCR (Interleukin 8- CXC motif chemokine receptor), JAK/STAT (Janus kinase/signal

transducers and activators of transcription), TGF-β and PI3K-Akt signaling pathways have been involved in the differentiation and the self-renewal of HER2+ breast CSCs (Shah and Osipo 2016). The role of Notch signaling has been described in detail in the later part of this chapter.

EMT and CSCs.

EMT is a phenomenon whereby the cells lose their differentiation characteristics of cell-cell adhesion and migration and instead acquire mesenchymal traits resulting in enhanced motility and invasion, and resistance to apoptosis. It is associated with the loss of epithelial markers such as E-cadherin, and upregulation of mesenchymal markers including N-cadherin, vimentin, and fibronectin. EMT was initially studied for its importance during normal development. For neoplastic cells, EMT seems to confer a distinct advantage that allows cells to disseminate to distant sites and initiate metastases. EMT is known to be involved in the formation of breast CSCs (Mani, Guo et al. 2008, Morel, Lievre et al. 2008). Following hormonal/chemotherapy, breast cancer cells show an upregulation of a gene signature associated with EMT. This is indicative of epithelial-mesenchymal plasticity in the development of drug resistance. The surviving cells have gene determinants like breast CSCs and exhibit epithelial marker cytokeratin and mesenchymal marker vimentin (Creighton, Li et al. 2009, Farmer, Bonnefoi et al. 2009, Li, Xu et al. 2009, Creighton, Chang et al. 2010). EMT-related signals are believed to arise from the stroma which then induces EMT-transcription factors, whose expression results in the acquisition of mesenchymal traits. Based on the transcription factor that is involved, the cells can enter a stem-like-cell state,

mesenchymal-like state or both. Even though not reported, accumulating evidence points to normal stem cells and CSCs arising in epithelial tissues with a mixture of epithelial and mesenchymal characteristics, suggesting that they have partially undergone EMT (Guo, Keckesova et al. 2012, Pattabiraman and Weinberg 2014). The gene expression profile of the epithelial and mesenchymal breast CSCs was found to be similar across various molecular subtypes of breast cancer and resembles that of basal and luminal stem cells observed in normal breast. It is the plasticity of breast CSCs that allow them to transition between EMT and mesenchymal to epithelial transition (MET) states, and undergo migration, invasion, and metastases at distant sites (Liu, Cong et al. 2014).

In contrast to the findings regarding the role of EMT in the formation of breast CSCs, a recent report by Xie *et al.* describes that the tumor initiating capacity of breast cancer cell lines is independent of the EMT status. In the study, they observed that the induction of EMT resulted in $CD44^{+}/CD24^{-/low}$ phenotype, enhanced cell proliferation, migration, resistance to radiation and doxorubicin in breast cancer cell lines. The observed traits were attenuated by MET. However, neither EMT or EMT affected the tumor initiating capacity of the breast cancer cell lines, as measured by the limiting dilution assay. (Xie, Ji et al. 2014).

CSCs and Tumor Microenvironment.

"The tumor microenvironment is the environment within which the tumor exists and its constituents are cancer associated fibroblasts, leukocytes, mesenchymal stem cells, lymphatic and blood endothelial cells, extracellular matrix and the signaling

molecules. The tumor microenvironment is believed to provide a niche to CSCs which helps them to thrive and maintain an immature phenotype. The microenvironment helps the CSCs to evade the immune system and to undergo EMT and thus metastasize. The microenvironment is responsible for the genetic changes and epigenetic variation. CSCs activate or secrete various factors to maintain their survival. The tumor microenvironment also plays a pivotal role in the regulation of bidirectional CSC and non-CSC switch (Plaks, Kong et al. 2015). This could be possibly attributed to EMT/MET plasticity that is exhibited by the tumor cells, to establish themselves in the changing microenvironment at the primary and metastatic sites (Gao, Vahdat et al. 2012).

Tumor niche consists of a combination of cells that carry wild type HER2 and the ones that harbor activating HER2 mutations in the tyrosine kinase domain. Recent work by Wang et al demonstrates that the mutations impact the tumor microenvironment and mediates the upregulation of growth factors such as Vascular Endothelial Growth Factor (VEGF), TGF-β and EGFR ligands like amphiregulin and TGF-α. The secreted growth factors promote autocrine and paracrine signaling which favors tumor growth (Wang, Yu et al. 2010).

Cancer associated fibroblasts are known to mediate trastuzumab resistance in HER2+ breast cancer through secretion of interleukin 6 (IL-6), expansion of CSCs and by activation of multiple signaling pathways like NF-kB, JAK/STAT3 and PI3K/AKT (Mao, Zhang et al. 2015).

HER2 is selectively expressed in the CSCs of the HER2- subtype of breast cancer and this involvement of HER2 is regulated by the tumor microenvironment. HER2 expression is induced by Receptor Activator of Nuclear factor Kappa-B (RANK) ligand produced by bone osteoblasts. Moreover, higher levels of HER2 are reported in the bone metastatic setting as compared to the primary tumor and the circulating tumor cells of HER2- breast cancer are known to express HER2. Currently, clinical trials are underway to evaluate the efficacy of using a RANK ligand inhibitor denosumab along with adjuvant therapy for the prevention of recurrence in the bone or any other part, in women with early stage breast cancer who are prone to recurrence (Korkaya and Wicha 2013, Shah and Osipo 2016)."

Notch Signaling: A Literature Review

Introduction.

Notch signaling pathway was first discovered in 1914 through a genetic mutation screen in *Drosophila melanogaster* (Dexter 1914). Soon after that, the mutant alleles were identified and the flies that were affected showed a "notched" winged margin that was passed on from parent to progeny (Morgan 1917). The Notch signaling functions in a contact dependent fashion and it acts as a mediator of cell-cell communication. The signals relayed through the pathway are found to be critical in various cellular processes such as determining cell fates, promoting differentiation programs during development and during maintenance of self-renewing adult tissues by stem/progenitor cell maintenance, and, regulating cell death and cell proliferation. As Notch signaling impacts cellular processes in a wide variety of tissues, aberrant gain or loss of signaling

through its components has been implicated in numerous developmental disorders as well as adult diseases (Kopan and Ilagan 2009).

Notch Signaling Pathway.

Notch signaling works in a contact dependent fashion to mediate the cell-cell communication. The Notch ligand present on the signal sending cell engages with the Notch receptor present on the signal receiving cell. There are five known Notch ligands in mammals – Jagged1, Jagged2, Delta-like ligand-1 (DLL1), DLL3 and DLL4, and there are four known Notch receptors – Notch1, Notch2, Notch3, and Notch4.

Notch receptors. While the fly has only one Notch receptor, and worms have two redundant Notch receptors, the four mammalian Notch receptors can play a redundant as well as unique roles. The extracellular domain of the Notch receptors comprises of 29-36 tandem EGF repeats, some of which mediate ligand interaction. Activating interaction with the ligand present on the neighboring cell (*trans-activation*) is mediated by repeats 11-12, whereas inhibitory interaction (*cis-inhibition*) with the ligand present on the same cell is mediated by repeats 24-29. Notch mediated lateral inhibition regulates binary cell fate choice by activating Notch signaling in the neighboring cells in the *trans* fashion and by preventing Notch signaling in the *cis* fashion if the ligand and receptor are co-expressed on the same cell. Lateral inhibition is found to be important during development and it plays a critical role in specifying differentiated cells from a sheet of undifferentiated cells in a spatially regulated manner (Artavanis-Tsakonas, Rand et al. 1999). The negative regulatory region (NRR) that prevents the receptor activation in the absence of ligand binding, is followed by the EGF repeats. The NRR

consists of three cysteine-rich Lin12-Notch repeats and a heterodimerization domain. Cleavage of the Notch receptor by furin like convertase S1 results in the formation of Notch extracellular domain-Notch transmembrane and intracellular domain (NECD-NTMIC) heterodimer held together by non-covalent interactions (Ca^{2+}) between the N- and C-terminal halves of the heterodimerization domain. The transmembrane domain is followed by 3-4 Arg/Lys residues that provide the stop signal. The Notch intracellular domain comprises the RBPjκ association module (RAM) domain, a long unstructured linker containing nuclear localization signal (NLS) that links the RAM domain with the seven ankyrin repeats (ANK domain), an additional bipartite NLS, a transcriptional activator domain (TAD) and the PEST domain (proline-, glutamate-, serine-, threonine-rich motifs), which is critical in maintaining the stability of notch intracellular domain (NICD) (Kopan and Ilagan 2009). While Notch1 has a strong TAD, Notch2 has a weak TAD (Radtke and Raj 2003). Notch3 and Notch4 lack a TAD. Notch4 is the least conserved Notch receptor, having short extracellular and intracellular domains (Wu and Bresnick 2007). Figure 3 shows the domain organization of Notch receptors and DSL ligands.

Notch ligands. The Notch ligands are single-pass type I transmembrane proteins characterized by three structural motifs: an N-terminal DSL (Delta/Serrate/LAG-2) motif, specialized tandem EGF repeats called the DOS domain (Delta and OSM-11-like proteins, and EGF-like repeats (both calcium binding and non-calcium binding)). Both DSL and DOS domains are involved in receptor binding (Komatsu, Chao et al. 2008). The N terminal domain is conserved amongst the ligands, and can be subdivided

into two regions – N1 which is cysteine-rich and N2 which is cysteine-free(Parks, Stout et al. 2006). The Jagged ligands are longer than the Delta like ligands, the length determined by the 6-16 EGF repeats present in the extracellular domain. Each EGF repeat contains 6 cysteine residues, which form disulfide bridges facilitating protein stabilization (Komatsu, Chao et al. 2008). A cysteine-rich region is located at the end of EGF repeats, with Jagged ligands having an extra cysteine-rich region (Vitt, Hsu et al. 2001). Jagged ligands and DLL1 contains a DOS domain, whereas DLL3 and DLL4 don't (Komatsu, Chao et al. 2008). The intracellular domain exhibits the lowest homology among different ligands, and it consists of a shorter cytoplasmic tail than the extracellular domain. Except for DLL3, all the ligands contain multiple lysine residues, which are the sites of modification by the E3 ubiquitin ligases. Most DSL ligands have a PDZ (postsynaptic density protein [PSD95], *Drosophila* disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1])-binding motif which aids in intracellular protein–protein interactions (Pintar, De Biasio et al. 2007).

Jagged1. JAGGED1 (*JAG1*) is located on chromosome 20 at cytogenetic location 20p12.2 and genomic location (GRCh37) chr20:10,618,331–10,654,693. It has 26 exons and it spans over 36kb. The protein contains 1218 amino acids (Oda, Elkahloun et al. 1997). The 21-amino acid signal peptide present in the N-terminal region of Jagged1 ensures its localization into the membrane. The EGF repeats 1 and 2 of Jagged1 are primarily important for increasing the affinity of Jagged1 with the Notch receptors (Chillakuri, Sheppard et al. 2012). In humans, JAG1 expression varies during development and amongst different tissues. In the adult, JAG1 is highly expressed in

the heart, placenta, pancreas and prostate with lower levels found in lung, liver, kidney, thymus, testis and leucocytes (Jones, Clement-Jones et al. 2000, Gasperowicz and Otto 2008). A higher level of JAG1 mRNA and protein is associated with poor overall survival of women with breast cancer (Reedijk, Odorcic et al. 2005, Dickson, Mulligan et al. 2007). IL-6, Notch3, and β-catenin are known to promote transcriptional activation of Jagged1 (Sansone, Storci et al. 2007, Rodilla, Villanueva et al. 2009, Chen, Stoeck et al. 2010). Also, Rac1 is known to facilitate STAT3 mediated Jagged1 transcription. (Zhao, Du et al. 2016)

Canonical Notch Signaling Pathway.

Canonical Notch signaling involves activation of the Notch receptor present on the signal receiving cell through engagement with the DSL ligand present on the signal sending cell. The Notch receptor is initially synthesized as a 300 kDa precursor in the endoplasmic reticulum and cleaved into two subunits in the *trans*-Golgi compartment, which is then cleaved by furin like convertase(s) through S1 cleavage. This cleavage results into a heterodimer consisting of an extracellular N-terminal fragment and a transmembrane plus intracellular domain C-terminal fragment, linked together by a non-covalent linkage. During the synthesis and trafficking of Notch receptors, the extracellular portion of the receptor undergoes extensive *N* and *O*-linked glycosylation, which is critical for the proper folding of the receptor, and its subsequent interactions with the ligands. Glycosylation of Notch by Fringe glycosyltransferase enhances the affinity of the Notch receptor for Delta ligands but reduces its affinity for Jagged ligands. The three Fringe proteins expressed in mammals include- Lunatic fringe, Manic fringe

and Radical fringe (Fortini 2009). Binding of the Notch receptor with the Notch ligand results in an E3 ubiquitin ligase (Neuralized 1/2 or Mindbomb1/2) mediated endocytosis of the ligand via mono-ubiquitylation, which provides the necessary pulling force to expose the ADAM10/TACE/kuz/SUP-17 cleavage site present in the extracellular portion of the transmembrane Notch C-terminal fragment. The S2 cleavage results in ectodomain shedding of the extracellular portion of the transmembrane portion of the Notch receptor at approximately 12 amino acids outside the transmembrane domain (Brou, Logeat et al. 2000, Mumm and Kopan 2000). This leads to the formation of the carboxyterminal fragment called Notch extracellular truncation (NEXT) (Reiss and Saftig 2009). The ligand and the extracellular portion of the Notch receptor is endocytosed into the signal sending cell, where it undergoes endosomal degradation or recycling. NEXT subsequently undergoes S3 and S4 cleavages by the aspartyl protease complex-γ-secretase, which leads the formation of NICD (Mumm, Schroeter et al. 2000). The γ-secretase complex consists of a catalytic subunit designated presenilin 1 or presenilin 2, a seven-pass transmembrane protein, and accessory subunits comprised of the transmembrane proteins nicastrin, anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN-2), a two-pass transmembrane protein. PEN-2 is stabilized by nicastrin and APH1, and it mediates endoproteolysis of presenilin. The cleavage can occur at the cell surface or within the endosomal trafficking (Lai 2002). In the absence of NICD, the Notch target genes are repressed by the formation of the complex between CSL (C promoter binding factor-1 [CBF-1], suppressor of hairless, Lag-1) family of transcription factors and co-repressors. When the NICD translocates to the nucleus,

the ankyrin-repeat motif of NICD docks with the Rel homology region of the DNA-binding factor CSL and forms a transcriptional activation complex. This leads to removal of co-repressors such as class I or II histone deacetylases, CBF-1-interacting repressor (CIR), SKI-interacting protein (SKIP), silencing mediator of retinoid and thyroid hormone receptor (SMRT), and SMRT/HDAC (histone deacetylase)-1-associated repressor protein (SHARP), and a recruitment of transcription factor co-activators (CoAs) such as mastermind-like 1–3 (MAML) protein. MAML further recruits the histone acetyltransferases, cyclic AMP (adenosine monophosphate) response element binding (CREB) protein CBP/p300 and p300/CBP-associated factor or general control non-depressible 5 (GCN5), which then leads to histone acetylation, unraveling of the DNA wrapped around the histones, and active transcription of Notch target genes like hairy and enhancer of split basic helix-loop-helix HES 1-7, HEY 1, 2, and HEY-L family of proteins (transcriptional repressors), Deltex 1-4, cyclinD1, c-Myc, p21, and anti-apoptotic Bcl-2 (Schroeter, Kisslinger et al. 1998).

Sustained Notch activation could be deleterious. Hence, it is critical to tightly regulate the NICD turnover to prevent sustained signaling for a very long period or at excessively high levels. NICD, during the process of transcriptional activation, is phosphorylated on its PEST domain by cyclin C cyclin-dependent kinase-8 complex (Cyc: CDK8) and glycogen synthase kinase 3β (GSK-3β) and is targeted by E3 ubiquitin ligase Sel10/Fbw7 for proteasome-mediated degradation. This results in the disassembly of the CSL/NICD/MAML ternary complex, allows the cell to become ligand-competent and resets the signaling for a new cycle of activation (Wu, Lyapina et al.

2001). Numb is a negative regulator of Notch signaling, and about 50% of breast cancers lose Numb mediated control of Notch signaling, due to ubiquitylation and proteasomal degradation of Numb (Pece, Serresi et al. 2004). Figure 4 provides an overview of the Notch signaling pathway.

Notch Signaling in Mammary Gland Development.

Adult stem cells are important for organ development and for tissue homeostasis. A single mouse mammary stem cell has been shown to reconstitute the entire mammary gland (Shackleton, Vaillant et al. 2006). The recent identification of the progenitor population proves that mammary epithelium comprises of stem cells which give rise to differentiated epithelium through lineage restricted intermediates (Stingl, Eaves et al. 2001, Sleeman, Kendrick et al. 2006, Asselin-Labat, Sutherland et al. 2007, Villadsen, Fridriksdottir et al. 2007). The mammary gland comprises of ductal and alveolar epithelial cells, and myoepithelial cells. Extracellular signals from cytokines and hormones are presumed to control mammary stem cell activity and their progressive lineage differentiation through specific regulatory pathways (Hennighausen and Robinson 2001).

Several signaling pathways involved in cell fate and cell differentiation decisions and stem cell maintenance are critical for the development of mammary gland. Notch signaling is involved in several cell-fate decisions that occur through ligand-receptor interactions. Activation of the receptor by a Notch ligand can lead to the specific fate of a cell, whereas the absence of activation leads to an alternate fate (Artavanis-Tsakonas, Matsuno et al. 1995).

The Notch receptors, ligands, and the target genes are implicated in various stages of mammary gland development. Notch signaling can play an important role in mammary gland development by acting on stem cells as well as progenitor cells, affecting their self-renewal and lineage specific differentiation (Dontu, Jackson et al. 2004). The steady state levels of Notch3, Jagged1, DLL3, and Hey2 mRNA were found to be high during various stages of postnatal mammary gland development (Raafat, Goldhar et al. 2011). During pregnancy, canonical Notch signaling regulates alveolar cell maintenance and basal cell proliferation (Buono, Robinson et al. 2006). The Notch1 receptor is found to be expressed in the luminal compartment and its ectopic expression in the basal compartment induces luminal cell commitment (Bouras, Pal et al. 2008). During embryonic development, Notch-1 expressing cells are multipotent and can produce all mammary lineages, whereas postnatally they exclusively maintain ERα negative lineage. The ERα negative lineage possesses high self-renewal capacity, as demonstrated by the ability of Notch-1 expressing cells to form the complete mammary gland comprising of myoepithelial and luminal cells (Rodilla, Dasti et al. 2015). Notch3 activation was found to be critical for the restriction of bipotent progenitors to luminal cell fate commitment, and this role of Notch3 was found to be non-redundant. In contrast to the role of Notch3, Notch4 gene expression is highest in the undifferentiated mammary progenitors, whereas the expression is downregulated when the cells become committed to luminal lineage (Raouf, Zhao et al. 2008). Constitutive activation of Notch4 *in vitro* inhibits differentiation of normal breast epithelial cells (Uyttendaele, Soriano et al. 1998). *In vivo*, the constitutively active form of Notch4 results in a failure to develop

normal mammary gland, and subsequently develops mammary tumors (Soriano, Uyttendaele et al. 2000).

Thus, various components of the Notch signaling cascade are involved in normal mammary development, and its aberrant activation can lead to breast cancer by deregulating the self-renewal of normal mammary stem cells.

Notch Signaling in Cancer.

Involvement of Notch signaling in human cancer was first demonstrated in T cell acute lymphoblastic leukemia (T-ALL) in 1991, where Notch1 was associated with chromosomal translocation (Ellisen, Bird et al. 1991). It was later observed that most T-ALL cases were associated with mutations in Notch1, resulting in ligand-independent proteolytic cleavage of Notch1, an increased stability of NICD, resulting in constitutive activation of Notch signaling. Unlike in T-ALL, mutations are not frequently observed in Notch genes in solid tumors. However, aberrant activation of Notch signaling has been reported in solid tumors, which can occur due to loss of negative regulators, such as Numb, or due to altered expression of Notch receptors and ligands, as observed in prostate cancer, colorectal cancer, glioblastoma, cervical cancer, pancreatic cancer, renal cell carcinoma and breast cancer (Ranganathan, Weaver et al. 2011, Olsauskas-Kuprys, Zlobin et al. 2013). Interestingly, the tumor suppressor role of Notch signaling has been noted in mouse keratinocytes, hepatocellular and pancreatic carcinoma, and in small cell lung cancer (Koch and Radtke 2007). This dual role of Notch signaling could be explained by the crosstalk of Notch signaling with other pathways, the stage of cancer progression and the role of the tumor microenvironment.

Notch Signaling and Breast Cancer.

Many components of the Notch signaling pathway were found to be elevated in invasive breast cancer, including DLL1,3,4, Jagged1-2, Notch receptors, HES and HEY gene transcripts (Mittal, Subramanyam et al. 2009). Enhanced co-expression of Jagged1 and Notch1 has been linked with poor prognosis in breast cancer (Reedijk, Odorcic et al. 2005, Dickson, Mulligan et al. 2007). Moreover, Numb, the negative regulator(Zhang, Wang et al. 2010) of Notch has been found to be lost in about 50% of breast cancers through ubiquitylation and proteasome-mediated degradation (Pece, Serresi et al. 2004). Notch signaling has been shown to be activated in ER+ and HER2+ breast cancer following drug treatment (Osipo, Patel et al. 2008, Rizzo, Miao et al. 2008). Triple negative breast cancers were found to be enriched with activating mutations within the PEST domain of Notch1, Notch2 and Notch3(Wang, Zhang et al. 2015). Conditional deletion of Lunatic Fringe in the mammary gland led to Jagged1 mediated Notch signaling and formation of basal like mammary tumors (Xu, Usary et al. 2012).

The role of Notch receptors in breast cancer is evident from the *in vivo* studies performed using murine transgenic models. Overexpression of Notch1 NICD and Notch3 NICD mediated by the mouse mammary tumor virus (MMTV) transgenic mice led to inhibition of mammary gland development and induction of mammary tumors (Hu, Dievart et al. 2006). MMTV insertion in the NOTCH1 gene led to overexpression of mutant Notch protein, which was capable of transforming HC11 mouse mammary epithelial cells *in vitro* and generating spontaneous tumors in vivo (Dievart, Beaulieu et

al. 1999). Notch1 was also found to be a mediator of oncogenic Ras signaling. Mutated Ras plays a central role in breast oncogenesis (Weijzen, Rizzo et al. 2002). Notch3 knockdown reduced osteoblast and TGF-β1 stimulated colony formation in breast cancer cells. Moreover, osteolytic lesions were reduced upon inoculation of cells with reduced Notch3 expression, suggesting that Notch3 plays a critical role in breast cancer derived bone metastasis (Zhang, Wang et al. 2010).

Furthermore, Notch4 NICD, when expressed under whey acidic promoter (WAP) or under MMTV promoter led to the formation of mammary tumors in mice.(Gallahan, Jhappan et al. 1996, Gallahan and Callahan 1997). Transgenic expression of 1.8 kb Notch4 RNA in non-malignant human mammary epithelial cell line MCF10A enabled the cells to grow in soft agar, indicating the transformative potential of Notch4 (Imatani and Callahan 2000). Moreover, Notch4 was found to promote the invasion of the extracellular matrix and enhance the tumorigenic potential of mammary epithelial cells *in vitro* (Soriano, Uyttendaele et al. 2000, Callahan and Raafat 2001).

Contrary to the role of other Notch receptors, Notch2 expression was found to be associated with better survival in breast cancer patients, with high expression related with well-differentiated and poorly proliferative tumors(Parr, Watkins et al. 2004). Moreover, constitutively active Notch2 NICD led to increased apoptosis in MDA-MB-231 cells. Moreover, Notch2 NICD suppressed tumor take and growth in the MDA-MB-231 xenograft model, suggesting that Notch2 signaling is a potent inhibitory signal in breast cancer (O'Neill, Urs et al. 2007).

Notch Signaling and Breast CSCs.

The Notch signaling pathway is the most extensively studied in relation to CSCs. And several Notch inhibitors are being developed for investigation. Notch signaling has been implicated in the self-renewal of CSCs in various malignancies and in the tumor-stroma and tumor-endothelium interactions in CSC niches in primary and metastatic tumors (Pannuti, Foreman et al. 2010, Gu, Rizzo et al. 2012). Increased Notch1 NICD accumulation and HES1 expression were observed in ductal carcinoma *in situ* as compared to the normal mammary tissue (Farnie, Clarke et al. 2007). Breast CSCs have enhanced Notch activation as compared to the bulk tumor cells, and inhibition of Notch signaling reduces breast CSCs and prevents mammosphere formation (Grudzien, Lo et al. 2010). Notch1 and Notch4 receptors were found to be differentially expressed in breast CSCs and differentiated cells. Notch1 signaling was found to be important in the activation of HER2, and thus HER2 mediated CSCs (Magnifico, Albano et al. 2009). Robert Clarke and his colleagues reported that the breast CSC activity is preferentially dependent on Notch4, rather than Notch1, and that Notch4 is required for tumor initiation. They observed that pharmacologic or genetic inhibition of Notch1 or Notch4 reduced CSC activity *in vitro* and tumor formation *in vivo.* However, Notch4 inhibition had a more pronounced effect and caused complete inhibition of tumor formation *in vivo.* They propose a model whereby Notch4 regulates exit of BCSCs into the proliferating progenitor population, whereas Notch1 activity regulates progenitor proliferation and luminal differentiation (Harrison, Farnie et al. 2010). Notch3/Jagged1 were found to be involved in the CSC self-renewal and survival under hypoxic

conditions (Sansone, Storci et al. 2007). Recently, it has been reported that *de novo* and acquired resistance to anti-estrogens is driven by the Jagged1-Notch4 axis dependent breast CSC activity (Simoes, O'Brien et al. 2015).

Crosstalk of Notch Signaling With Other Signaling Pathways in Breast Cancer.

Notch signaling is important for cell proliferation, differentiation, cell survival, vasculogenesis, and angiogenesis. Notch expression is regulated by hypoxia and inflammatory cytokines like leptin, IL-6, and IL-8. Notch signaling is known to crosstalk with developmentally conserved Hedgehog and Wnt signaling pathways, with growth factors related signaling pathways including HER2/RTK signaling, platelet derived growth factor (PDGF)/ platelet derived growth factor receptor (PDGFR) signaling, TGF-β signaling and VEGF/ vascular endothelial growth factor receptor (VEGFR) signaling. Moreover, janus kinase/signal transducers and activators of transcription (Jak/STAT) signaling, Ras signaling, PI-3K/Akt signaling, mTOR signaling, nuclear factor-κB (NF-κB) signaling, hypoxia inducible factor (HIF) signaling, ER signaling as well as various micro-RNAs are known to crosstalk with Notch signaling pathway (Guo, Liu et al. 2011). We are going to focus on the crosstalk of Notch signaling with HER2 signaling, ER signaling, and polyoma enhancer activator 3 (PEA3). It is vital to identify the crosstalk of Notch signaling with other pathways critical for breast cancer, as it will allow the identification of novel biomarkers and lead to the development of better combinatorial therapies that would be more effective in preventing drug resistance and tumor recurrence.

Crosstalk between Notch1 and HER2 has been elucidated by our group and various others since. Low Notch activity was observed in HER2 overexpressing breast cancer cell lines. HER2 inhibition in the cells that were sensitive to trastuzumab resulted in an increase in Notch1 activity and trastuzumab resistant cells were found to express higher Notch1 and endogenous Notch1 targets. Treatment of trastuzumab sensitive or resistant cells with a gamma-secretase inhibitor (GSI) plus trastuzumab or Notch1 small interfering RNA (siRNA) resulted in a decrease in cell proliferation and an increase in apoptosis, suggesting that increased activation of Notch1 followed by anti-HER2 therapy might be responsible for survival and drug resistance (Osipo, Patel et al. 2008). In an *in vivo* model, we have shown that the Notch activation resulting from HER2 inhibition is responsible for tumor recurrence and that the combination of GSI and trastuzumab prevents tumor recurrence (Pandya, Meeke et al. 2011). A recent report found that Notch signaling is upregulated in tumor cells following HER2 inhibition, that Notch signaling remains activated in a subset of dormant tumor cells that survive after HER2 inhibition, that activation of Notch signaling accelerates tumor recurrence, and that Notch inhibition impairs tumor recurrence (Abravanel, Belka et al. 2015).

In contrast to this, Chen *et al.* have shown that Notch signaling could regulate HER2 expression as the HER2 promoter contains CSL binding sequences (Chen, Fischer et al. 1997). Another group has shown that downregulation of Notch1 by siRNA didn't affect cell proliferation of HER2+ breast cancer cell lines (Yamaguchi, Oyama et al. 2008). Magnifico *et al.* demonstrated that HER2 overexpressing cells have active Notch1 and that inhibition of Notch1 by siRNA or by GSI resulted in a decrease in

mammosphere formation and downregulation of HER2 expression. These studies reveal the importance of crosstalk between HER2 and Notch signaling and the need to investigate this crosstalk in more detail.

Crosstalk between Notch and ER was described by Rizzo *et al.* They found that in ERα+ breast cancer cells, 17 beta-estradiol inhibits Notch1 NICD levels, reduces notch activity and affects the cellular distribution of Notch1. Inhibition of ERα by tamoxifen or raloxifene resulted in reactivation of Notch activity. Notch1 induced Notch4. In clinical specimens, they observed that Notch4 expression correlated with higher levels of Ki-67, and thus cell proliferation. The combination of GSI and tamoxifen was found to be more effective in the regression of T47D: A18 induced xenograft tumors, indicating that the combination of Notch inhibitors and the anti-estrogens may be more effective in the treatment of ERα+ breast cancers (Rizzo, Miao et al. 2008). Another study revealed that Notch1 can activate ERα dependent transcription through IKKα in breast cancer cells, suggesting the possibility of a feedback loop controlling the Notch-ERα crosstalk (Hao, Rizzo et al. 2010). A recent report reveals that the treatment with antiestrogens results in the enrichment of breast CSCs through Jagged1-Notch4 activation mechanism in xenograft PDX tumors and patient-derived samples. The study suggests that the combination therapy involving antiestrogens along with Jagged1-Notch4 inhibition will be more beneficial in overcoming anti-estrogen resistance in human breast cancers (Simoes, O'Brien et al. 2015). Overall, the crosstalk between Notch and the ERα signaling pathway has a significant role in human breast tumor biology and importantly resistance to anti-estrogens.

For the triple negative subtype of breast cancer, PEA3, an Ets family transcription factor, has been shown to activate the transcription of Notch1 and Notch4. While PEA3 recruitment on the Notch-1 promoter was independent of AP-1 activity, PEA3 recruitment to the Notch-4 promoter was dependent on AP-1 activity: positively regulated by c-JUN and Fra-1 and negatively regulated by c-Fos. Combination therapy targeting PEA3 and Notch was shown to be beneficial for the treatment of a more aggressive triple negative subtype of breast cancer (Clementz, Rogowski et al. 2011).

Conclusion

CSCs have been implicated in drug resistance, radiation resistance, metastasis and tumor recurrence. They're identified in several solid tumors, including breast cancer. They have been shown to promote self-renewal, give rise to progeny that is different from them, and utilize various signaling pathways. There have been reports regarding the plasticity of CSCs and non-CSCs, suggesting that it would be critical to develop a combinatorial regimen that targets both CSCs and non-CSCs. This combinatorial approach would be critical for the complete elimination of tumor, and for the improvement of survival and quality of life of cancer patients.

In the context of HER2+ breast cancer, our group has identified a novel crosstalk between HER2 and Notch signaling. We have found that HER2 restricts Notch activation and that inhibiting HER2 by using anti-HER2 agents results in an increase in Notch activation, drug resistance, and tumor recurrence. Another study revealed that Notch signaling remains active in a subset of dormant tumor cells following HER2 inhibition, that the Notch activation is responsible for tumor recurrence, and that

inhibition of Notch signaling impairs tumor recurrence. Together, these studies support a role for Notch signaling in the formation of CSCs in HER2+ breast cancer. There have been reports about the involvement of Notch1 and Notch4 receptors in the formation of CSCs in HER2+ breast cancer. In the current study, we intend to identify the mechanism by which Notch signaling leads to the formation of CSCs in HER2+ breast cancer. This would help in the identification of the novel biomarkers and in the development of newer drug targets for the inhibition of Notch signaling.

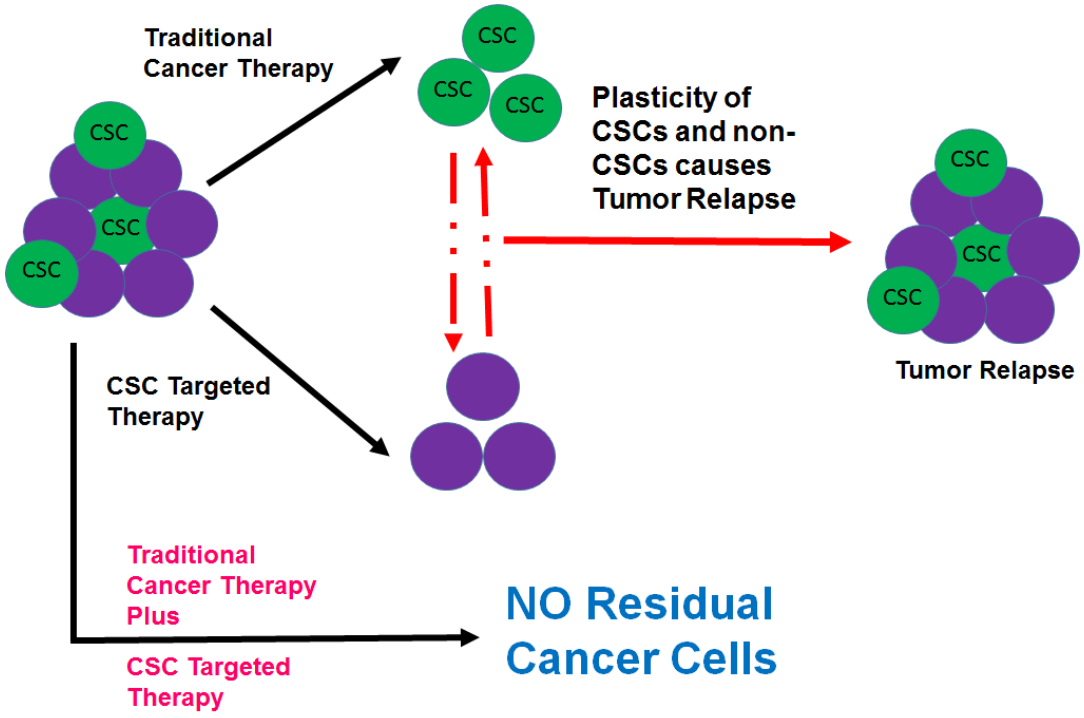


Figure 2: The CSC Hypothesis.

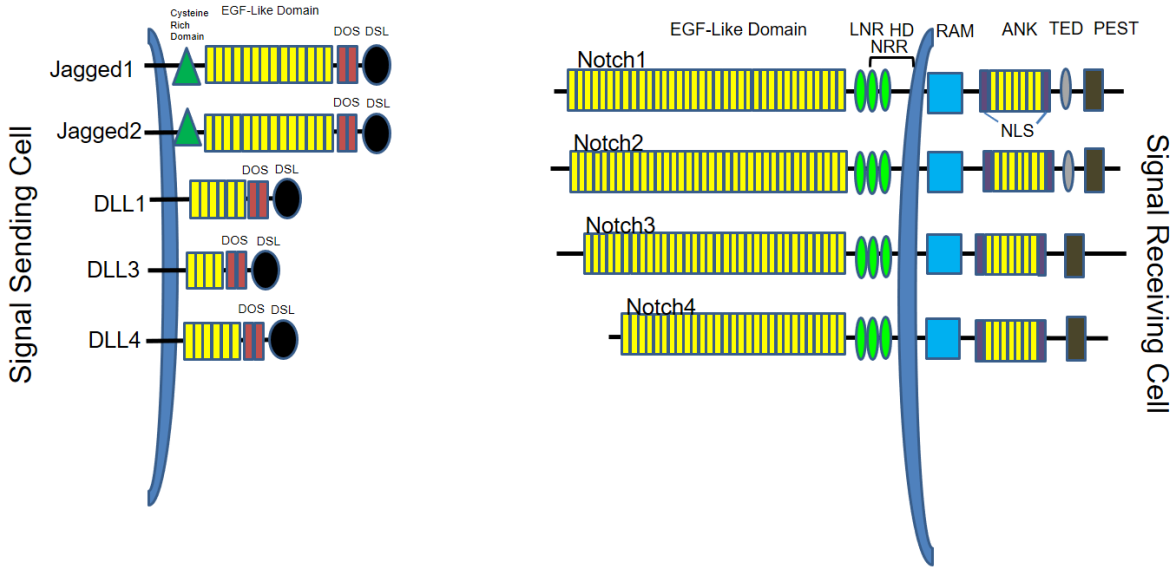


Figure 3: Domain Organization of Notch Receptors and DSL Ligands.

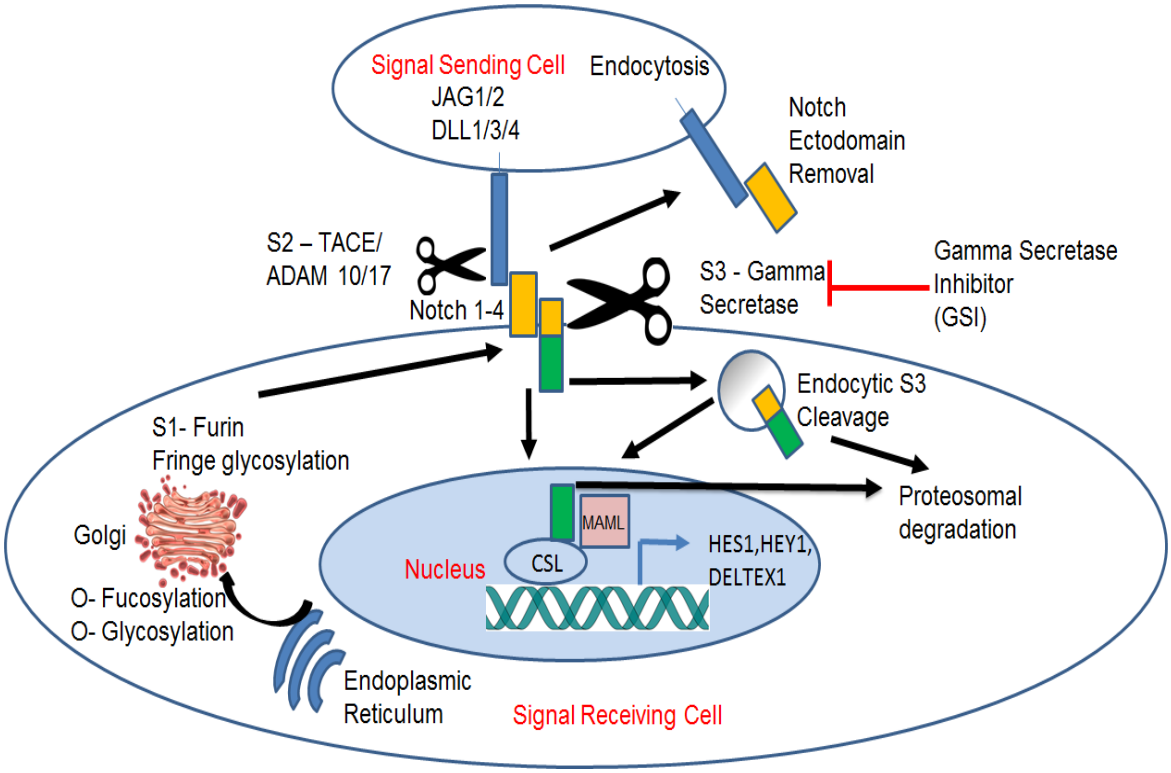


Figure 4: The Notch Signaling Pathway.

CHAPTER III

MATERIALS AND METHODS

Cell Culture

HCC1954, MDA-MB-453 and MCF7 cells were purchased from American Type Culture Collection (Manassas, VA). HCC1954 and MDA-MB-453 are HER2+ breast cancer cell lines. MCF7 is a luminal A subtype cell line and it lacks HER2 overexpression. MCF7 cells overexpressing HER2, designated as MCF7-HER2, were generously gifted by Dr. Mien-Chie Hung from The University of Texas MD Anderson Cancer Center in Houston, Texas. HCC1954 and MDA-MB-453 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Scientific, Waltham, MA), whereas MCF7 and MCF7-HER2 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Thermo Fischer Scientific, Waltham, MA). The cell culture medium was supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 1% L-glutamine (2 mM, Thermo Fischer Scientific, Waltham, MA) and 1% non-essential amino acids (100 µM, Invitrogen, Carlsbad, CA). All the cell lines were authenticated using short tandem repeat allelic profiling (DCC Medical). All the cell lines were maintained by incubating them at 37^0C with 95% O_2 and 5% CO_2.

Drugs, Chemicals, and Reagents

Gamma-Secretase Inhibitors

GSIs act by preventing the final proteolytic cleavage of the Notch receptor which is mediated by the gamma-secretase complex and thus prevents Notch activation. MRK-003 GSI was provided by Merck Oncology & Co. (Whitehouse Station, NJ). MRK-003 GSI is a small molecule inhibitor and it acts by binding to the binding pocket of presenilin, the catalytic subunit of gamma-secretase complex. For *in vitro* studies, 5 mM MRK-003 GSI stock solution was prepared by dissolving it in dimethyl sulfoxide (DMSO). The working concentration was 5 µM and the prepared drug was stored at -80^{0}C for future use.

Lapatinib

Lapatinib is a dual HER2-EGFR tyrosine kinase inhibitor. It was purchased from Selleck Chemicals (Houston, TX, Cat. S1028). For *in vitro* studies, 4 mM stock concentration of lapatinib was prepared by dissolving it in DMSO. The working concentration was 2 µM and the prepared drug was stored at -80^{0}C for future use.

Matrigel

Basement membranes are thin extracellular matrices underlying cells *in vivo*. Corning® Matrigel® Basement Membrane Matrix (Tewksbury, MA, Cat. 354234) is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen. Corning Matrigel Basement Membrane Matrix also contains TGF-β,

epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumor. Matrigel has been used for the CSC model and its use has been shown to enhance tumor growth rates *in vivo.*

RNA Interference and Transfection Reagents

Jagged1 stealth siRNA having two different sequences was purchased from Thermo Fisher Scientific (Waltham, MA, Cat. HSS176254 and HSS176255). HER2 siRNA was purchased from Origene (Rockville, MD, Cat. SR301443). Notch1 siRNA was purchased from Dharmacon (Lafayette, CO, Cat. HSS107248). Notch3 and Notch4 siRNAs were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, Cat. sc-37135 and sc-40137). Non-targeting scrambled control siRNA was purchased from Qiagen (Germantown, MD, Cat. 1027281). The transfection reagents Lipofectamine 3000 (Cat. L3000015) and Lipofectamine RNAimax (Cat. 13778150) were purchased from Thermo Fisher Scientific (Waltham, MA). Lipofectamine 3000 was used for Jagged1 knockdown and Lipofectamine RNAimax was used to knockdown HER2, Notch1, Notch3, and Notch4. The siRNAs were reconstituted with RNAse free water to yield a stock concentration of 10 µM. The final working concentration of the siRNA was 10 nM. For Jagged1 siRNA transfection, 17 µl siRNA and 17 µl of lipofectamine 300 were used in a 60mm plate. For HER2, Notch1, Notch3 and Notch4 siRNAs transfection, 20 µl of siRNA and 20 µl RNAimax was used in a 60mm plate. The transfection was performed according to the manufacturer's protocol. The sequences of each of the siRNA used is listed below -

siRNA	Sequence (5'-3')
Jagged1 A	GATAACTGTGCGAACATCACATTTA
Jagged1 B	CGCGACGAGTGTGACACATACTTCA
HER2	rGrCrCrArArCrArArArGrArArArUrCrUrUrArGrArCrGrAAG
Notch1	ACGAAGAACAGAAGCACAAAGGCGG
Notch3	GUCAGAAUUGUGAAGUGAAtt CUCGUCAGUUCUUAGAUCUtt CCUCUCAUUUCCUUACACUtt
Notch4	GAACCUGGAUGACUGUAUUtt GCAGGCAUAUGGGAUGUAAtt CCACUGCUAUCGCUAUUUAtt
Scrambled Control	Sequence held by Qiagen

Table 3: siRNA Sequences.

Antibodies

Phospho-HER2 (Tyr 1221/1222) (6B12) antibody was purchased from Cell Signaling Technology (Danvers, MA, Cat.2243). Total HER2 antibody (e2-4001 + 3B5) was purchased from Thermo Fisher Scientific (Waltham, MA, Cat. MA5-14057). Jagged1 (C20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, Cat. sc-6011). Notch3 antibody was purchased from Abcam (Cambridge, MA, Cat. ab23426). Notch1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, Cat. sc-376403). Notch4 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, Cat. sc-5594). Beta Actin (AC-15) antibody was purchased from Sigma-Aldrich (St. Louis, MO, Cat. A5441). Beta Actin was used as endogenous control for all the western blots. Secondary antibodies - donkey anti-mouse IgG-HRP (1:5000, Cat. sc-2314), donkey anti-rabbit IgG-HRP (1:2000, Cat. sc-2313),

and donkey anti-goat IgG-HRP (1:2000, Cat. sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western Blot Analysis

HCC1954, MDA-MB-453, MCF7 and MCF7-HER2 cells were transfected with siRNA and/or treated with 2 µM lapatinib for 4 days. The cells were maintained in the incubator at 37^0C with 95% O_2 and 5% CO_2. Followed by the transfection and/or treatment, the medium was aspirated and the cells were washed twice using ice cold phosphate-buffered saline (PBS). Depending upon the cell confluency and the size of the plate, 150 µl-300 µl of Triton X-100 lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% Triton X-100,150 mM sodium chloride (NaCl), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM sodium fluoride (NaF), 1 protease inhibitor cocktail tablet) was used to lyse the cells. The cells were then scraped using cell scraper, and the lysate was collected into Eppendorf tubes. The collected cell lysate was allowed to sit on ice for 10 minutes. Followed by that, the cells were sonicated thrice for 10 seconds each using the Sonic Dismembrator (Model 100, Thermo Fischer Scientific, Waltham, MA). After the sonication, the Eppendorf tubes were centrifuged at maximum speed for 30 minutes at 4^0C. The supernatant was collected after centrifugation, and the protein concentration was determined using Pierce bicinchoninic acid (BCA) Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, Cat. 23225). To 10 µl of test or standard protein sample (triplicates), 200 µl of the reagent mix (50 parts reagent A and 1 part reagent B) was added in the well of a 96 well

plate. Standards were prepared by dissolving bovine serum albumin (BSA) in PBS (Standards – 0 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml). The plate was incubated for 30 mins at 37^0C and was read using a 96 well plate reader at 562 nm. Based on the standard curve generated, the protein concentration of the test samples was determined. Based on the protein concentration, protein samples were prepared (20 µg – 50 µg) using 2X or 4X Laemmli buffer (BioRad, Hercules, CA, Cat. 1610737 or 1610747) and beta-mercaptoethanol (Thermo Fischer Scientific, Waltham, MA, Cat. BP-176-100). The prepared samples were boiled at 95^0C for 5 minutes. The denatured protein samples were then run on 7% tris-acetate gels (Thermo Fisher Scientific, Waltham, MA, Cat. EA0358BOX) along with HiMark Prestained protein standard (Thermo Fisher Scientific, Waltham, MA, Cat. LC5699) at 150V for 60 minutes. 20X NuPAGE Tris-Acetate SDS Running Buffer (Thermo Fisher Scientific, Waltham, MA, Cat. LA0041) was diluted using deionized water for the usage. The separated proteins were then transferred onto Polyvinylidene difluoride (PVDF) membrane. The transfer was performed at 40V for 120 minutes using 1X transfer buffer (NuPAGE® 20x Transfer buffer was diluted to 1x in deionized water, 20% methanol). The 20x transfer buffer was purchased from Invitrogen (Cat. NP006 Grand Island, NY). The transfer assembly included 3 sponges presoaked in transfer buffer for 30 mins, 1 filter paper, the gel, PVDF membrane presoaked in methanol and rinsed in water and transfer buffer, 1 filter paper and 3-4 presoaked sponges. After the transfer, the membrane was rewetted in methanol and water and was blocked using 5% non-fat dry milk, 20% Roche buffer or 5% BSA (used for phosphorylated proteins) for 1 hour at room temperature. TBST (5

mM Tris-HCl, 5 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, and 0.2% NP-40 at pH 8.0) was used for the dilution of non-fat dry milk or BSA, whereas tris-buffered saline (TBS) was used for the dilution of Roche buffer. After the blocking, the membrane was incubated overnight with the primary antibody at 4^0C with constant shaking. The primary antibody was diluted at the recommended concentration using the diluent that was used for blocking. The following day, the membrane was washed thrice using TBST. Each wash was for 10 minutes and the blots were shaken vigorously at room temperature. After the three washes, the secondary antibody was added and the membrane was incubated at room temperature for 1 hour, with constant rocking. The dilution buffer for the secondary antibody was the same as the one used for primary antibody. Followed by this, the membrane was again washed for three times using TBST as mentioned before. After the final wash, the proteins were detected by covering the membrane using enhanced chemiluminescence (ECL) Western blotting substrate (Pierce, Rockford, IL) or SuperSignal® West Dura (Thermo Fischer Scientific, Waltham, MA) added in 1:1 volume. The membrane was incubated for 1 minute at room temperature. The protein bands were visualized after allowing the membrane to expose onto the X-ray film in the dark room for the necessary timeframe.

To reprobe the membrane, it was washed in TBST at room temperature for 10 minutes. The membrane was then stripped using Restore Plus Western Blot Stripping buffer (Thermo Fischer Scientific, Waltham, MA). The membrane was covered with the stripping buffer and was incubated for 20 minutes at 37^0C. Followed by this, the

membrane was washed three times using TBST as mentioned before. The membrane was then blocked and probed with the primary antibody of interest.

Protein	Blocking Reagent	Antibody Dilution
Jagged1	20% Roche	1:1000
Notch1	20% Roche	1:500
Notch3	20% Roche	1:1000
Notch4	20% Roche	1:1000
Total HER2	5% nonfat dry milk	1:1000
Phospho-HER2 (Tyr 1221/1222)	5% BSA	1:500
Beta Actin	5% nonfat dry milk	1:5000

Table 4: Western Blotting Specifications.

Flow Cytometry

HCC1954 (250k cells/well – 6 well plate) and MDA-MB-453 cells (350k cells/well-6 well plate for treatment and 700k cells/well-60 mm plate for transfection) were either transfected with HER2 siRNA for 48 hours or treated for four days with 2 µM lapatinib and the impact of genetic or pharmacologic inhibition of HER2 on the surface expression of Jagged1 was assessed by using flow cytometry. Scrambled siRNA and DMSO were used as negative controls. For 4 days of lapatinib treatment, HCC1954 cells were trypsinized and replated after 2 days at a similar density. This was done to prevent the cells from becoming overconfluent. MCF7 (40k cells/well-6 well plate) and MCF7 HER2 (40k cells/well-6 well plate) were similarly treated for 4 days with 2 µM lapatinib using DMSO as a control.

The cultured cells were harvested using Cellstripper (Corning Cellgro, Manassas, VA, Cat. 25-056-CI). The harvested cells were neutralized using DMEM and the cell

suspension was centrifuged at 1300 rpm for 3 minutes. Followed by this, the cell pellet was resuspended in 2 ml flow cytometry staining buffer (R&D Systems, Minneapolis, MN, Cat. FC001) and the cell suspension was transferred into FACS tube. The cells were then washed twice with 2 ml flow cytometry staining buffer by centrifuging the cell suspension at 1300 rpm for 3 minutes. After the second wash, the excess staining buffer was aspirated, while leaving about 250 µl of buffer and the cell pellet. The cells were then stained using biotinylated human Jagged1 primary antibody (R&D Systems, Minneapolis, MN, Cat. BAF1277). About 6 µl of the primary antibody was added to the tube containing 100k-1 million cells. The cell suspension containing the antibody was then incubated for 45 minutes at room temperature. Followed by the incubation, the cells were washed twice using the flow cytometry staining buffer by centrifuging them at 1300 rpm for 3 minutes. After the second wash, excess staining buffer was aspirated, leaving behind around 250 µl. To this cell suspension, APC conjugated secondary antibody (BioLegend, San Diego, CA, Cat. 405207) was added. All the subsequent steps were performed in the dark. The secondary antibody was diluted 20-fold using flow cytometry staining buffer and then about 8 µl of the antibody was added to the tube containing 100k – 1 million cells. The tubes were then incubated at room temperature for 45 minutes. After the incubation, the tubes were washed twice with flow cytometry staining buffer as described before. The cell pellet was finally resuspended in 250 µl of flow cytometry staining buffer for analysis by using BD FACS Canto II (BD BioSciences, San Jose, CA). Data was attained using BD FACSDiva software. Data analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR).

For CD44 High/CD24 low assay, HCC1954 cells were harvested and stained per the protocol mentioned above. The cells were stained with APC conjugated CD44 (BioLegend, San Diego, CA, Cat. 103012)/ FITC conjugated CD44 (BioLegend, San Diego, CA, Cat. 103021) and PE conjugated CD24 (BioLegend, San Diego, CA, Cat. 311106), and were analyzed as mentioned above.

For assessing the surface expression of Notch receptors, Notch1-PE (BioLegend, San Diego, CA, Cat. 352106), Notch3-PE (BioLegend, San Diego, CA, Cat. 345406), and Notch4-PE (BioLegend, San Diego, CA, Cat. 128407) antibodies were used.

Cell Sorting

HCC1954 cells were treated with 2 µM lapatinib for 4 days, and were stained with biotinylated Jagged1 (40 µl) and streptavidin-APC antibodies (5 ul undiluted) as described before. To sort sufficient cells, 3 T150 flasks (4 million cells/flask) were used for DMSO treatment and 7-8 T150 flasks (4.5 million cells/flask) were used for lapatinib treatment. Followed by the staining, the cells were sorted based on Jagged1 surface expression (DMSO Jagged1-low and lapatinib Jagged1-high) by using BD FACSAria cell sorter (BD BioSciences, San Jose, CA). The cells were sorted into 24 well plate containing mammosphere medium (35k cells/well) supplemented with DMSO/ 5 µM GSI for performing mammosphere assay. The cells were sorted into the FACS tube for performing aldefluor assay, for CD44 high/CD24 low assessment, and for detecting the expression of Notch target genes. Followed by the sort in the tubes, the cell suspension was centrifuged at 1300 rpm for 3 minutes, and the cells were then used for performing

the necessary assay. The detailed protocols for mammosphere assay, aldefluor assay, CD44 high/CD24 low assessment and detection of Notch target genes using real-time polymerase chain reaction (PCR) have been described.

A control experiment was performed whereby HCC1954 cells (3 T150 flasks) were stained for the detection of Jagged1 surface expression as mentioned above, and Jagged1 high and Jagged1 low cells were sorted (25k cells/well) into 24 well plate containing mammosphere medium supplemented with DMSO/ 5 µM GSI. Mammosphere formation was assessed as described in the protocol.

Aldefluor Assay

ALDH1 is one of the 19 ALDH isoforms expressed in humans and is believed to be responsible for ALDH activity of CSCs. ALDH is a detoxifying enzyme and is responsible for the oxidation of intracellular aldehydes. Aldefluor kit (STEMCELL Technologies, Vancouver, Canada, Cat. 01700) was used to perform the aldefluor assay to detect the CSC population based on the enzymatic activity of ALDH1 isoform.

Two tubes containing 200k sorted/ unsorted HCC1954 cells suspended into 1ml of aldefluor assay buffer were prepared – a test tube that received 5 µl ALDH1 substrate, and the control tube that received 10 ul N, N-diethylaminobenzaldehyde (DEAB), the inhibitor of aldehyde dehydrogenase and 5 µl substrate. Followed by this, the tubes were incubated at 37^{o}C for 45 minutes in the dark. After the incubation, the tubes were put on the ice and were centrifuged for 5 mins at 250g. The supernatant was then removed and the cell pellet was resuspended, into 0.5 ml Aldefluor assay buffer.

The tubes were then put on the ice and were analyzed using flow cytometry as mentioned before.

Mammosphere Assay

Preparation of Methyl Cellulose Based Mammosphere Medium.

196 ml of DMEM-F12 medium (Gibco, Gaithersburg, MD, Cat. 11039021) was warmed at 60^{o}C and added to a bottle containing 2 grams of methyl cellulose. The contents were then allowed to stir at 60^{o}C for about 2.5 hrs. until methyl cellulose is uniformly mixed. Followed by this, the DMEM-F12 methyl cellulose medium was stirred overnight at 4^{o}C. The following day, 4 ml B-27 supplement and 4 µl recombinant hEGF (Sigma-Aldrich, Milwaukee, WI, Cat. E-9644) was added to the medium. The medium was then stirred at 4^{o}C for about 30 mins and then transferred into centrifuge tubes. The tubes were then spun at 9500 rpm in Beckman rotor at 4^{0}C for 30 mins. The supernatant (mammosphere medium) was poured into 50 ml conical tubes after centrifugation. The tube containing mammosphere medium was incubated in a bead bath at 37^{0}C for 2-3 hours prior to use. Excess mammosphere medium was stored at -20^{0}C.

Mammosphere Assay Protocol.

To perform mammosphere assay, the cells were harvested using trypsin or sorted. The single cell suspension was prepared and 100,000 cells/well (6 well ultra low attachment plate) or 35,000/25,000 cells/well (24 well ultra low attachment plate) were added into the mammosphere medium containing vehicle control DMSO or 5 µM MRK-003 GSI.

To study the effect of Jagged1, Notch1, Notch3 or Notch4 knockdown, 600k HCC1954 cells were plated into 60mm plate. 3 plates each were used for transfection of scrambled control siRNA or Jagged1, Notch1, Notch3, or Notch4 siRNA. The transfection was performed as mentioned before. After the transfection, the cells were harvested and were replated for DMSO or lapatinib treatment for 4 days. After the treatment, 100k cells in a single cell suspension was used for the mammosphere assay and the remaining cells were used to perform western blot. The western blot protocol is described later.

After the addition or sorting of the cells, the mammosphere plate was rocked back and forth several times to ensure that the cell suspension is evenly distributed across the well. The mammosphere plates were then left in the incubator at 37^0C and 5% CO_2 for seven days. After seven days, the mammospheres were harvested and counted.

To harvest the mammospheres, about 2 ml PBS was added to the well containing mammospheres. The mixture of PBS and mammospheres was pipetted gently a few times and was transferred into 15 ml conical tube. This process was repeated 3-4 times until all the mammospheres were harvested. The plate was observed under the microscope to ensure that all the mammospheres were harvested. The 15 ml conical tube containing the mammosphere suspension was spun down at 1500 rpm for 5 minutes. Followed by this, the supernatant was aspirated and 2 ml PBS was added to the mammosphere pellet. The mix was then transferred to a 2ml tube and was spun at 5000 rpm for 5 mins. One more wash with 2 ml PBS was performed and

then the mammosphere pellet was again resuspended in 2ml PBS (2 ml PBS was used as resuspension volume if 100k cells were plated. For 35,000 sorted cells, 500 µl PBS was used for resuspension). After resuspension, 50 µl was transferred into a well of a 96 well plate. 350 µl PBS was then added to the well of 96 well plate. Followed by this, the well containing mammospheres was observed under a microscope and five pictures were taken at 4X magnification. The pictures contained measurement scale. Taking five pictures ensured that the pictures captured all the mammospheres that were present in the well. The pictures were then transferred into PowerPoint and the mammospheres were manually counted, based on the scale present in the picture. The size cut-off was 50 µm i.e. all the spheres ≥ 50 µm were counted as mammospheres. Based on resuspension volume, the dilution factor was used to count the total mammospheres. %MFE was defined as

100*(Number of mammospheres/ number of cells plated).

***In Vivo* Study**

HCC1954 cells were treated with either vehicle control i.e. DMSO or 2 µM lapatinib for 4 days and then were sorted on the basis of Jagged1 surface expression. The sorted cells were diluted in PBS and mixed with Matrigel (1:1 ratio) before injecting them into mammary fat pads of ovariectomized FoxN1 nu/nu athymic nude mice (Harlan Sprague-Dawley, Madison, WI). 100 µl of cell suspension was injected at each site. During the study, each mouse and its tumor were tracked using an ear tag. To perform limiting dilution assay to detect the frequency of CSC formation, the mice were randomized into following groups–

100/ 1k/ 10k Vehicle Control i.e. DMSO Jagged Low cells/site (n=8 or 10 per group)

100/ 1k/ 10k Lapatinib Jagged High cells/site (n=8 or 10 per group)

One week after the injection, the tumor formation was assessed weekly. Tumors were measured by using Vernier calipers and the tumor area (l x w) was calculated and graphed. At the end of seven weeks, the number of mice that developed tumor ≥ 40 mm^2 were counted and the tumor initiating potential was calculated by using the ELDA software (Hu and Smyth 2009). All the protocols for the animal study were approved by Loyola University's Institutional Animal Care and Use Committee (IACUC).

Immunohistochemical Staining of Human HER2+ Breast Tumors

The HER2+ breast tumor tissue was allowed to adhere by placing the tissue microarray sections in oven at 58^{o}C to 60^{o}C. The sections were deparaffinized using xylene, rehydrated through graded ethanol and washed with PBS prior to 1X Reveal treatment in a Decloaking Chamber (Biocare Medical) for antigen retrieval. The procedure was performed according to the manufacturer's protocol. Followed by the PBS rinse for 15 minutes, the sections were soaked in 3%H_2O_2 in PBS for 20 minutes to quench endogenous peroxidase activity. To block non-specific binding, the sections were incubated for one hour in 3% normal rabbit serum (Vector Laboratories) in PBS at room temperature. After blocking, the sections were probed with primary Jagged1 antibody prepared in PBS containing 1.5% normal rabbit serum for one hour and were incubated in a hydrated chamber at room temperature. Tissue Microarray (TMA) sections incubated with 1µg/ml normal goat IgG (Santa Cruz Biotechnologies, CA) were used as negative controls. Pilot experiments were performed to determine the best

antibody concentration and to minimize the background. Following extensive washing, antigen-antibody complexes were detected using the Vectastain Elite ABC kit (Vector Laboratories, CA) as per the manufacturer's protocol. Staining was performed with ImmPactTM DAB peroxidase substrate kit (Vector Laboratories, CA). Sections were then counterstained with Gill's hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting under a coverslip using Cytoseal XYL. The levels of Jagged1 protein expression in each specimen were scored per the extent (percent of stained cells) and intensity of staining. The score for the extent of the IHC stained area was scaled as 0 for no IHC signal at all and 1 for 10–80 tumor cells stained. Stained slides were sent to Nottingham, England where they were scanned. High-resolution images were uploaded to the Nottingham web-accessible scoring site, and were scored by 2 independent investigators (He Zhu and Lucio Miele). Intensity scores (0-1), percent staining scores and H-Scores were uploaded as Excel spreadsheets and survival analysis was performed using SPSS.

Real-Time PCR

HCC1954 cells were treated for four days with 2 μM lapatinib. Followed by this, the cells were sorted on the basis of Jagged1 surface expression as mentioned before. For unsorted cells, the total RNA was extracted according to the manufacturer's protocol using RiboPure RNA Purification kit (Ambion, Austin, TX, Cat. AM1924). For the cells that were sorted on the basis of Jagged1 surface expression, RNeasy Plus Micro Kit (Qiagen, Germantown, MD, Cat. 74034) was used for RNA extraction, as this kit is more suitable for small number of cells. After the extraction, the RNA yield was

determined by using the NanoDrop Spectrophotometer (Thermo Fischer Scientific, Waltham, MA). cDNA was synthesized through reverse transcription by using 1 µg RNA in 100 µl volume containing 1X RT buffer, 5.5 mM MgCl2, 500 µM dNTPs, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitors, and 1.25 U/µl RT enzyme (MultiScribe™ Reverse Transcriptase Kit, Applied Biosystems, Foster City, CA, Cat. N8080234). For reverse transcription, the parameters were as follows: 10 minutes at 25^0C, 30 minutes at 48^0C, 5 minutes at 95^0C, 60 minutes at 25^0C, and at 4^0C until use. After the cDNA was synthesized, real-time PCR was performed using iTaq™ SYBR® Green Supermix with ROX (BioRad, Hercules, CA).to detect the transcript levels of Notch receptors and Notch target genes. In a 96 well plate, 2.5 µl of prepared cDNA was added to 22.5 µl of mastermix containing 2X SYBR Green Universal Master Mix, and 50 µM forward and reverse primers of the intended target. The assay was performed in triplicates. The plate was then sealed to prevent evaporation and quantitative Real Time PCR was performed using following parameters: the initial denature temperature was for 10 minutes at 95^0C; PCR cycling for 40 cycles was carried out for 10 seconds at 95^0C, and for 45 seconds at 60^0C. A melt curve was added after completion of the 40 cycles set by the StepOnePlus thermocycler manufacturer (Applied Biosystems, Foster City, CA). At the end of the run, relative expression of various genes in different samples was calculated based on the C_t value. C_t value indicates the number of cycles that were required to detect a real signal from the sample. Lower C_t value is suggestive of more abundant gene expression in the sample, as fewer cycles were required to generate a real signal. Similarly, higher C_t value is indicative of less abundant gene expression in

the sample. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used as endogenous control for normalizing the C_t values. Relative fold change in the gene expression between different samples was calculated on the basis of $2^{(-\text{Delta Delta } C_t)}$ method-

Delta C_t sample = (C_t value of gene of interest (sample) - C_t value of HPRT(sample))

Delta C_t control = (C_t value of gene of interest (control) - C_t value of HPRT(control))

Delta Delta C_t = (Delta C_t sample - Delta C_t control)

Fold change in gene expression = $2^{(-\text{Delta Delta } C_t)}$

The primer sequences that were used for detecting the gene expression of various genes are listed below:

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
Notch1	ATCAACGCCGTAGATGACC	TTGTTAGCCCCGTTCTTCAG
Notch2	TGGTTCAGAGAAAACATACA	TCCACTTCATACTCACAGTTGA
Notch3	TCCTGGCTACAATGGTGATAAC	ATTAATCTCGCAGAGCACCC
Notch4	TGCAGGCATATGGGATGTAA	CATCCCCACAGTGGAGTTCT
Jagged1	GACTCATCAGCCGTGTCTCA	TGGGGAACACTCACACTCAA
HES1	CGGACATTCTGGAAATGACA	CATTGATCTGGGTCATGCAG
HEY1	TCATTTGGAGTGTTGGTGGA	CTCGCACACCATGATCACTT
CYCLIND1	GTCCATGCGGAAGATCGTC	GCGGTCCAGGTAGTTCATG
HES4	GAGCGCGTATTAACGAGAGCCT	CTCACGGTCATCTCCAGGATGT
C-MYC	CCTACCCTCTCAACGACAGC	CTCTGACCTTTTGCCAGGAG
HEY2	AAAAGGCGTCGGGATCG	AGCTTTTTCTAACTTTGCAGATCC
HPRT	ATGAACCAGGTTATGACCTTGAT	CCTGTTGACTGGTCATTACAATA

Table 5: Primer Sequences.

Statistical Analysis

Most of the experiments were performed three times and the results were reported as Mean± Standard Deviation (S.D.). For data involving two comparisons,

Microsoft Excel was used to perform a two-tailed Student's *t*-test. Multiple comparisons were assessed using analysis of variance (ANOVA) (Tukey's test for multiple comparisons) and GraphPad Prism 6 was used for the analysis. All the graphs were generated using GraphPad Prism 6.

For real-time PCR, the graphs were plotted based on the Relative Quantitation (RQ) value, and the error bars represented S.D.. To determine the statistical significance, delta C_t values were used for performing the *t*-test.

ELDA software was used to calculate CSC frequency for the limiting dilution *in vivo* study.

Kaplan-Meier curve for the overall survival of women suffering from HER2+ breast cancer was created through GraphPad Prism 6 and the data was analyzed with a log-rank Mantel-Cox test.

CHAPTER IV

HYPOTHESIS AND SPECIFIC AIMS

Despite improved prognosis for women with the HER2-positive subtype of breast cancer due to the availability of targeted therapy, drug resistance, and tumor recurrence remains a major concern. About 20-50% of the women that are suffering from HER2+ metastatic breast cancer exhibit intrinsic resistance whereas 10-15% of the women treated with trastuzumab plus adjuvant chemotherapy develop acquired resistance within the first year of drug treatment (Hudis 2007, Valabrega, Montemurro et al. 2007)

Previous findings suggest that HER2 restricts Notch activation and inhibition of HER2 using inhibitors such as trastuzumab or lapatinib results in Notch1 activation (Osipo, Patel et al. 2008). Studies reveal that high levels of Notch1 receptor and its ligand Jagged1 correlate with worst prognosis for women with breast cancer (Reedijk, Odorcic et al. 2005). We have previously shown that Notch1 and Jagged1 contribute to trastuzumab resistance (Osipo, Patel et al. 2008, Pandya, Wyatt et al. 2016). Furthermore, the *in vivo* work revealed that dual blockade of HER2 and Notch activation prevents HER2+ tumor recurrence suggesting the critical role of Notch activation in a subset of cells for HER2+ tumor recurrence (Pandya, Meeke et al. 2011). Moreover, there have been reports about the roles of Notch1, Notch3 or Notch4 receptors for the survival of breast CSCs (Yao, Rizzo et al. 2011). However, the exact mechanism by which Notch activation upon HER2 blockade leads to the formation of a subset of cells

responsible for drug resistance and tumor recurrence [also known as CSCs] is yet unknown.

Since Notch signaling works in a contact dependent fashion with the ligand being expressed on the signal sending cell and the receptor being expressed on the signal receiving cell, we hypothesized that HER2 restricts Notch activation through its ligand Jagged1 as high levels of Jagged1 are sufficient to predict poor outcome in women with breast cancer (Reedijk, Pinnaduwage et al. 2008). Previous work suggests that increased Notch activation in response to HER2 blockade is dependent on Jagged1 expression. Furthermore, dual blockade of HER2 and Jagged1 inhibited cell proliferation *in vitro* and reversed drug resistance *in vivo* (Pandya, Wyatt et al. 2016). Taken together, these results suggest that Jagged1-mediated Notch activation upon HER2 inhibition leads to cell proliferation, drug resistance and possibly tumor recurrence. In addition, previous work has shown that Jagged1 is involved in the survival and self-renewal of breast CSCs from ER+ or the triple negative basal- like subtype of breast cancer (Sansone, Storci et al. 2007, Xu, Usary et al. 2012). Based on the preliminary data, I hypothesize that-

HER2 blockade of HER2+ breast cancer cells enriches a Jagged1-positive subset of CSCs that are resistant to anti-HER2 treatment and responsible for tumor initiation.

The hypothesis will be tested by following aims-

Specific Aim 1-Determine whether surface expression of Jagged1 is critical for the formation of CSCs.

Specific Aim 2-Assess the ability of Jagged1 high subpopulation to form a tumor *in vivo* and to measure its CSC potential.

Data from our studies will delineate the mechanism by which Notch activation upon HER2 inhibition leads to the formation of CSCs in HER2+ breast cancer. It will elucidate for the first time that HER2 inhibits the formation of CSCs by limiting the expression of Jagged1 on the cell surface and thus Jagged1 mediated Notch activation. Moreover, our studies would identify Jagged-1 as a potential novel therapeutic target for preventing the enrichment of CSCs that mediate drug resistance and tumor recurrence in HER2+ breast cancer. Furthermore, our studies would provide a preclinical proof of concept for future clinical trials using HER2 inhibitor in combination with Notch pathway inhibitor (Jagged1 targeted therapy) to inhibit the enrichment of CSCs and thus prevent drug resistance and tumor recurrence in HER2+ breast cancer.

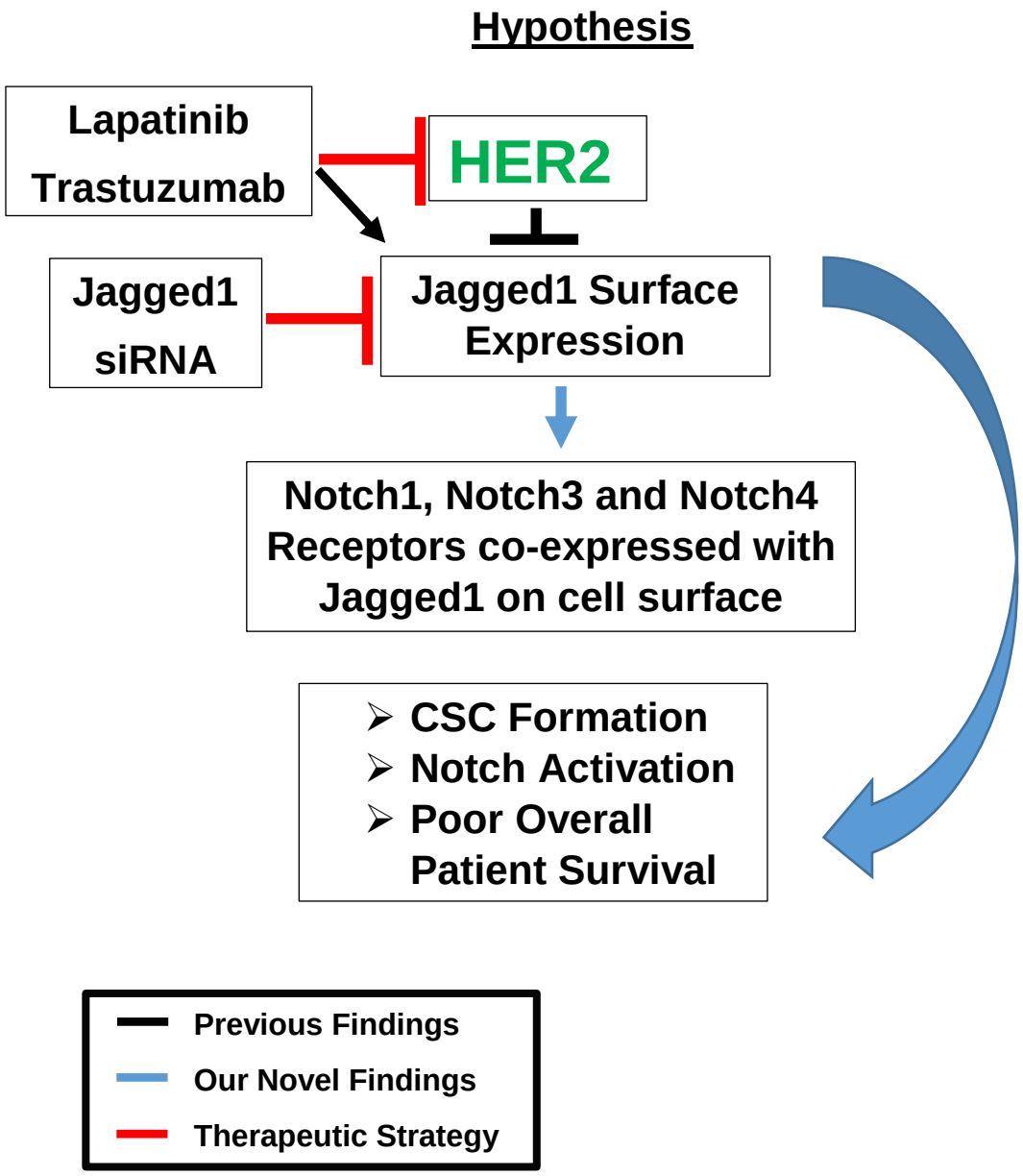


Figure 5: Hypothesis of the Investigation.

HER2 restricts Jagged1 surface expression. Inhibition of HER2 results in an increase in Jagged1 on cell surface leading to higher Notch activation, CSC formation and poor overall patient survival. Dual inhibition of Jagged1 and HER2 will effectively destroy bulk cells as well as CSCs, and thus improve overall patient survival.

CHAPTER V

RESULTS

Specific Aim 1–Determine Whether Surface Expression of Jagged1 Is Critical for the Formation of CSCs

Specific Aim 1 will be tested by following subaims:

Aim 1A-Determine whether Jagged1-high subpopulation upon lapatinib treatment results in an increase in the formation of CSCs as compared to Jagged1-low subpopulation in the absence of lapatinib treatment. CSCs would be assessed by %MFE, aldehyde dehydrogenase activity and the levels of CD44 High/CD24 Low.

Aim 1B-Assess whether Jagged1 is necessary for lapatinib mediated enrichment of mammospheres in the HER2+ subtype of breast cancer.

Aim 1C-Measure the degree of Notch activation in Jagged1-high subpopulation upon lapatinib treatment and in the Jagged1-low subpopulation in the absence of lapatinib treatment.

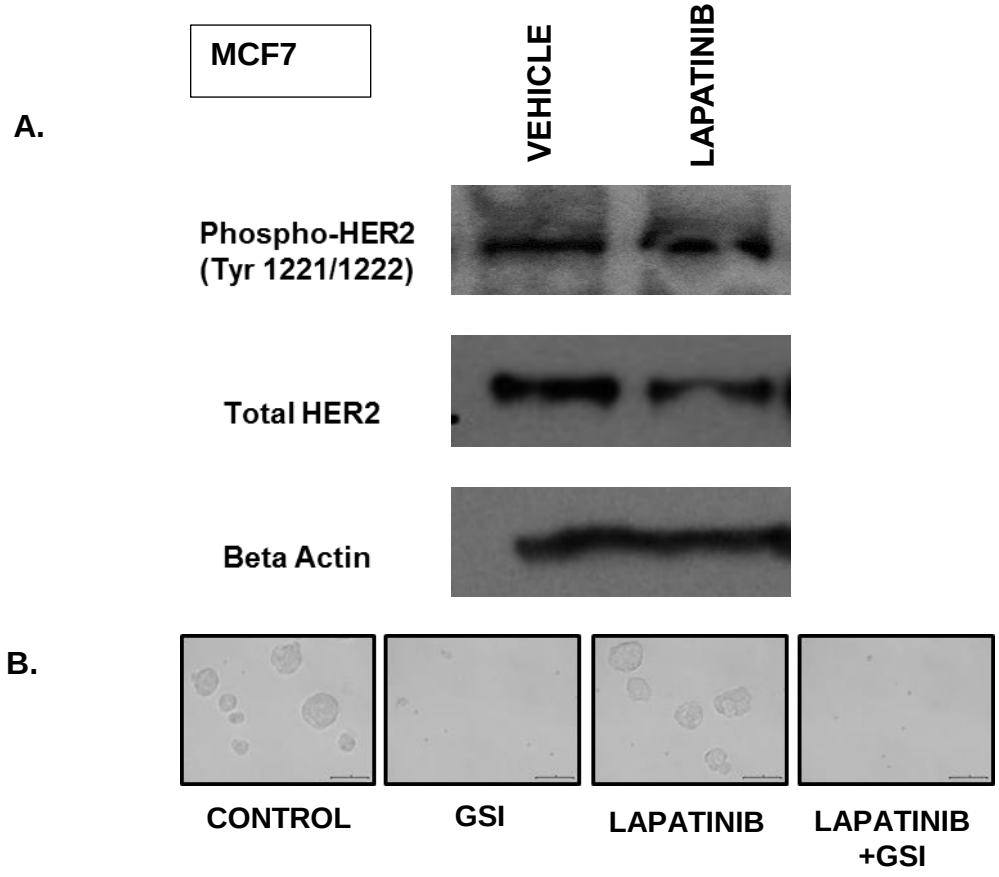
Aim 1D-Identify the Notch receptor that interacts with Jagged1 to cause lapatinib mediated enrichment of mammospheres in the HER2+ subtype of breast cancer.

Since Notch activation was required for tumor recurrence, we wanted to test if Notch activation is necessary for the formation of CSCs derived from HER2+ breast cancer cells.

To address this, HCC1954, MCF7, and MCF7-HER2 cells were treated for 4 days with 2 µM lapatinib. HCC1954 is a HER2+ breast cancer cell line. MCF7 cells do not overexpress HER2, whereas MCF7-HER2 cells constitutively overexpress HER2. DMSO was used as a vehicle control. In order to ensure that lapatinib was able to effectively inhibit the tyrosine kinase activity of HER2, protein was harvested and Western blot was performed to detect phosphor-HER2 (Tyr 1221/1222), total HER2 and Beta actin in MCF7 (Figure 6A), MCF7-HER2 (Figure 6D), and HCC1954 cells (Figure 6G). Beta actin was used as a loading control.

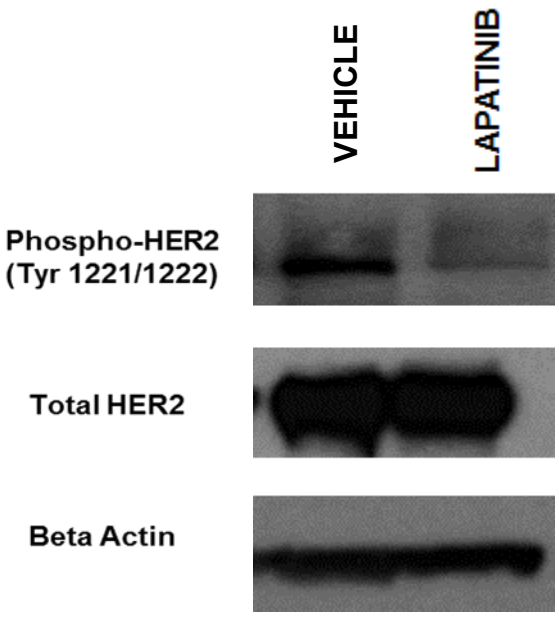
For the mammosphere assay, after the drug treatment, the cells were harvested and the total number of cells present in the single cell suspension were counted. 50,000 MCF7 cells/MCF7- HER2 cells and 100,000 HCC1954 cells (DMSO or lapatinib treated) were plated in an ultra-low attachment plate containing mammosphere medium supplemented with 5 µM GSI or vehicle control DMSO. The mammosphere formation was assessed after 7 days and representative pictures were taken [MCF7 (Figure 6B), MCF7-HER2 (Figure 6E), and HCC1954 (Figure 6H)]. The mammospheres were then collected and %MFE was determined. The results showed that HER2 inhibition using lapatinib results in an increase in survival of CSCs as measured by %MFE only in HER2+ breast cancer cells [MCF7-HER2 (Figure 6F) or HCC1954 (Figure 6I)] and not in non-overexpressing cells (MCF7) (Figure 6C). Mammosphere formation of all cells was prevented using a GSI, confirming reports that Notch activation is mediated by gamma-secretase cleavage. This suggested that HER2 inhibition resulted in

enhancement of CSC formation, as assessed by mammopshere forming efficiency, and this increase was dependent on Notch activation.



MCF7-HER2

D.



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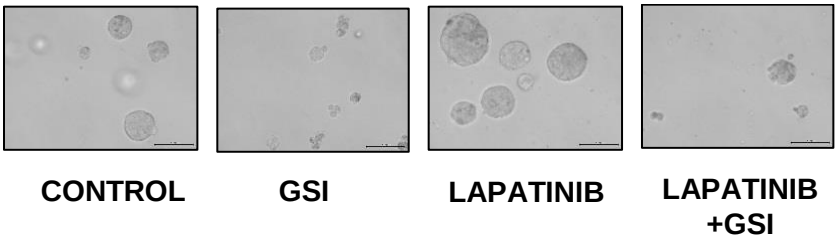

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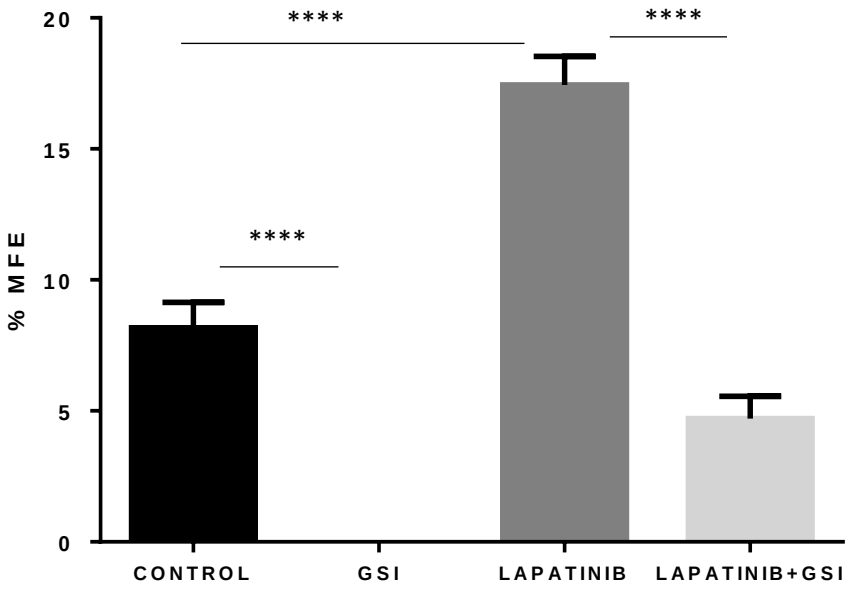

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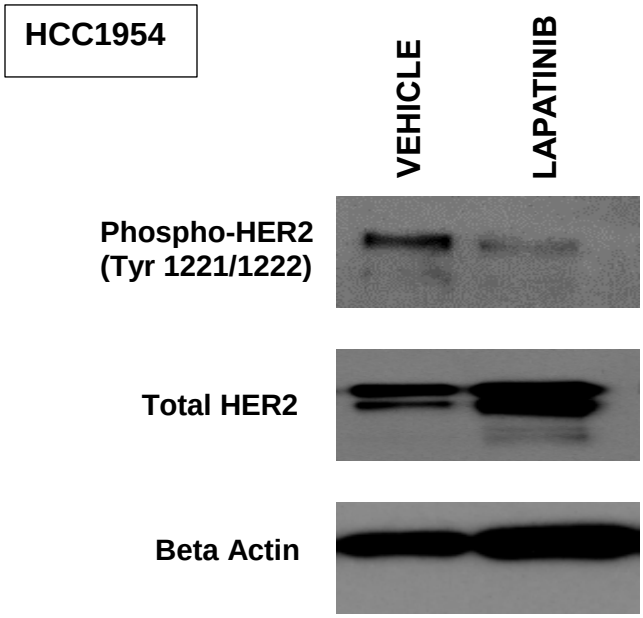
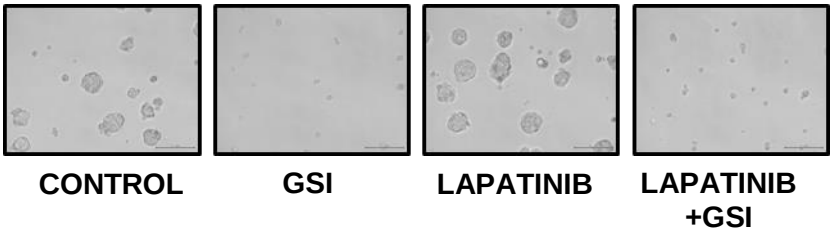

HCC1954
VEHICLE
LAPATINIB
Phospho-HER2
(Tyr 1221/1222)
Total HER2
Beta Actin

H.


CONTROL
GSI
LAPATINIB
LAPATINIB
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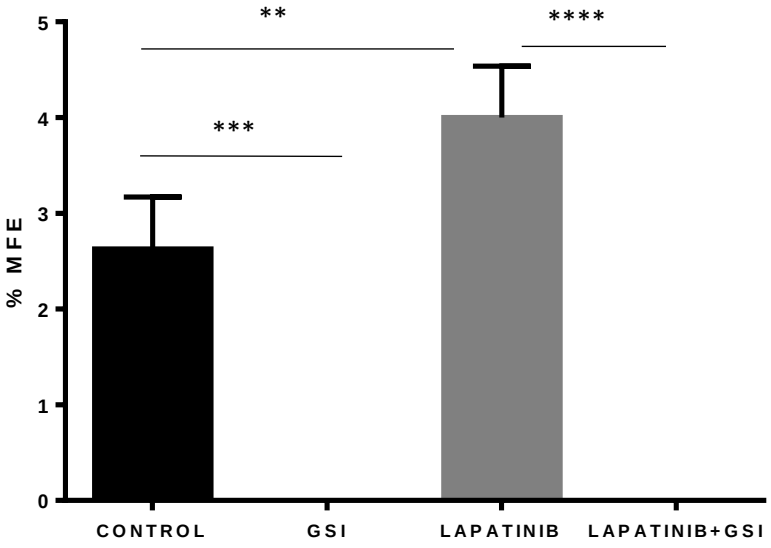
I.


**
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% MFE
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Figure 6: Lapatinib Mediated Inhibition of HER2 Tyrosine Kinase Activity Results in Notch Activation Dependent Increase in Mammosphere Formation.

MCF7 cells (HER2-, i.e. lacks HER2 overexpression), MCF7-HER2 cells (HER2+), and HCC1954 cells (HER2+) were treated with vehicle control i.e. DMSO/ 2 µM lapatinib for 4 days and mammosphere forming assay was performed.

(A) MCF7, (D) MCF7-HER2, and (G) HCC1954. Representative western blot showing the effect of lapatinib treatment on the levels of phosphorylated HER2 (Tyr 1221/1222) and total HER2. Beta actin was used as a loading control.

(B) MCF7, (E) MCF7-HER2, and (H) HCC1954.Effect of lapatinib treatment on mammosphere formation was assessed after 7 days. The pictures are representative of three independent experiments.

(C) MCF7, (F) MCF7-HER2, and (I) HCC1954.The %MFE was determined and the data represents the average of three independent experiments. Error bars represent S.D. and the statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Since the data supported the hypothesis that HER2 restricted the Notch activation, and previously we showed that Jagged1 was required for the Notch activation (Pandya, Wyatt et al. 2016), the question that we asked was – does HER2 affect the surface expression of Jagged1? Higher Jagged1 mRNA and protein levels do predict poor prognosis for women with breast cancer (Dickson, Mulligan et al. 2007). To address this question, HER2+ cells: HCC1954, MDA-MB-453, MCF7-HER2 and HER2-low MCF7 cells were treated with 2 µM lapatinib for 4 days, and the surface expression of Jagged1 was measured by using flow cytometry. Figure 7 demonstrates the effectiveness of lapatinib treatment on phosphorylation of HER2 in MDA-MB-453 cells. Figure 6A, 6D and 6G shows the effect of lapatinib treatment on HER2 phosphorylation in MCF7, MCF7-HER2 and HCC1954 cells respectively. HER2 inhibition using lapatinib

resulted in an increase in the surface expression of Jagged1 in HCC1954 (Figure 8A), MDA-MB-453 (Figure 8B), and MCF7 HER2 cells (Figure 8D). No effect of lapatinib was observed in the MCF7 cells that do not overexpress HER2 but have higher levels of EGFR (Figure 8C). This would suggest that the increase in Jagged1 surface levels observed with lapatinib treatment is specifically due to inhibition of HER2 tyrosine kinase activity and not by lapatinib mediated inhibition of EGFR tyrosine kinase activity. To genetically inhibit HER2, HCC1954 and MDA-MB-453 cells were transfected with a HER2 siRNA or scrambled control siRNA for 48 hours. Followed by the transfection, the cells were harvested and the surface expression of Jagged1 was measured by flow cytometry (Figure 9A and Figure 9C). Western blot was performed to assess the efficiency of HER2 knockdown (Figure 9B and Figure 9D). Similar to pharmacologic inhibition of HER2, genetic depletion of HER2 resulted in an increase in surface expression of Jagged1, suggesting that HER2 restricts the surface expression of Jagged1.

As HER2 inhibition led to an increase in the surface expression of Jagged1, we wanted to test whether there was an increase in the Jagged1 mRNA and total Jagged1 protein. For this purpose, HCC1954 cells were treated for 4 days with 2 μM lapatinib and real-time PCR, and Western blot was performed to detect the levels of Jagged1 mRNA and Jagged1 protein respectively. There was an increase in the Jagged1 mRNA upon lapatinib mediated HER2 inhibition (Figure 10A). However, Jagged1 protein decreased upon lapatinib treatment (Figure 10C). As lapatinib treatment affected the levels of beta actin, Ponceau staining was also performed to ensure that the loading

was even across the samples (Figure 10B). These results reveal that even though the total protein is decreased, there is an increase in the surface levels of Jagged1 protein. The results suggest that this might be a trafficking mediated event, causing either decreased endocytosis of Jagged1 from the cell surface or enhanced recycling of Jagged1 from the endosome onto the cell surface.

Aim 1 is based on the hypothesis that the increase in surface expression of Jagged1 upon HER2 inhibition leads to an increase in the Notch signaling dependent formation of CSCs.

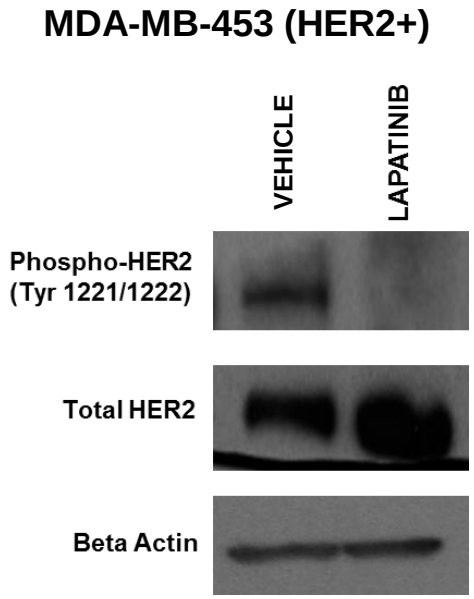


Figure 7: Lapatinib Effectively Inhibits Tyrosine Kinase Activity of HER2 in MDA-MB-453 Cells.

MDA-MB-453 cells were treated with vehicle control i.e. DMSO/ 2 µM lapatinib for 4 days. To ensure that lapatinib is able to effectively inhibit the phosphorylation of HER2, protein was harvested and western blot was performed to detect the phosphorylation of HER2 (Tyr 1221/Tyr 1222), total HER2 and Beta actin. Beta actin was used as a loading control.

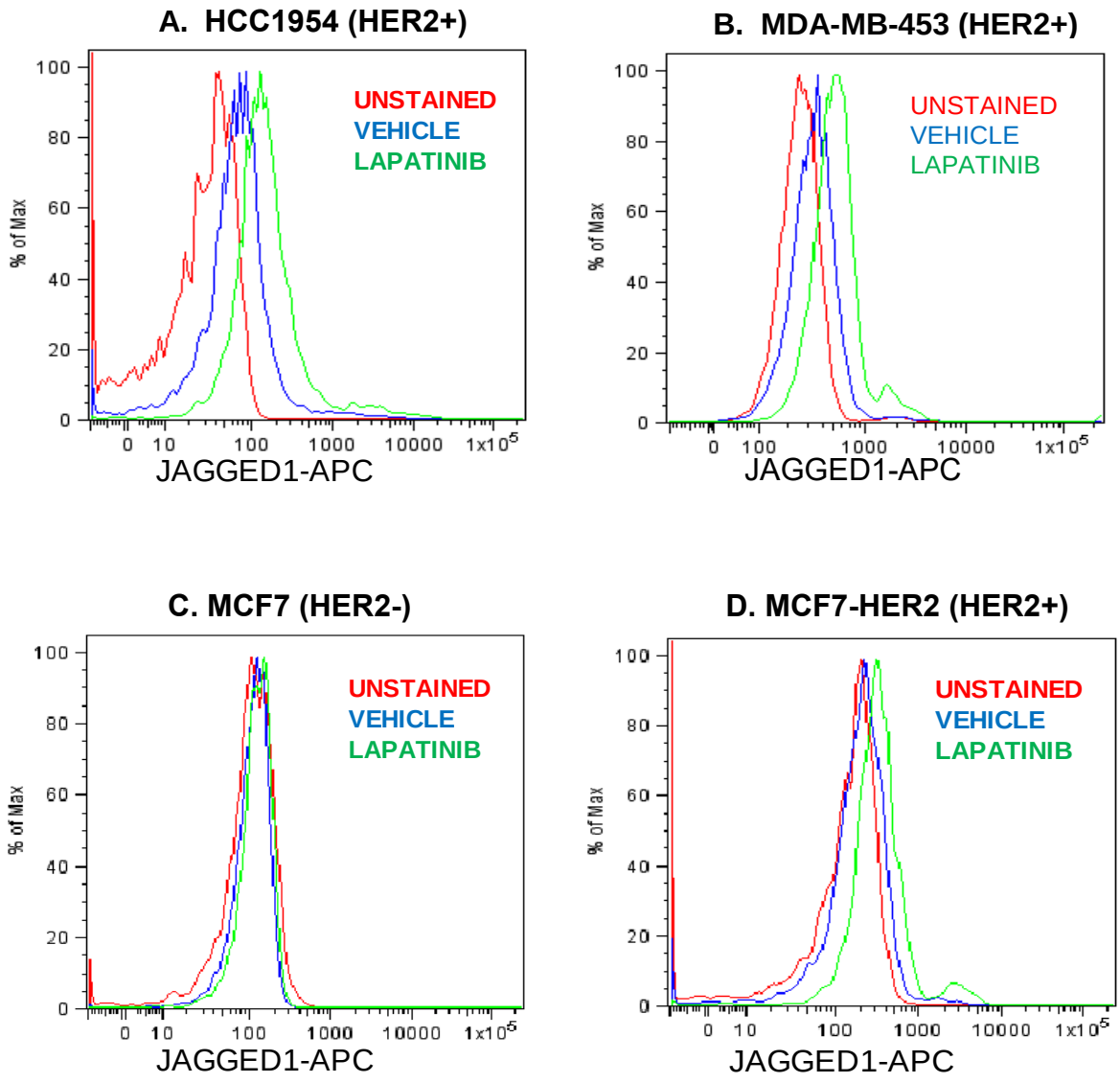


Figure 8: Pharmacological Inhibition of HER2 Increases the Surface Expression of Jagged1.

Various breast cancer cell lines were treated with vehicle DMSO/ 2 μM lapatinib for 4 days and the surface expression of Jagged1 was measured by using flow cytometry.

(A) Flow cytometry analysis for Jagged1 surface expression in HCC1954 cells. The picture is representative of three independent experiments. Similar study was performed using MDA-MB-453 (B), MCF7 (C), and MCF7-HER2 cells (D).

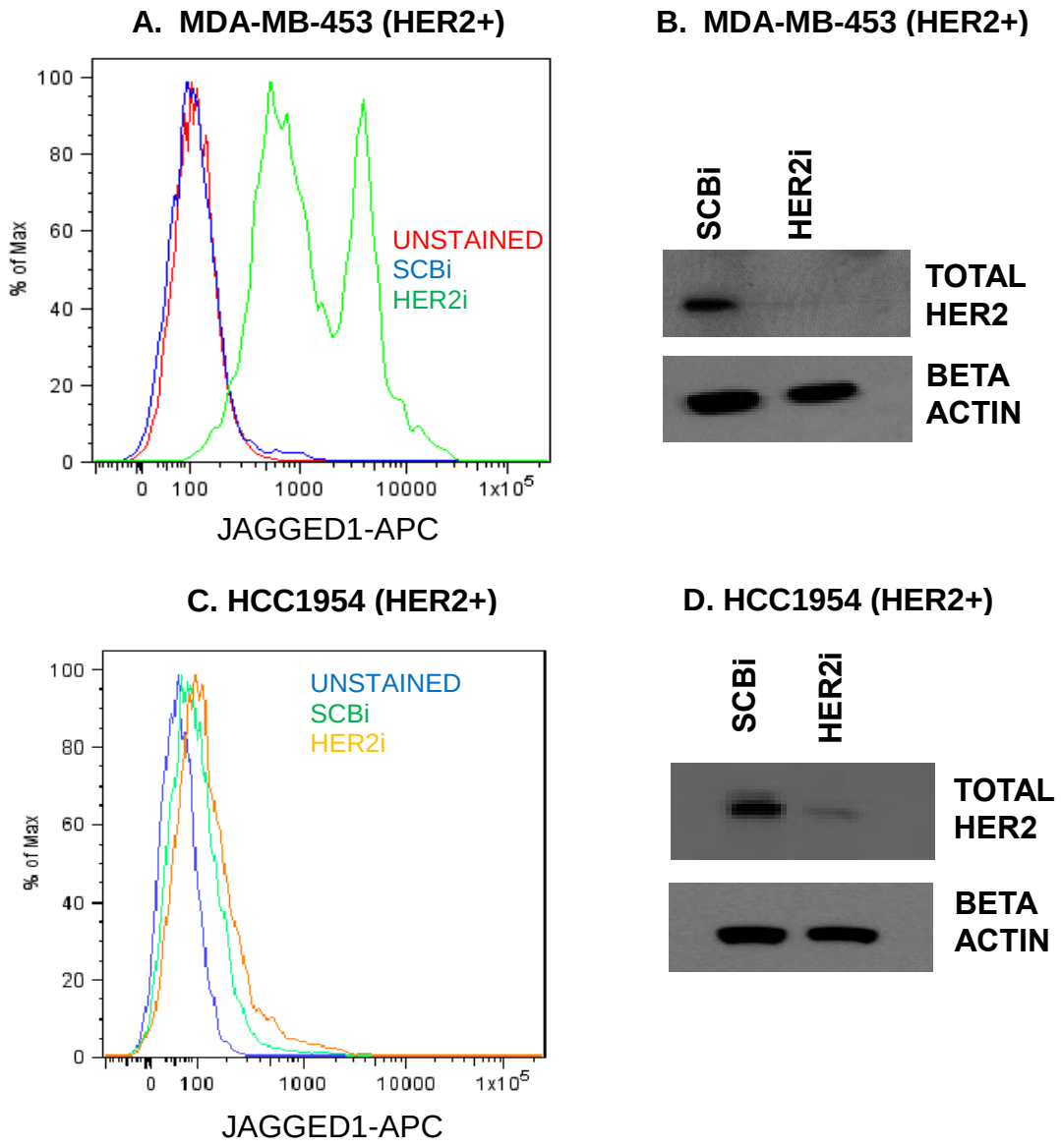


Figure 9: Genetic Inhibition of HER2 Increases the Surface Expression of Jagged1.

HER2 was knocked down in MDA-MB-453 and HCC1954 cells and the effect of HER2 knockdown on Jagged1 surface expression was measured my flow cytometry.

(A) MDA-MB-453 cells and (C) HCC1954 cells were transfected with HER2/scrambled control siRNA for 48 hours. The cells were then stained with Jagged1 and the surface expression of Jagged1 was measured by flow cytometry. The picture is representative of three independent experiments.

(B) Representative total HER2 western blot showing the efficiency of HER2 knockdown in MDA-MB-453 cells. Beta actin was used as a loading control. The experiment was performed independently for three times.

(D) Similarly, the efficiency of HER2 knockdown in HCC1954 cells was determined.

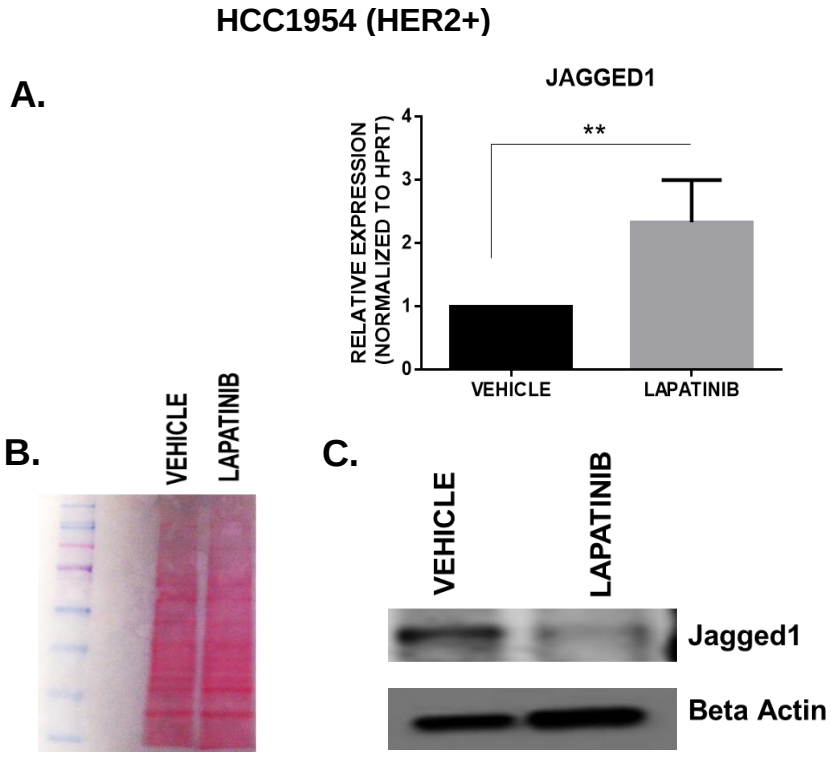


Figure 10: Effect of Lapatinib Mediated HER2 Inhibition on mRNA and Protein Levels of Jagged1.

HCC1954 cells were treated with 2 µM lapatinib for 4 days and the effect of lapatinib treatment on the transcript and protein levels of Jagged1 was assessed by real-time PCR and Western blot respectively.

(A) Followed by the lapatinib treatment, HCC1954 cells were harvested, RNA was extracted, cDNA was synthesized, and real-time PCR was performed to detect the transcript levels of Jagged1. Error bars represent S.D. and the statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis. **p<0.01.

(B) To determine the effect of lapatinib treatment on the protein levels of Jagged1, the cells were harvested and Western blot was performed. Beta actin was used as a

loading control. Based on previously performed experiments, lapatinib treatment affected the levels of Beta actin. Hence, Ponceau staining was also performed after the transfer to ensure that the loading was even across the samples. Panel B shows even loading between the samples as determined by Ponceau staining. The image is representative of three independent experiments.

(C) Panel C shows the Western blot that was performed to detect the effect of lapatinib treatment on the protein levels of Jagged1. Beta actin was used as a loading control. The figure is representative of three independent experiments.

Aim 1A-Determine Whether Jagged1-High Subpopulation Upon Lapatinib Treatment Results in an Increase in the Formation of CSCs as Compared to Jagged1-Low Subpopulation in the Absence of Lapatinib Treatment. CSCs Would be Assessed by %MFE, Aldehyde Dehydrogenase Activity and the Levels of CD44 High/CD24 Low.

To assess whether the enrichment of Jagged1 on the cell surface was necessary for the formation of CSCs, HCC1954 cells were treated with 2 μM lapatinib or vehicle DMSO for 4 days. The cells were harvested after the treatment and stained for Jagged1.The Jagged1 stained cells were sorted through the cell sorter. 20% of the cells expressing the least Jagged1 (as determined by the bell-shaped curve) upon vehicle treatment, and 20% of the cells expressing highest Jagged1 upon lapatinib treatment were sorted. This kind of sort provided two extremes of Jagged1 levels – vehicle treated Jagged1-low cells and lapatinib treated Jagged1-high cells. Propidium iodide (1 μg/ml) was used during the sort to exclude the dead cells from the assay. Various markers of CSCs, i.e. mammosphere formation, levels of aldehyde dehydrogenase, and the levels of CD44 high/CD24 low were assessed after the sort. These are the classic CSC markers that are routinely used in the field of breast cancer.

Jagged1-high subpopulation shows enhanced mammosphere formation upon HER2 inhibition, as compared to Jagged1-low subpopulation in the absence of HER2 inhibition. To determine the effect of Jagged1 surface expression on the formation of mammospheres, 35,000 vehicle Jagged1-low and lapatinib Jagged1-high cells were sorted into 24 well ultra-low attachment plates

containing mammosphere medium supplemented with or without 5 μM GSI (Figure 11A). The mammosphere formation was assessed after 7 days and representative prictures were taken. The pictures are indicative of three independent experiments (Figure 11B). The mammopsheres were then harvested and the mammopshere forming efficiency was determined. The results revealed a significant increase in mammosphere formation efficiency by the Jagged1-high cells as compared to Jagged1-low cells. The addition of GSI to the mammosphere medium prevented the mammosphere formation, confirming that the formation of mammospheres was dependent on Notch activation (Figure 11C). The difference in mammosphere formation between vehicle treated Jagged1-low cells and lapatinib treated Jagged1-high cells was more striking than the difference observed when vehicle and lapatinib treated unsorted cells were used for the mammosphere formation, implying that the surface expression of Jagged1 is required for the formation of mammospheres and thus survival of CSCs.

A control experiment was performed to address whether higher Jagged1 surface expression independent of HER2 inhibition was necessary to form mammospheres, and thus CSCs. To test this, untreated HCC1954 cells were harvested and were stained for surface Jagged1. 20% of the cells that express the least Jagged1 and 20% of the cells that express the highest Jagged1 on the cell surface were sorted into mammosphere plates containing mammosphere medium supplemented with or without 5 μM GSI (Figure 12A). Mammosphere formation was assessed and pictures were taken after 7 days. The pictures are indicative of three independent experiments (Figure 12B). No to little mammospheres were observed under Jagged1-high or Jagged1-low conditions,

and GSI completely prevented mammosphere formation (Figure 12C). These results suggest that both HER2 inhibition and increase in Jagged1 are necessary to enhance mammosphere formation. Importantly, these results also suggest that the inherently Jagged1-high cell population in HER2+ breast cancer cells might not be sufficient to enhance mammosphere formation.

Jagged1-high subpopulation upon HER2 inhibition shows an enhancement of aldehyde dehydrogenase positive CSCs, as compared to Jagged1-low subpopulation in the absence of HER2 inhibition. To assess the effect of Jagged1 surface expression on the levels of ALDH positive CSCs, vehicle treated Jagged1-low and lapatinib treated Jagged1-high cells were sorted into FACS tubes. Aldefluor assay was performed after cell sorting. MDA-MB-231 cells (Triple Negative breast cancer cells) were used as a negative control (Figure 13A) and MDA-MB-468 cells (Triple Negative breast cancer cells) were used as a positive control for the assay (Figure 13B). Jagged1-high cells from lapatinib treatment showed significantly higher levels of ALDH, as compared to vehicle treated, Jagged1-low cells (Figure 13C-E). This contrasts with what was observed for the unsorted cells, as lapatinib treatment reduced ALDH levels in unsorted cells, although the decrease was not significant (Figure 14A-C). These results signify the importance of the surface expression of Jagged1 for the formation of ALDH positive CSCs.

Upon HER2 inhibition, Jagged1-high subpopulation doesn't show a significant change in the levels of CD44 high/CD24 low CSCs, as compared to Jagged1-low subpopulation in the absence of HER2 inhibition. To determine whether lapatinib treatment which resulted in high Jagged1 surface expression affected levels of CD44/CD24, vehicle treated Jagged1-low and lapatinib treated Jagged1-high cells were sorted, stained with CD44 and CD24 antibodies, and assessed by flow cytometry (Figure 15D-E). MDA-MB-231 cells were used as a positive control (Figure 15B) and MDA-MB-468 cells served as a negative control (Figure 15C) for the assay. Vehicle treated Jagged1-low cells had a very small fraction of CD44 high/CD24 low cells, which was further reduced in lapatinib treated Jagged1-high cells. Similar results were observed when the assay was performed using unsorted cells (Figure 16A-C). These results would imply that HER2 inhibition mediated increase in Jagged1 surface expression does not enrich CSCs that express CD44 high/CD24 low.

The discrepancy observed between the two CSC markers could be attributed to a recent report which shows that ALDH is a better marker for epithelial like CSCs, and CD44 high/CD24 low is a better marker for mesenchymal-like CSCs (Liu, Cong et al. 2014). This would imply that by inhibiting HER2, we have enriched a subpopulation of epithelial like CSCs that have higher Jagged1 on the cell surface.

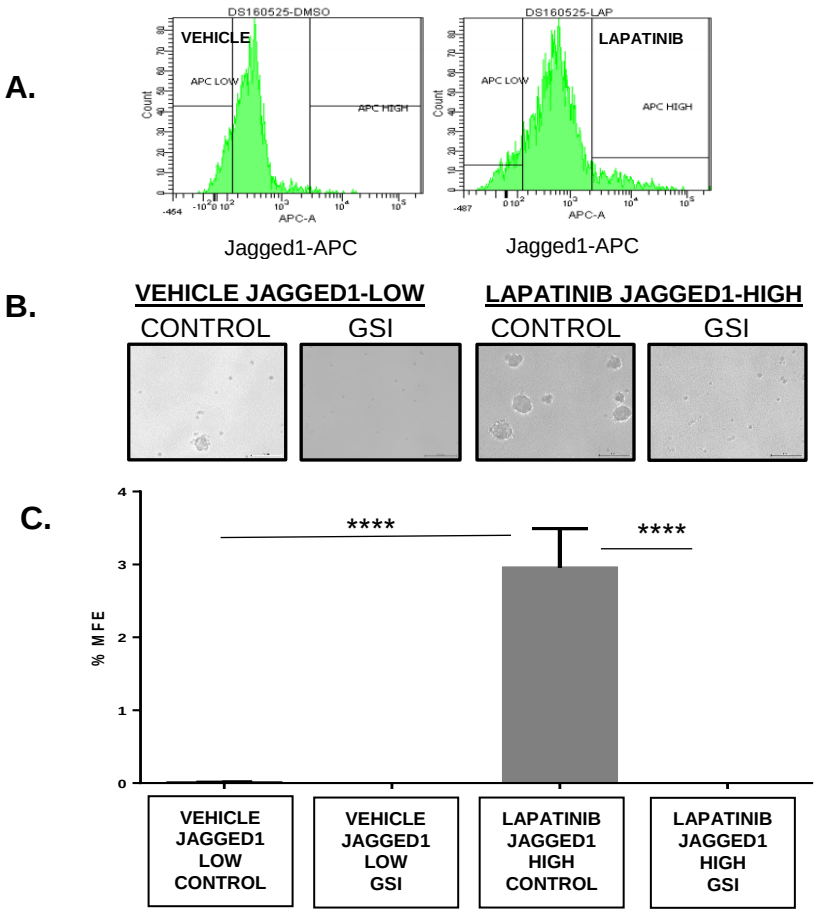


Figure 11: Jagged1-High Subpopulation Upon HER2 Inhibition Shows Enhanced Mammosphere Formation.

(A) HCC1954 cells were treated with 2 µM lapatinib for 4 days. Followed by the treatment, the cells were stained with Jagged1. The stained cells were sorted such that - 20% of the cells that expressed least Jagged1 on the cell surface under vehicle condition and 20% of the cells that expressed highest Jagged1 upon lapatinib treatment were included (as shown above).

(B) 35,000 cells were sorted into mammosphere plates containing mammosphere media supplemented with or without 5 µM GSI. The mammosphere pictures were taken after 7 days and are representative of three independent experiments.

(C) Mammopshere were harvested after 7 days and the %MFE was determined. The columns represent the average %MFE and the error bars represent the S.D. of three independent experiments. The statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

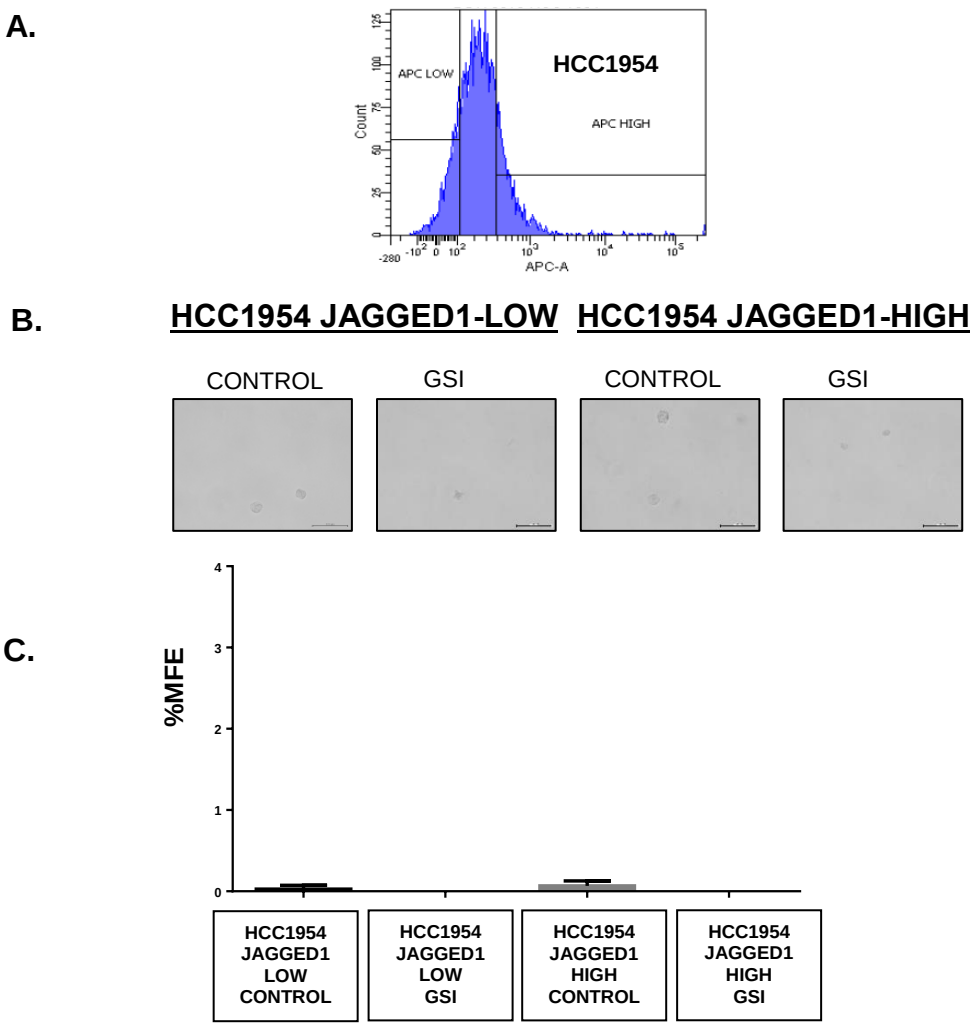


Figure 12: Jagged1-High Subpopulation Without HER2 Inhibition Doesn't Show Enhanced Mammosphere Formation.

(A) HCC1954 cells were harvested and stained with Jagged1. The stained cells were sorted such that - 20% of the cells that expressed least Jagged1 on the cell surface under vehicle condition and 20% of the cells that expressed highest Jagged1 upon lapatinib treatment were included (as shown above).

(B) 25,000 cells were sorted into mammosphere plates containing mammosphere media supplemented with or without 5 µM GSI. The mammosphere pictures were taken at the end of 7 days, and are representative of three independent experiments.

(C) The mammospheres were harvested after 7 days and the %MFE was determined. The columns represent average %MFE and the error bars represent the S.D. of three independent experiments. The statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis.

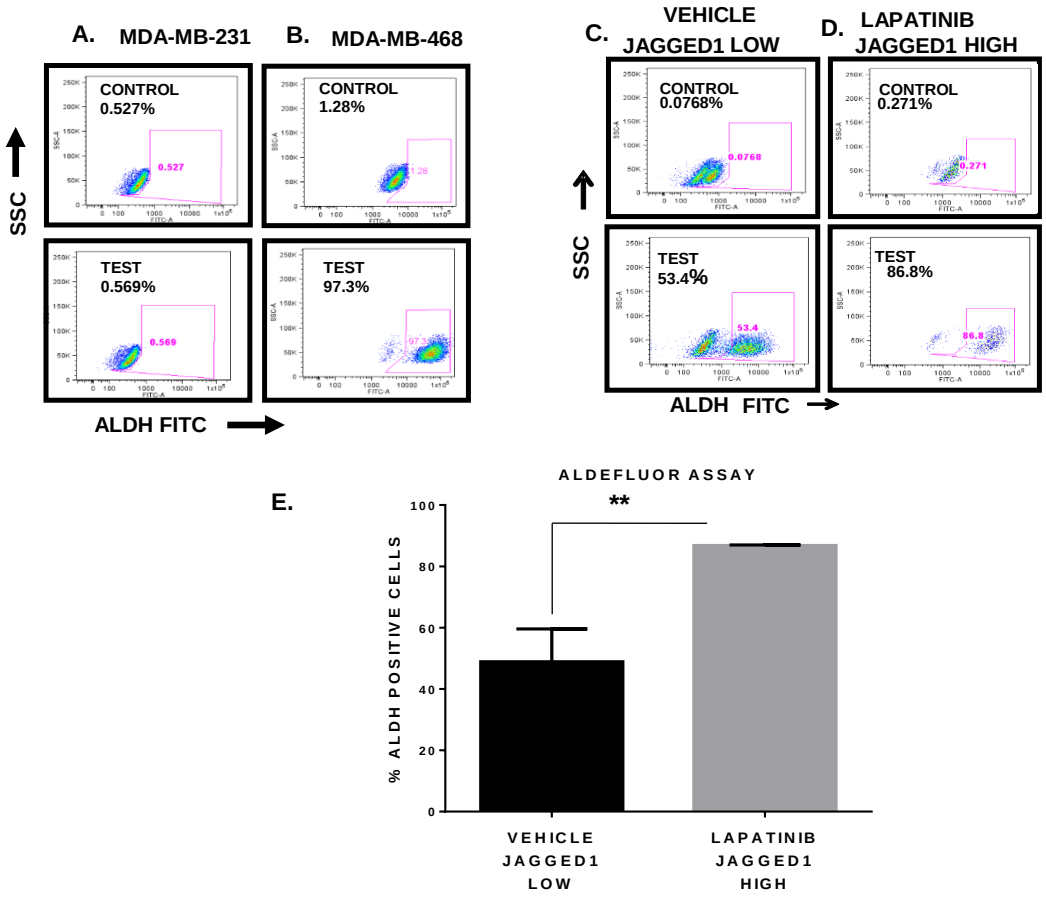


Figure 13: Jagged1-High Subpopulation Upon HER2 Inhibition Shows Increased Levels of ALDH1 Positive CSCs.

(A-B) Aldefluor assay was performed to dectect the %ALDH1+ CSCs. DEAB was used in the control sample to inhibit ALDH1 and it acted as a negative control. MDA-MB-231 and MDA-MB-468 cells were used as negative control and positive control respectively. The pictures are representative of three independent experiments.

(C-D) HCC1954 cells were treated with 2 μM lapatinib for 4 days. Followed by the treatment, the cells were stained with Jagged1. The stained cells were sorted such that 20% of the cells that expressed least Jagged1 on the cell surface under vehicle condition and 20% of the cells that expressed highest Jagged1 upon lapatinib treatment were included (as described before). 200,000 cells from each sample were used to perform the aldefluor assay. The pictures are representative of three independent experiments.

(E) The bar graph represents the average of three independent experiments. Error bars represent S.D. and the statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis. * $p<0.05$, ** $p<0.01$.

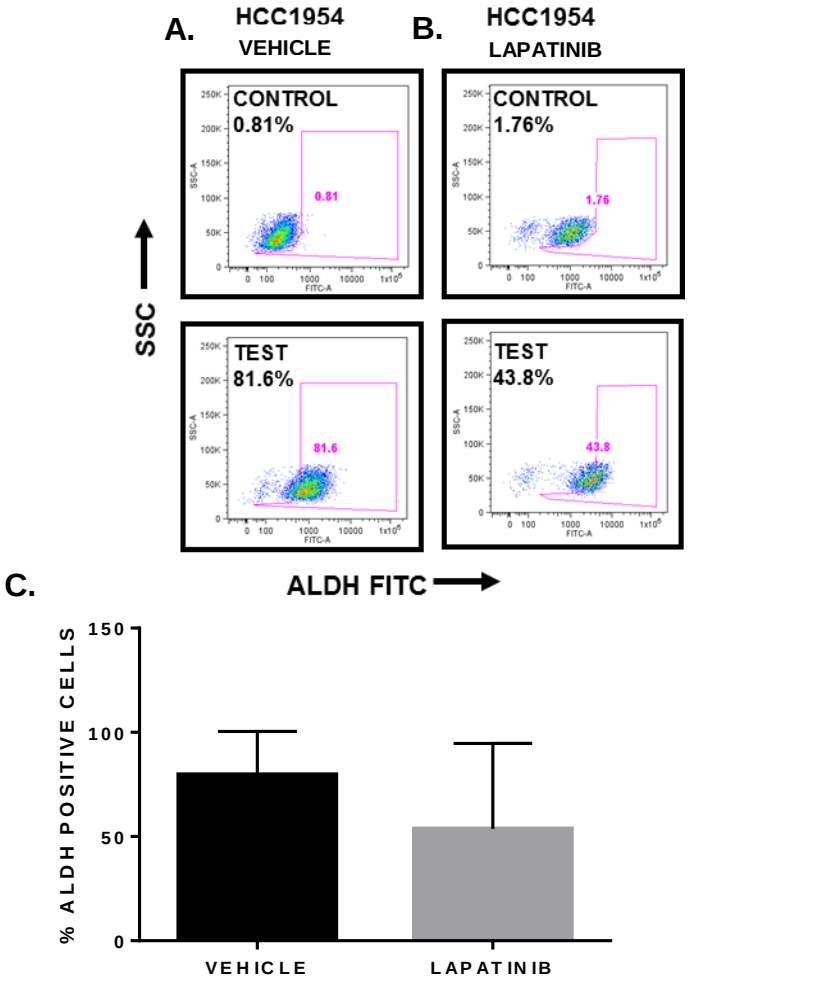


Figure 14: Lapatinib Mediated HER2 Inhibition Does Not Significantly Affect the Levels of ALDH1 Positive CSCs in Unsorted Cells.

(A and B) HCC1954 cells were treated with vehicle control i.e. DMSO or 2 µM lapatinib for 4 days. Followed by the treatment, the cells were harvested and aldefluor assay was performed, as described in the methods section. DEAB was used in the control sample to inhibit ALDH1 and it acted as a negative control. 200,000 cells from each sample were used to perform the assay. Values mentioned along with the dot plots indicate the percentage of ALDH1+ population. The pictures are representative of three independent experiments

(C) The bar graph represents the average and the error bars represent S.D. of three independent experiments. The statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis.

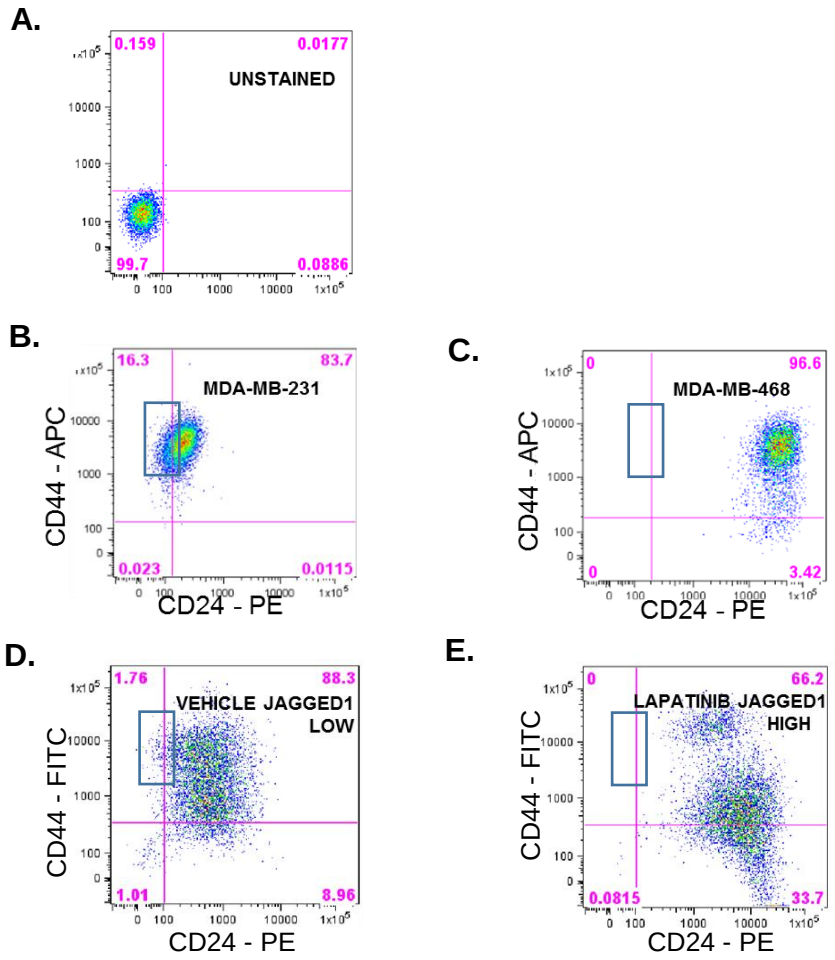


Figure 15: Jagged1-High Subpopulation Upon HER2 Inhibition Does Not Show a Marked Difference in the Levels of CD44 High/ CD24 Low CSCs.

HCC1954 cells were treated with 2 µM lapatinib for 4 days. Followed by the treatment, the cells were stained with Jagged1. The stained cells were sorted such that- 20% of the cells that expressed least Jagged1 on the cell surface under vehicle condition and 20% of the cells that expressed highest Jagged1 upon lapatinib treatment were included (as described before). Followed by the sort, the cells were stained with CD44-FITC and CD24-PE. Appropriate compensation controls were used and flow cytometry was performed to detect the levels of CD44 high/CD24 low. The pictures are representative of three independent experiments.

(A) Unstained Control

(B-E) Surface expression of CD44 and CD24, as assessed by flow cytometry. MDA-MB-231 cells were used as a positive control and MDA-MB-468 cells were used as a negative control.

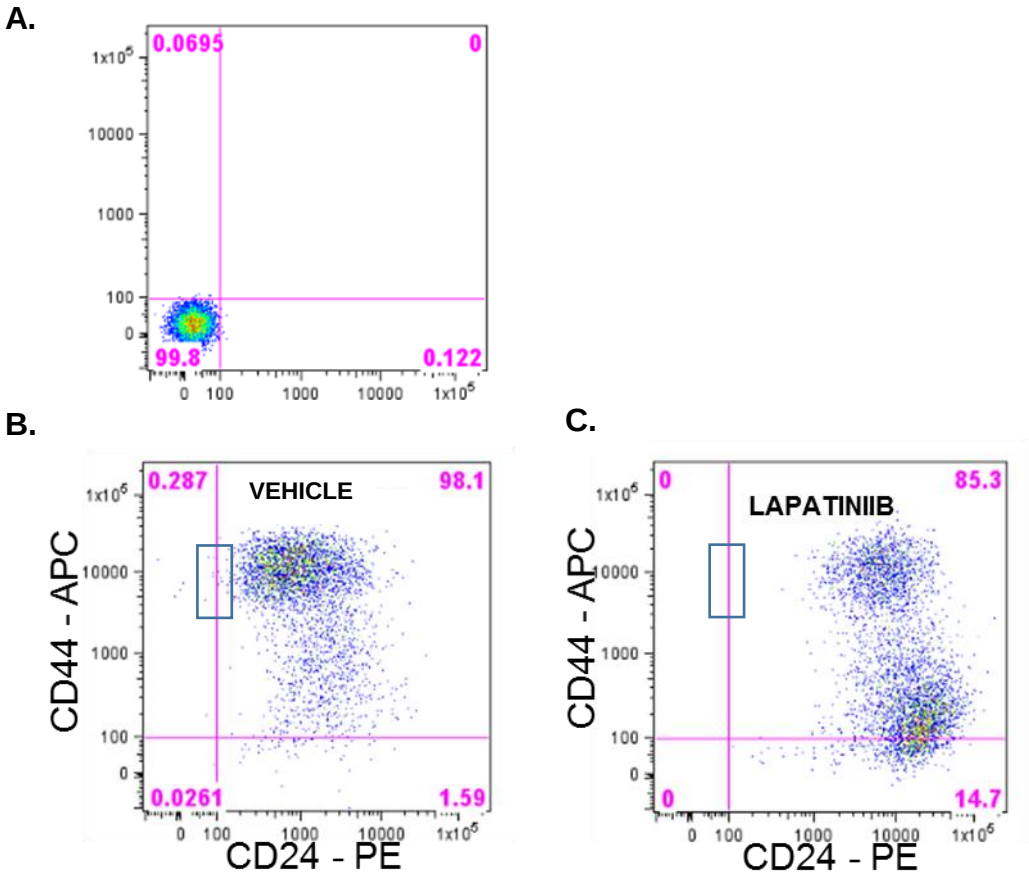


Figure 16: Lapatinib Mediated HER2 Inhibition Does Not Significantly Affect the Levels of CD44 High/CD24 Low CSCs in Unsorted Cells.

HCC1954 cells were treated with 2 µM lapatinib for 4 days. The cells were stained with CD44-APC and CD24-PE after the treatment, and flow cytometry was performed to detect the levels of CD44 high/CD24 low. The pictures are representative of three independent experiments.

(A) Unstained control.

(B and C) Surface expression of CD44 and CD24 when HCC1954 cells are treated with vehicle control i.e. DMSO or lapatinib for 4 days.

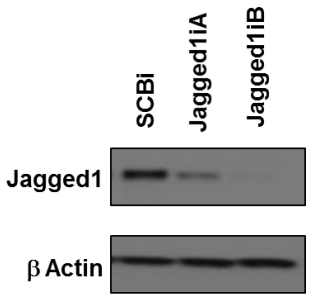
Aim 1B-Assess Whether Jagged1 is Necessary for Lapatinib Mediated Enrichment of Mammospheres in the HER2+ Subtype of Breast Cancer.

Since the results indicated that HER2 inhibition results in an increase in Jagged1 expression on the cell surface, and that the subpopulation of cells expressing higher Jagged1 on the cell surface is enriched with CSCs, we wanted to determine if Jagged1 was required for this effect.

Jagged1 is necessary for lapatinib mediated enrichment of mammospheres. Jagged1 knockdown using siRNA was used to determine whether Jagged1 expression is necessary for the HER2 inhibition-mediated enrichment of mammospheres. In order to ensure specificity and rule out the possibility of any off-target effects, two different siRNAs directed against Jagged1 were used. The cells were harvested and replated after transfection. The next day they were treated with vehicle DMSO or 2 μM lapatinib. The treatment was performed for 4 days. A second transfection was performed after 2 days of treatment to ensure that the transient knockdown lasts for 4 days of treatment. Protein was harvested at the end of 4 days and Western blot was performed to assess the efficiency of the Jagged1 knockdown. Beta actin was used as a loading control. Both the siRNAs were able to effectively knock down Jagged1 at the protein level (Figure 17A). The cells were harvested after treatment and 100,000 individual cells were plated into each well of a 6 well mammosphere plate containing mammosphere media. Mammospheres were harvested after 7 days and the %MFE under each condition was determined.

Under the scrambled control conditions, lapatinib treatment resulted in an enrichment of mammospheres. However, upon Jagged1 knockdown, lapatinib treatment failed to increase mammosphere formation. Jagged1 knockdown without lapatinib treatment did not significantly reduce mammosphere formation (Figure 17B-E). These results suggest that when HER2 is inhibited, Jagged1 enriched subpopulation of cells is required for the formation of the mammospheres, and thus survival of CSCs.

A.


SCBi
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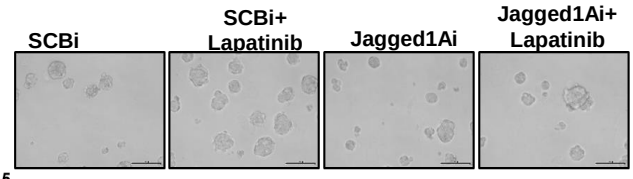
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SCBi+ Lapatinib
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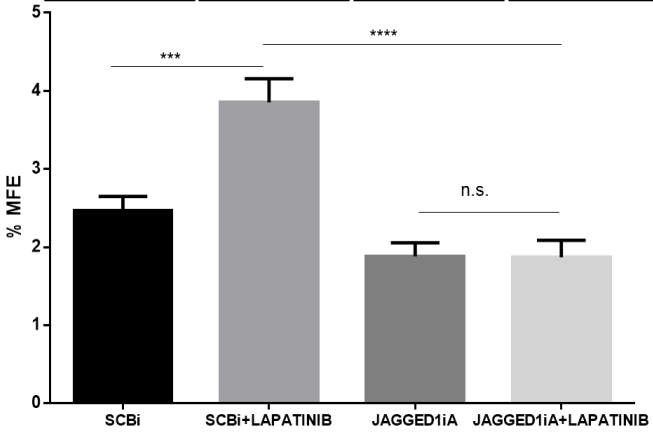
C.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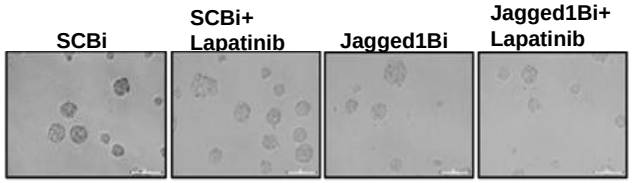
SCBi
SCBi+ Lapatinib
Jagged1Bi
Jagged1Bi+ Lapatinib

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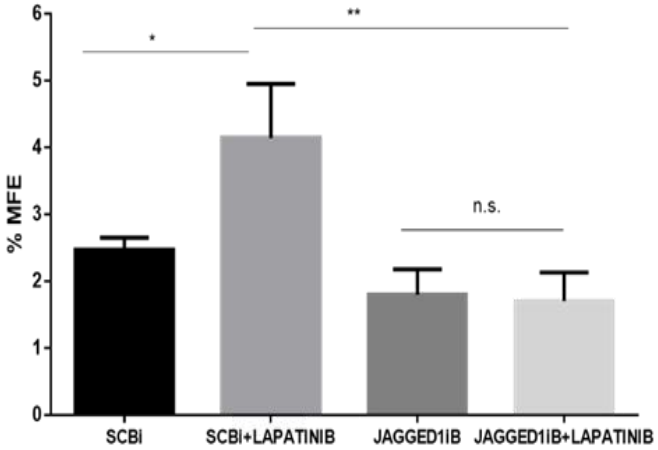
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Figure 17: Jagged1 Is Necessary for Lapatinib Mediated Enrichment of Mammospheres.

(A) HCC1954 cells were transfected with non-targeted scrambled control siRNA or Jagged1 siRNA for 48 hours. To rule out the possibility of any off-target effect of siRNA, two different sequences of siRNA targeting Jagged1 were used (Jagged1 A and Jagged1 B). Followed by the transfection, the cells were treated for 4 days with vehicle DMSO or 2 μM lapatinib. The second transfection was performed after 2 days of treatment to ensure that the transient transfection lasts until 4 days. The cells were harvested after treatment and Jagged1 knockdown was determined by western blot. Beta actin was used as a loading control.

(B, D) For the mammosphere assay, the cells were harvested after the treatment, and 100,000 single cells were plated into 6 well ultra-low attachment plate containing mammosphere media. The mammospheres were harvested after 7 days and the mammosphere pictures were taken. The pictures are representative of three independent experiments.

(C, E) The mammospheres were harvested after 7 days and the %MFE under each condition was determined. The mammosphere images are representative of three independent experiments. Error bars represent S.D. and the statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. * $p<0.05$, ** $p<0.01$.

Aim 1C-Measure the Degree of Notch Activation in Jagged1-High Subpopulation upon Lapatinib Treatment and in the Jagged1-Low Subpopulation in the Absence of Lapatinib Treatment.

Previous work by Osipo et al. demonstrated that HER2 inhibition results in an increase in Notch activation (Osipo, Patel et al. 2008). Since HER2 inhibition resulted in an increase in membrane Jagged1 expression, we wanted to test the status of Notch activation in the subset of cells that expressed high membrane Jagged1.

The Jagged1-high subpopulation in the presence of lapatinib mediated HER2 inhibition has higher Notch activation, as compared to the Jagged1-low subpopulation, in the absence of HER2 inhibition. To assess the Notch activation in the Jagged1-high subpopulation, HCC1954 cells were treated with 2 μM lapatinib for 4 days and then the cells were sorted based on Jagged1 membrane expression. Followed by this, the RNA was extracted using QIAGEN RNeasy micro kit. The extracted RNA was quantified and cDNA was synthesized using reverse transcription. Real-time PCR was performed to detect Notch activation by using primers directed against the Notch target gene transcripts. Jagged1- high subpopulation of cells in response to lapatinib showed a significant increase in the transcript levels of most Notch targets (Hes1, Hes4, Hey2, CyclinD1, and c-Myc), as compared to vehicle treated, Jagged1-low subpopulation (Figure 18A-F). Upon HER2 inhibition, significant Notch activation was observed in unsorted cells as well (Hes1) (Figure 19).

Besides Notch targets, the transcript levels of Notch receptors were measured in the two subpopulations as well. Upon HER2 inhibition, the Jagged1-high subpopulation

showed a significant increase in the expression of Notch1 and Notch3 receptors, as compared to the Jagged1-low subpopulation in the absence of HER2 inhibition. The expression of Notch2 and Notch4 receptors also increased in the Jagged1-high subpopulation. However, the increase was not significant (Figure 20). The expression of the Notch2 receptor was the lowest, and hence the relative fold change in the expression of all other receptors was normalized against vehicle/-Jagged1-low-Notch2.

As mentioned before, Notch signaling functions in a contact dependent manner. To our surprise, the ligand expressing cells showed higher Notch activation. The Notch receptor is activated by the ligand on the signal sending cell. However, if the Notch receptor and the Notch ligand are present on the same cell, then it leads to inhibition of Notch signaling, and this phenomenon is known as *cis* inhibition (del Alamo, Rouault et al. 2011). To address this, we performed flow cytometry to detect the surface expression of Notch receptors (Notch1, Notch3, and Notch4). Since Notch2 was the least expressed and was barely detected by real-time PCR, Notch2 was not used for any further investigation. The results revealed that all the cells that express Jagged1 on the membrane also express Notch1 (Figure 21B-C), Notch3 (Figure 21D-E), and Notch4 (Figure 21F-G) on the surface. This suggests that HER2 inhibition selects for a subpopulation of cells that have high membrane Jagged1, as well as Notch receptors on the surface and that Jagged1 on the membrane of one cell could interact with Notch receptor on another cell within the same subpopulation.

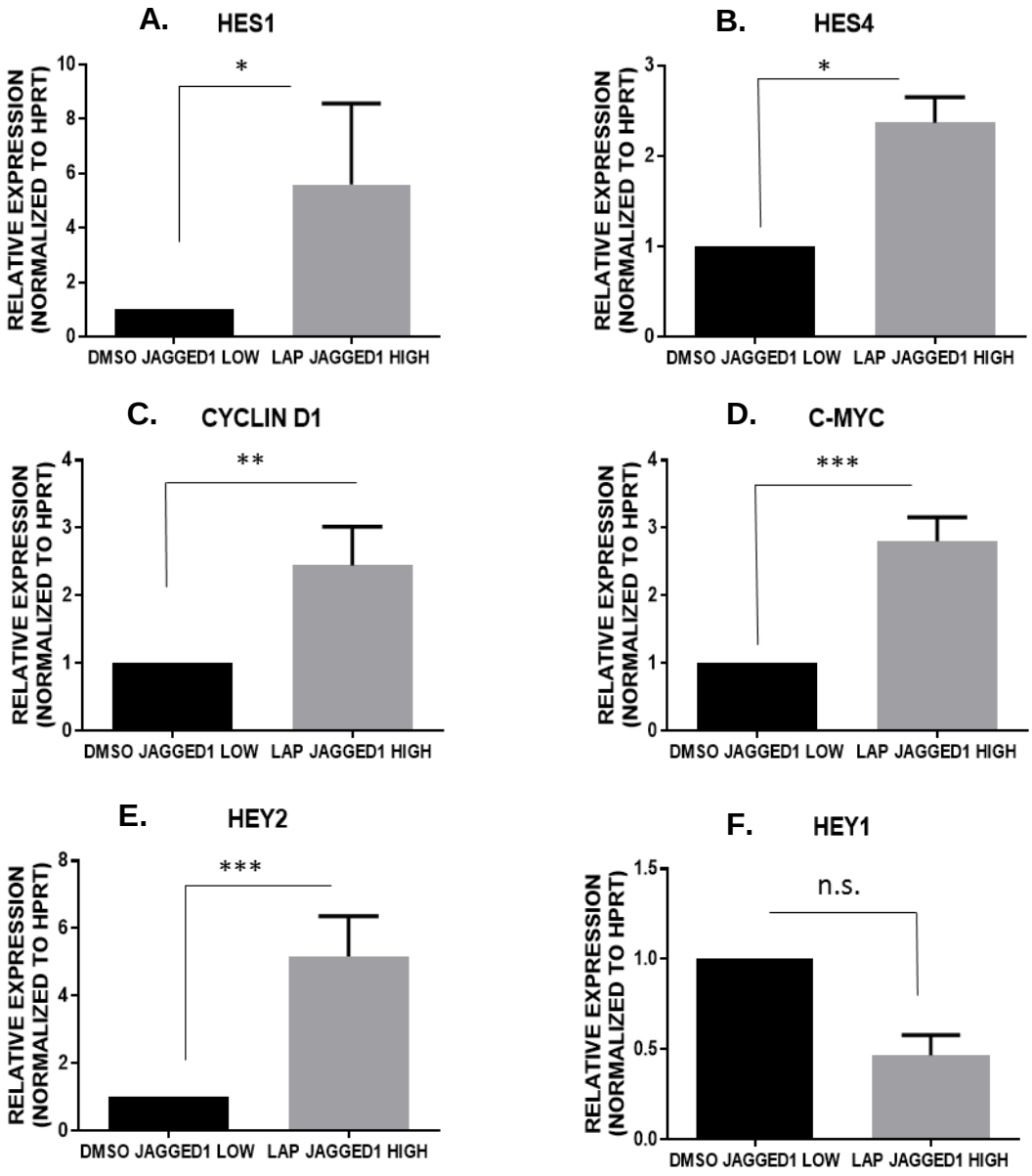


Figure 18: Jagged1-High Subpopulation in the Presence of Lapatinib Mediated HER2 Inhibition Has Higher Notch Activation, as Compared to Jagged1-Low Subpopulation in the Absence of HER2 Inhibition.

HCC1954 cells were treated with 2 μM lapatinib for 4 days. Followed by the treatment, the cells were stained with Jagged1. The stained cells were sorted such that - 20% of the cells that expressed least Jagged1 on the cell surface under vehicle control condition i.e. DMSO and 20% of the cells that expressed highest Jagged1 upon

lapatinib treatment (LAP) were included (as described before). RNA was extracted from the sorted cells using QIAGEN RNeasy Micro kit. cDNA was synthesized from the extracted RNA and real- time PCR was performed to detect the transcript levels of Notch target genes.

(A) *HES1* (B) *HES4* (C) *CYCLIND1* (D) *c-MYC* (E) *HEY2* and (F) *HEY1*. HPRT was used as an endogenous control. Columns represent the average gene expression (normalized to HPRT) and the error bars represent S.D. of three independent experiments. The statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis. * p<0.05, **p<0.01, ***p<0.001.

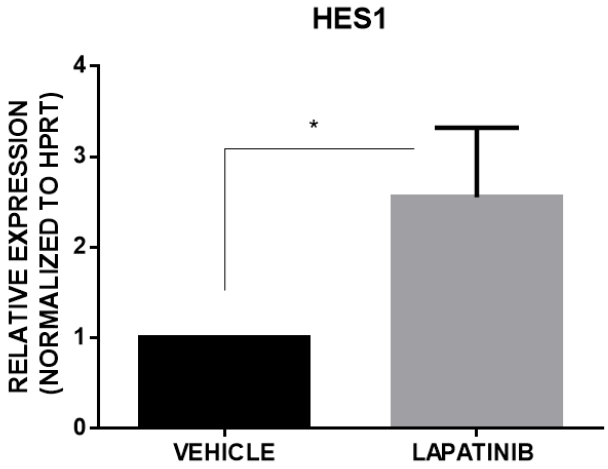


Figure 19: HER2 Inhibition Results in an Increase in Notch Activation.

HCC1954 cells were treated for 4 days with vehicle DMSO or 2 μM lapatinib. Followed by the drug treatment, the cells were harvested and real-time PCR was performed to detect the transcript levels of Notch targets. The graph above shows HES1 levels under the two conditions. HPRT was used as endogenous control. Columns represent the average gene expression (normalized to HPRT) and the error bars represent S.D.. The statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis. * p<0.05.

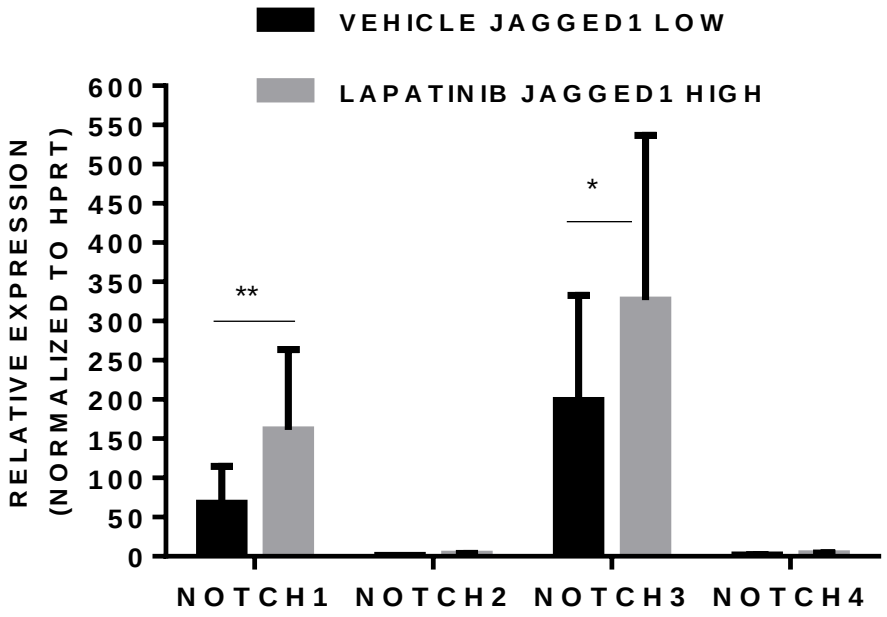


Figure 20: Jagged1-High Subpopulation Has Increased Expression of Notch1 And Notch3 Receptors.

HCC1954 cells were treated with 2 μM lapatinib for 4 days. Followed by the treatment, the cells were stained with Jagged1. The stained cells were sorted such that - 20% of the cells that expressed least Jagged1 on the cell surface under vehicle condition and 20% of the cells that expressed highest Jagged1 upon lapatinib treatment were included (as described before). Followed by the sort, RNA was extracted from the cells using QIAGEN RNeasy Micro kit. cDNA was synthesized from the extracted RNA and real-time PCR was performed to detect the transcript levels of Notch target genes. HPRT was used as an endogenous control. Real-time PCR results show means of fold changes between the two subpopulations, normalized against vehicle Jagged1-low-Notch2. Error bars represent S.D. and the statistical significance was calculated using unpaired *t* test. GraphPad Prism was used for the analysis. * $p<0.05$, ** $p<0.01$.

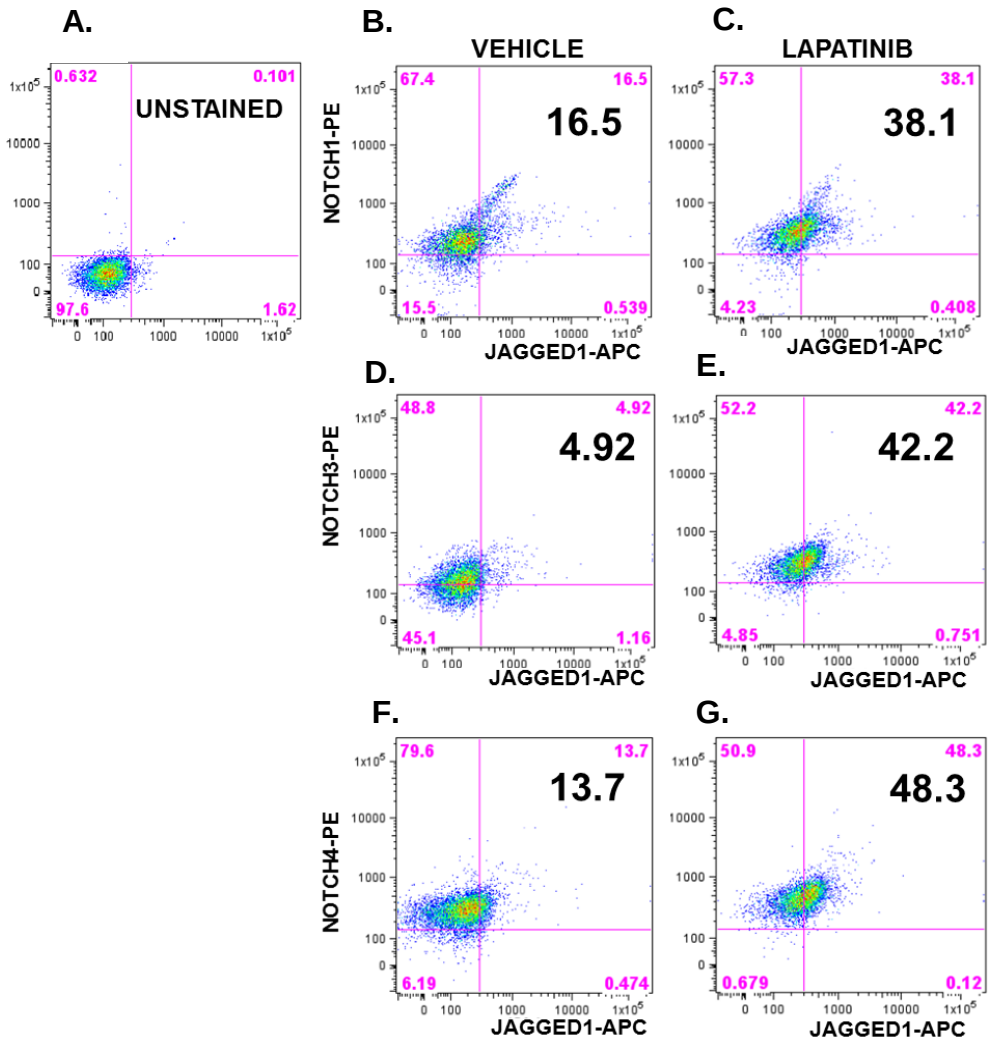


Figure 21: Jagged1 Is Co-Expressed Along with Notch1, Notch3, And Notch4.

HCC1954 cells were treated with vehicle control DMSO or 2 µM lapatinib for 4 days. Followed by the drug treatment, the cells were harvested and were divided into 3 sets of tubes and were stained accordingly-
I Jagged1 and Notch1 staining II Jagged1 and Notch3 staining III Jagged1 and Notch4 staining. Followed by the staining, the surface expression of Jagged1 and the Notch receptors with or without the lapatinib treatment was assessed by flow cytometry. Appropriate compensation controls were used. The pictures are representative of three independent experiments.

(A) Unstained Control
(B) Vehicle-Jagged1+Notch1
(C) Lapatinib-Jagged1+Notch1
(D) Vehicle-Jagged1+Notch3
(E) Lapatinib-Jagged1+Notch3
(F) Vehicle-Jagged1+Notch4
(G) Lapatinib-Jagged1+Notch4

Aim 1D-Identify the Notch Receptor That Interacts with Jagged1 to Cause Lapatinib Mediated Enrichment of Mammospheres in the HER2+ Subtype of Breast Cancer.

As Notch signaling works in a contact dependent fashion, we wanted to determine the Notch receptor that Jagged1 engages with, to mediate Notch signaling. Based on the real-time PCR data, mRNA levels of all the Notch receptors were increased in the Jagged1-high subpopulation, the levels of Notch1 and Notch3 being significantly high in the Jagged1-high subpopulation. Flow cytometry data showed that Jagged1 is co-expressed with Notch1, Notch3, and Notch4. Jagged1 could interact with one or multiple Notch receptors to mediate Notch activation and enhance formation of CSCs. There have been reports elucidating the involvement of Notch1, Notch3, and Notch4 receptors in the formation of breast CSCs (Sansone, Storci et al. 2007, Magnifico, Albano et al. 2009, Harrison, Farnie et al. 2010).

The question that we asked was: Which receptor is required in the engagement with Jagged1 to mediate lapatinib induced enrichment of mammospheres, and thus CSCs? To address this question, HCC1954 cells were transfected with scrambled control siRNA or the siRNA targeted against Notch1, Notch3, or Notch4 individually for 48 hours. Followed by the transfection, the cells were treated with vehicle control or 2 μM lapatinib for four days. A second transfection was performed after two days, to ensure that the transient transfection lasts until four days of treatment. Followed by the treatment, the knockdown was assessed by either Western blot or flow cytometry. Notch3 knockdown was detected by Western blot. Beta actin was used as a loading

control (Figure 22 A). To assess Notch1 knockdown (Figure 23 A-B) and Notch4 knockdown (Figure 24 A-B), flow cytometry was performed as the signal by Western blot was weak and non-specific.

For performing mammosphere assay, the cells were harvested after the treatment, and 100,000 individual cells were plated in a 6 well ultra-low attachment plate containing mammosphere media. The mammospheres were harvested after 7 days and the %MFE was determined under each condition.

In agreement with previous data, we saw lapatinib mediated enrichment of mammospheres under control conditions. Notch3 knockdown significantly increased mammosphere formation as compared to scrambled control in the absence of lapatinib treatment. Notch3 knockdown showed no significant change in the mammosphere formation as compared to scrambled knockdown in the presence of lapatinib treatment (Figure 22 C).

Notch1 knockdown did not significantly affect the mammosphere formation under control or lapatinib treated conditions (Figure 23 D). Notch4 knockdown did not show any effect on the mammosphere formation under control or lapatinib treated conditions (Figure 24 D). Based on the Western blot and flow cytometry data, the knockdown of Notch1 and Notch4 was not efficient. Hence, it is hard to conclude anything regarding the role of Notch1 and Notch4 receptors in the lapatinib mediated enrichment of mammospheres. Future investigation should involve creating stable cell lines using the CRISPR approach to effectively knockout Notch1 and Notch4. The stable cell lines can

then be used to test the role of Notch1 and Notch4 in mediating lapatinib mediated enrichment of mammospheres.

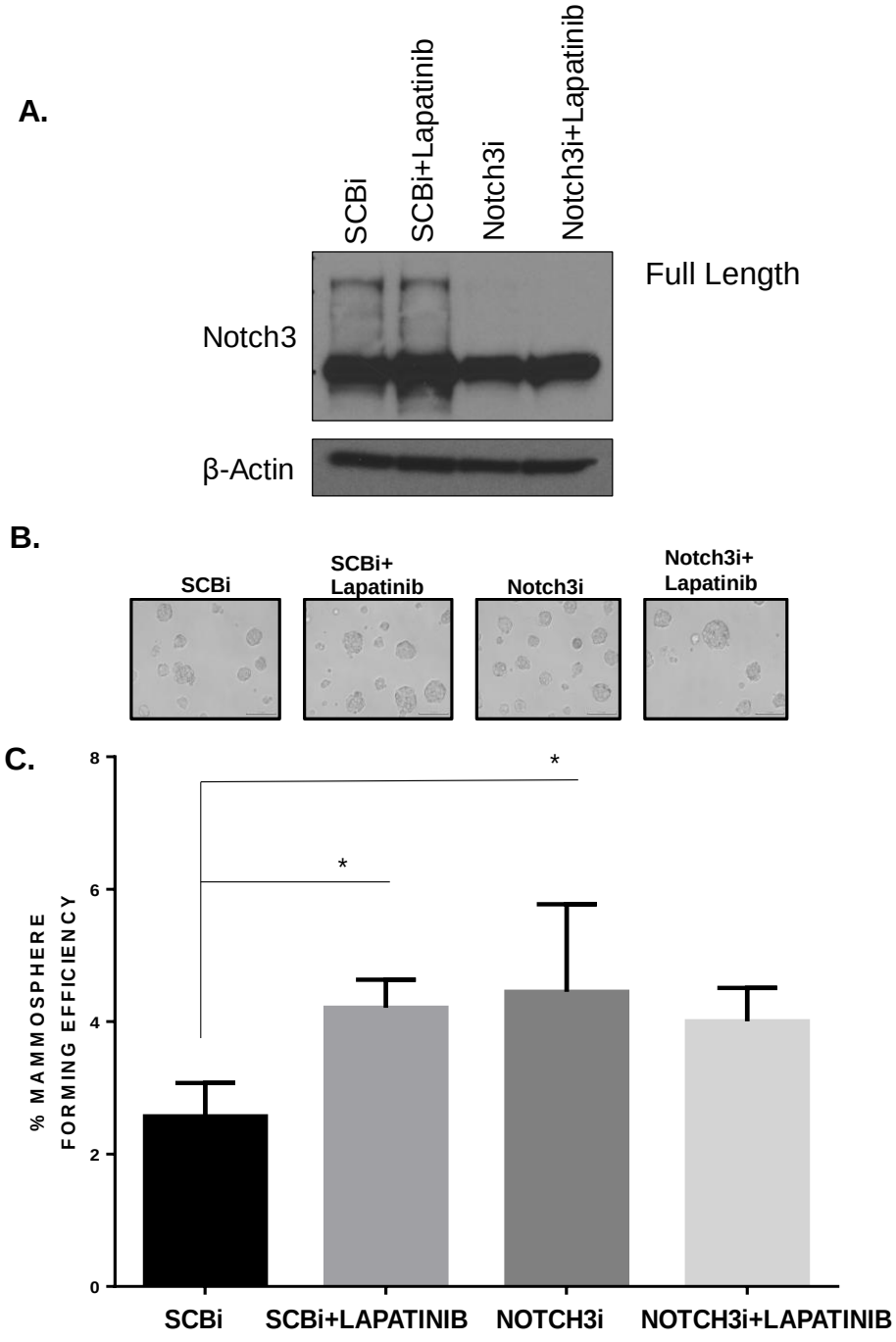


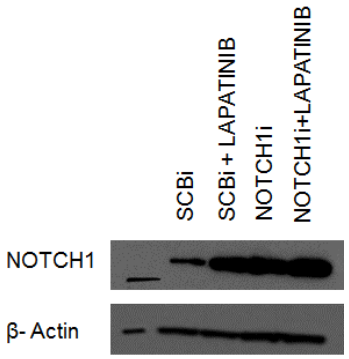
Figure 22: Effect of Notch3 Knockdown on the Lapatinib Mediated Enrichment of Mammospheres.

(A) HCC1954 cells were transfected with non-targeted scrambled control siRNA or Notch3 siRNA for 48 hours. After the transfection, the cells were treated for 4 days with vehicle DMSO or 2 μM lapatinib. Followed by the treatment, the cells were harvested and Notch3 knockdown was determined by western blot. Beta actin was used as a loading control.

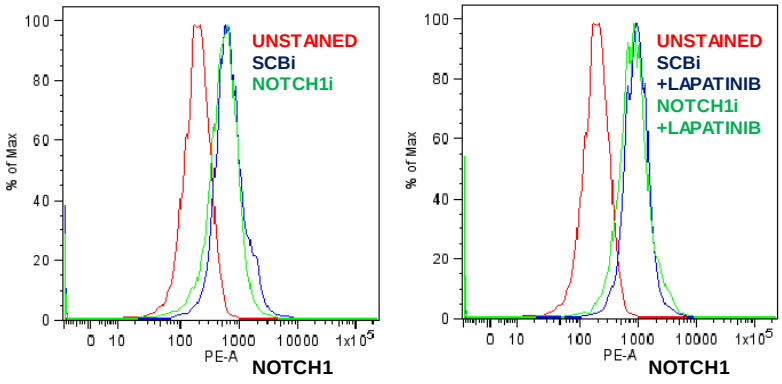
(B) For the mammosphere assay, the cells were harvested after the treatment, and 100,000 single cells were plated into 6 well ultra-low attachment plate containing mammosphere media. At the end of 7 days, the mammopshere pictures were taken. The pictures are representative of three independent experiments.

(C) The mammospheres were harvested after 7 days and the %MFE under each condition was determined. The columns represent the average %MFE and the error bars represent S.D. of three independent experiments. The statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. * $p<0.05$

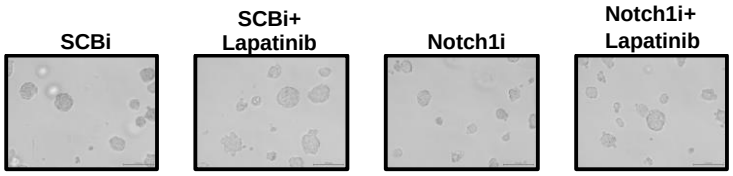
A.


SCBi
SCBi + LAPATINIB
NOTCH1i
NOTCH1i+LAPATINIB
NOTCH1
β- Actin

B.


UNSTAINED
SCBi
NOTCH1i
% of Max
PE-A
NOTCH1
UNSTAINED
SCBi
+LAPATINIB
NOTCH1i
+LAPATINIB
% of Max
PE-A
NOTCH1

C.


SCBi
SCBi+ Lapatinib
Notch1i
Notch1i+ Lapatinib

D.

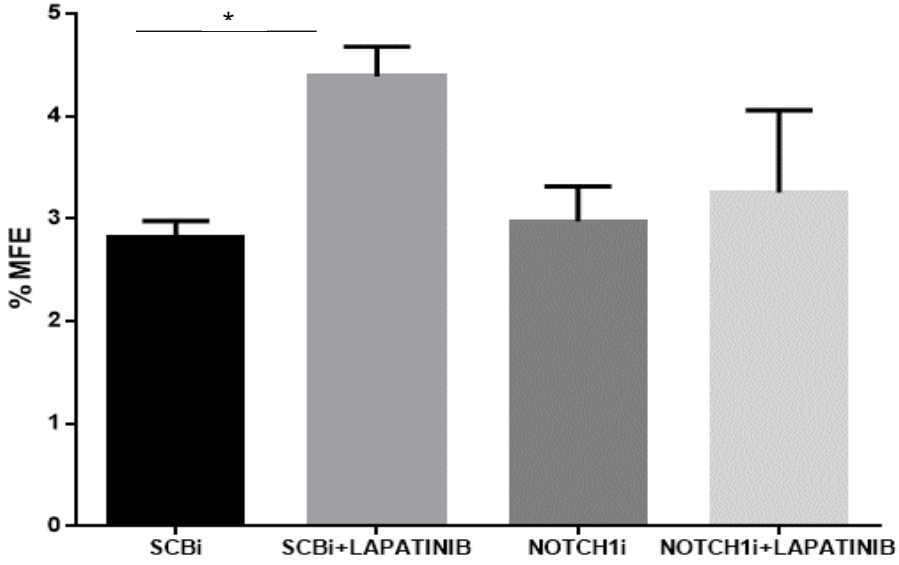

*
% MFE
SCBi
SCBi+LAPATINIB
NOTCH1i
NOTCH1i+LAPATINIB

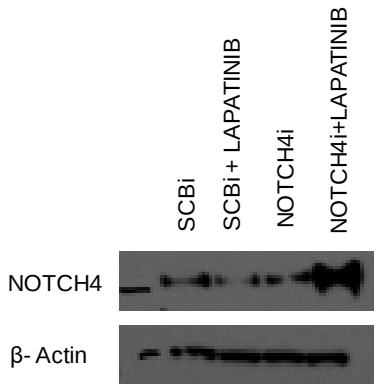
Figure 23: Effect of Notch1 Knockdown on the Lapatinib Mediated Enrichment of Mammospheres.

HCC1954 cells were transfected with non-targeted scrambled control siRNA or Notch1 siRNA for 48 hours. After the transfection, the cells were treated for 4 days with vehicle DMSO or 2 μM lapatinib. The cells were harvested after the treatment and Notch1 knockdown was determined by western blot (A) or flow cytometry (B). Beta actin was used as a loading control for western blot.

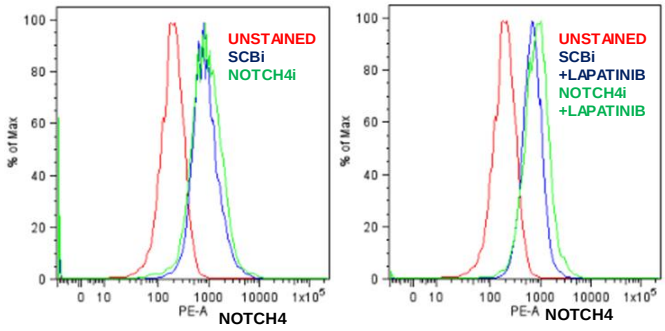
(C) For the mammosphere assay, the cells were harvested after the treatment, and 100,000 single cells were plated into 6 well ultra-low attachment plate containing mammosphere media. At the end of 7 days, the mammosphere pictures were taken. The pictures are representative of three independent experiments.

(D) The mammospheres were harvested after 7 days and the %MFE under each condition was determined. The columns represent the average %MFE and the error bars represent the S.D. of three independent experiments. The statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. * $p<0.05$

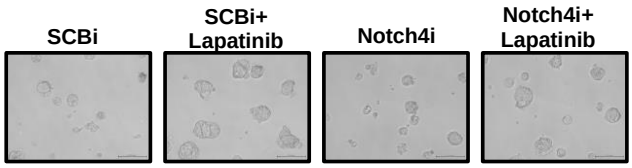
A.


SCBi
SCBi+ LAPATINIB
NOTCH4i
NOTCH4i+LAPATINIB
NOTCH4
β- Actin

B.


UNSTAINED
SCBi
NOTCH4i
% of Max
PE-A
NOTCH4
UNSTAINED
SCBi
+LAPATINIB
NOTCH4i
+LAPATINIB
% of Max
PE-A
NOTCH4

C.


SCBi
SCBi+ Lapatinib
Notch4i
Notch4i+ Lapatinib

D.

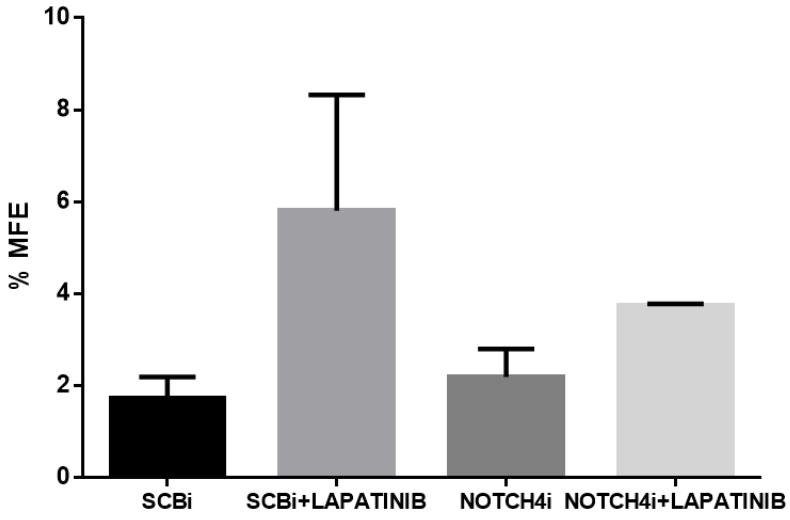

% MFE
SCBi
SCBi+LAPATINIB
NOTCH4i
NOTCH4i+LAPATINIB

Figure 24: Effect of Notch4 Knockdown on the Lapatinib Mediated Enrichment of Mammospheres.

HCC1954 cells were transfected with non-targeted scrambled control siRNA or Notch4 siRNA for 48 hours. After the transfection, the cells were treated for 4 days with vehicle DMSO or 2 μM lapatinib. The cells were harvested after the treatment and Notch4 knockdown was determined by western blot (A) or flow cytometry (B). Beta actin was used as a loading control for western blot.

(C) For the mammosphere assay, the cells were harvested after the treatment, and 100,000 single cells were plated into 6 well ultra-low attachment plate containing mammosphere media. At the end of 7 days, the mammosphere pictures were taken. The pictures are representative of two independent experiments.

(D) The mammospheres were harvested after 7 days and the %MFE under each condition was determined. The columns represent the average %MFE and the error bars represent the S.D. of two independent experiments. GraphPad Prism was used for plotting the graph.

Specific Aim 2- Assess the Ability of Jagged1-High Subpopulation to Form a Tumor *In Vivo* and to Measure its CSC Potential

As mentioned before, another name for CSC is TIC. Hence, the true assessment of CSC relies on the ability of the cells to form a tumor *in vivo.* It is done by using limiting dilution assay, which is considered as a gold standard in the breast cancer field for the assessment of true CSC frequency. It involves using log fold dilution of the cell population and determining tumor take at each dilution. The idea is that if the cell population is truly enriched with CSCs, it'll have a higher CSC frequency and the ability to form the tumor with a lower cell number. Researchers have used as low as 10 cells for the assessment of CSC frequency (Cheung, Chuang et al. 2016). Using bulk tumor cells, 1-10 million cells are required to form the tumor.

Based on our *in vitro* data, HER2 inhibition enriches for a Jagged1-high subpopulation of cells that have increased capacity to form mammospheres and have enhanced aldefluor activity. Moreover, Jagged1 was found to be necessary for the enrichment of CSCs when HER2 was inhibited. To determine if the Jagged1-high subpopulation is indeed enriched with CSCs, we performed a pilot *in vivo* experiment using 10,000 cells sorted on the basis of Jagged1 surface expression.

Jagged1-high subpopulation shows 100 percent tumor take *in vivo* with 10,000 sorted cells. For the *in vivo* study, HCC1954 cells were treated with vehicle or 2 µM lapatinib. After the treatment, the cells were harvested, stained, and were sorted for Jagged1 surface expression, as mentioned before. Followed by sorting, 10,000 cells were diluted in PBS and mixed with Matrigel (1:1 ratio). The cell

suspension was then injected into the mammary fat pad of ovariectomized FoxN1 nu/nu athymic nude mice. The mice were monitored weekly for tumor take in each group (tumors ≥ 40 mm^2 were considered palpable and measured). The experiment was continued for up to 10 weeks. Based on tumor take, a Kaplan Meier curve for % tumor free mice was plotted. The group of mice that were injected with 10,000 lapatinib treated Jagged1-high cells showed 100 percent tumor take (5/5) i.e. zero percent tumor free mice. The group that was injected with 10,000 vehicle treated Jagged1-low cells showed 33.3 percent tumor take (2/6) i.e. 66.6 percent tumor free mice. The difference in the tumor take between the two groups was found to be statistically significant, as determined by the Log-rank (Mantel-Cox) Test (P = 0.0051) (Figure 25).

Normally, 1-2.5 million HCC1954 cells are injected into the mammary fat pad for *in-vivo* studies. These results of the study suggested that Jagged1-high subpopulation of cells were enriched with CSCs, as 100 percent tumor take was observed with as few as 10,000 Jagged1-high sorted cells.

Jagged1-high subpopulation has significantly higher CSC frequency as compared to the Jagged1-low subpopulation, and Jagged1 neutralizing antibody did not reduce the CSC frequency of either subpopulation. The pilot study convincingly showed that upon HER2 inhibition, the Jagged1-high subpopulation is indeed enriched with CSCs. To determine the CSC frequency of the Jagged1-high and Jagged1-low subpopulations, limiting dilution assay was performed *in vivo.* Also, the effect of blocking Jagged1 on CSC frequency of both the subpopulations was assessed by using a neutralizing antibody against Jagged1 (CTX-014). HCC1954

cells were sorted based on the membrane expression of Jagged1 i.e. vehicle treated Jagged1-low subpopulation and lapatinib treated Jagged1-high subpopulation. Followed by sorting, either 100, 1000 or 10,000 cells were injected into the mammary fat pad of ovariectomized athymic nude mice and tumor take was assessed up to 8 weeks (tumors ≥ 40 mm^2 were considered palpable and measured). One week after injecting the cells, the mice were randomized into control (Figure 26 A-B) and Jagged1 neutralizing antibody (CTX-014) treatment groups (Figure 26 C-D). 10 mg/kg CTX-014 or PBS control was injected intraperitoneally (i.p.) once a week. The antibody acts by preventing the binding of Jagged1 and Jagged2 with Notch receptors. There were four groups of each dilution of 100, 1000 and 10,000 cells-

i. Vehicle Jagged1-low control

ii. Vehicle Jagged1-low CTX-014

iii. Lapatinib Jagged1-high control

iv. Lapatinib Jagged1-high CTX-014

Based on the tumor take in each group, the CSC frequency was calculated using ELDA software (Table 6).

The results from ELDA revealed that the Jagged1-high subpopulation after lapatinib treatment had significantly higher CSC frequency (1/2180, p-value=0.000413) as compared to vehicle treated Jagged1-low subpopulation (1/12638). Interestingly, CTX-014 treatment significantly increased the CSC frequency of vehicle Jagged1-low subpopulation (1/3279, p-value=0.0166). CTX-014 did not significantly affect the CSC frequency of the lapatinib Jagged1-high subpopulation (1/3357, p value 0.377). The *in*

vivo results conclusively demonstrated the high tumor initiating potential of the Jagged1-high subpopulation after HER2 inhibition.

Also, average tumor area and average tumor weight assessment of the groups at each dilution i.e. 100 cells, 1000 cells and 10,000 cells was performed. A line graph of number of cells injected versus average tumor area (Figure 27) or number of cells injected versus average tumor weight (Figure 28) was plotted for each group. However, there was high variation in the tumor size within each group at each dilution. Due to very high S.D. amongst the samples within each group, no significant difference in average tumor area or average tumor weight was observed between the groups.

The Jagged1 neutralizing antibody (CTX-014) was not found to be effective, and it significantly increased the CSC frequency of the vehicle, Jagged1-low subpopulation. This could be attributed to suboptimal binding of the antibody to Jagged1 or rapid clearance of the antibody from the murine immune system. Development of antibodies against CTX-014 or clonal selection of Jagged1-low cells by CTX-014 treatment are other possible reasons for the ineffectiveness of the CTX-014 antibody.

Higher Jagged1 surface expression predicts poor overall survival in women suffering from HER2+ breast cancer. To assess the importance of membrane Jagged1 on survival of women with HER2+ breast cancer, we examined the University of Nottingham breast cancer cohort, prepared as tissue microarray. 145 HER2+ breast cancer tissues were stained for Jagged1 and assessed for outcome based on Kaplan-Meier analysis. Based on the staining intensity, the samples were classified into different groups, high Jagged1 (membrane/cytoplasmic/nuclear: staining

intensity 2-3) or low Jagged1 (membrane/cytoplasmic/nuclear: staining intensity 0-1) (Figure 29A-D). The cumulative survival of each patient was assessed. The cumulative survival was defined as the time from the onset of surgery to breast cancer related death. The results revealed that there was no significant correlation between cytoplasmic or nuclear Jagged1 (Figure 29F-G) and the overall cumulative survival (log-rank *P* = 0.186 for nuclear Jagged1 and log-rank *P*=0.914 for cytoplasmic Jagged1). However, there was a significant inverse correlation between membrane Jagged1 staining and overall cumulative survival i.e. higher Jagged1 on the membrane significantly predicted poor overall survival in women with HER2+ breast cancer (log-rank *P*=0.01) (Figure 29E). Table 7 depicts the case processing summary for membrane Jagged1 (Table 7A), nuclear Jagged1 (Table 7B), and cytoplasmic Jagged1 (Table 7C).

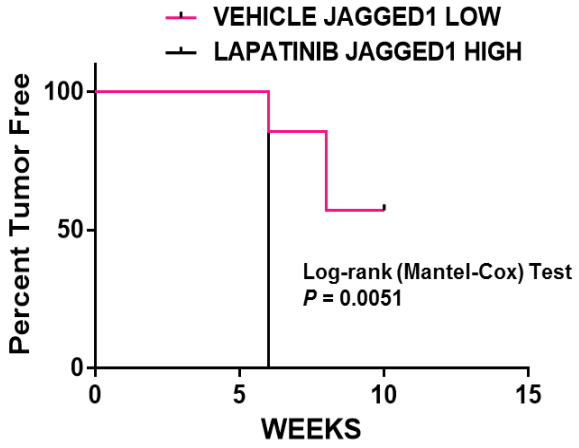


Figure 25: Jagged1-High Subpopulation Has Higher CSC Potential (Pilot Experiment).

To assess the ability of Jagged1-high subpopulation to form tumors, vehicle treated Jagged1-low cells and lapatinib treated Jagged1-high cells were sorted, as described before. 10,000 sorted cells were resuspended into matrigel (1:1 matrigel:PBS) and were then injected into mammary fat pad of athymic nude mice, and tumor take was

assessed for up to 10 weeks. Based on the tumor take, Kaplan-Meier curve was plotted and the statistics were performed using Log-rank (Mantel-Cox) test.

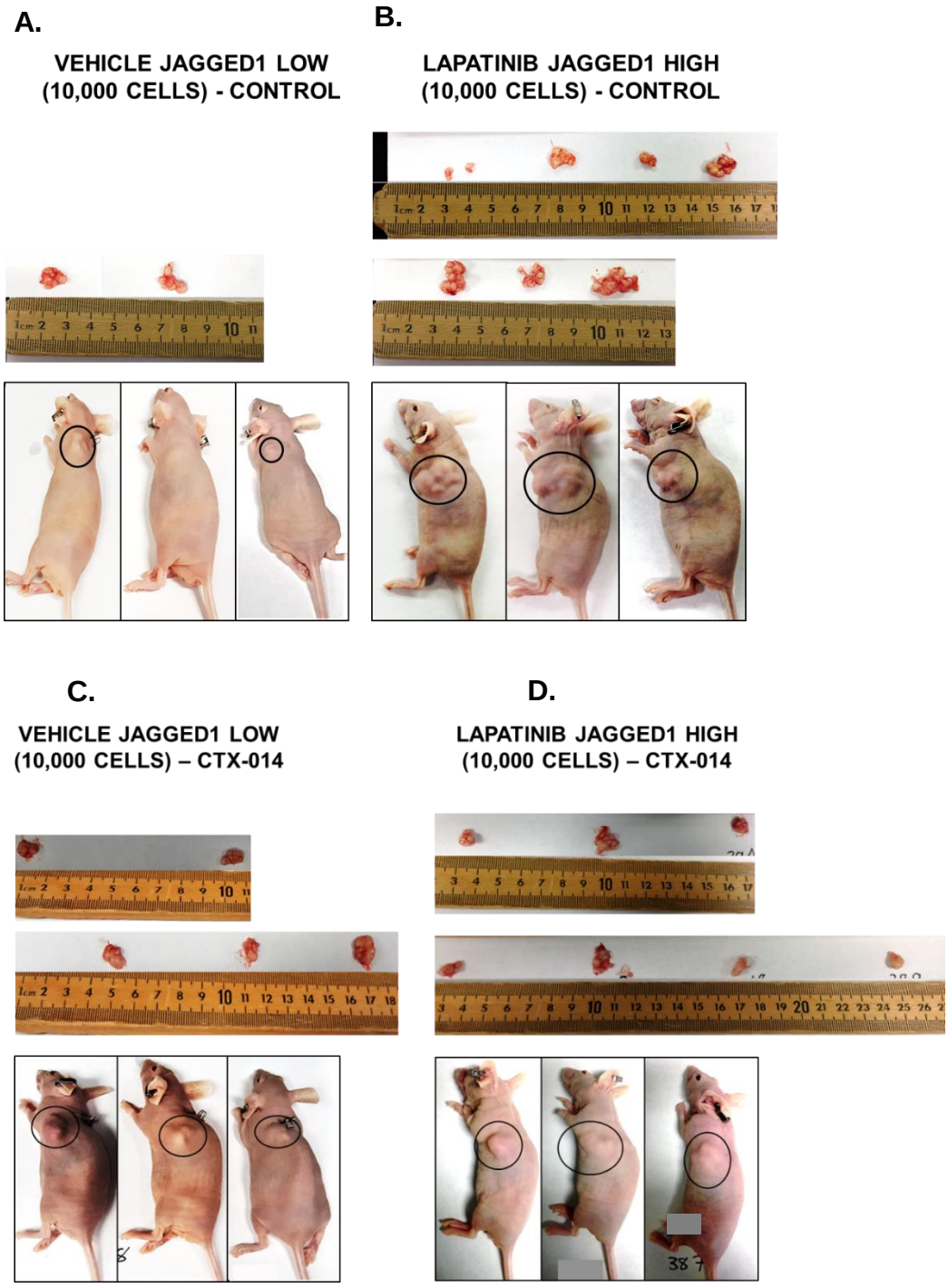


Figure 26: Jagged1-High Subpopulation Has Higher CSC Potential.

To assess the true CSC potential of the Jagged1-high subpopulation, vehicle Jagged1-low subpopulation and lapatinib treated Jagged1-high subpopulation of cells were sorted, as described before. Followed by the sort, 10,000, 1,000 or 100 cells were resuspended into matrigel (1:1 matrigel:PBS) and injected into mammary fat pad of athymic nude mice. The tumor take was assessed for each dilution of cells for 8 weeks and tumors ≥ 40 mm^2 were considered for the CSC frequency estimate. To determine the effect of Jagged1 on CSC frequency, the mice were randomized for control or Jagged1 neutralizing antibody (CTX-014) treatment. The treatment was started after one week of injecting the cells. There were four groups of each dilution of 100, 1000 and 10,000 cells- i vehicle Jagged1-low-control, ii vehicle Jagged1-low-CTX-014, iii lapatinib Jagged1-high-control, iv lapatinib Jagged1-high-CTX-014. CSC frequency was calculated using ELDA software.

The above figure shows the representative images of mice and tumors from the vehicle Jagged1-low-control (A), lapatinib Jagged1-high-control (B), vehicle Jagged1-low-CTX-014 (C), and lapatinib Jagged1-high-CTX-014 (D).

Group	Limiting Dilution			CSC Frequency
	100	1000	10,000	
VEHICLE JAGGED1 LOW - CONTROL	1/7	3/8	2/8	1/12638 (1/5174-1/30870)
VEHICLE JAGGED1 LOW – CTX-014	0/7	4/7	5/6	1/3279 (1/1342-1/8013)
LAPATINIB JAGGED1 HIGH-CONTROL	3/10	5/10	7/8	1/2180 (1/966-1/4921)
LAPATINIB JAGGED1 HIGH – CTX-014	1/8	3/8	7/8	1/3357 (1/1517-1/7431)

Table 6: Impact of Jagged1 Neutralizing Antibody (CTX-014) on the CSC Frequency Estimate of the Jagged1 Subpopulations.

To assess the true CSC potential of the Jagged1-high subpopulation, vehicle Jagged1-low subpopulation and lapatinib treated Jagged1-high subpopulation of cells were sorted, as described before. Followed by the sort, 10,000, 1,000 or 100 cells were resuspended into matrigel (1:1 matrigel:PBS) and injected into mammary fat pad of athymic nude mice. The tumor take was assessed for each dilution of cells for 8 weeks and tumors ≥ 40 mm^2 were considered for the CSC frequency estimate. To determine the effect of Jagged1 on CSC frequency, the mice were randomized for control or Jagged1 neutralizing antibody (CTX-014) treatment. The treatment was started after one week of injecting the cells. There were four groups of each dilution of 100, 1000 and 10,000 cells- i Vehicle Jagged1-low-control, ii Vehicle Jagged1-low-CTX-014, iii Lapatinib Jagged1-high-control, iv Lapatinib Jagged1-high-CTX-014. CSC frequency was calculated using ELDA software. The table reveals the CSC frequency estimate calculated using ELDA software.

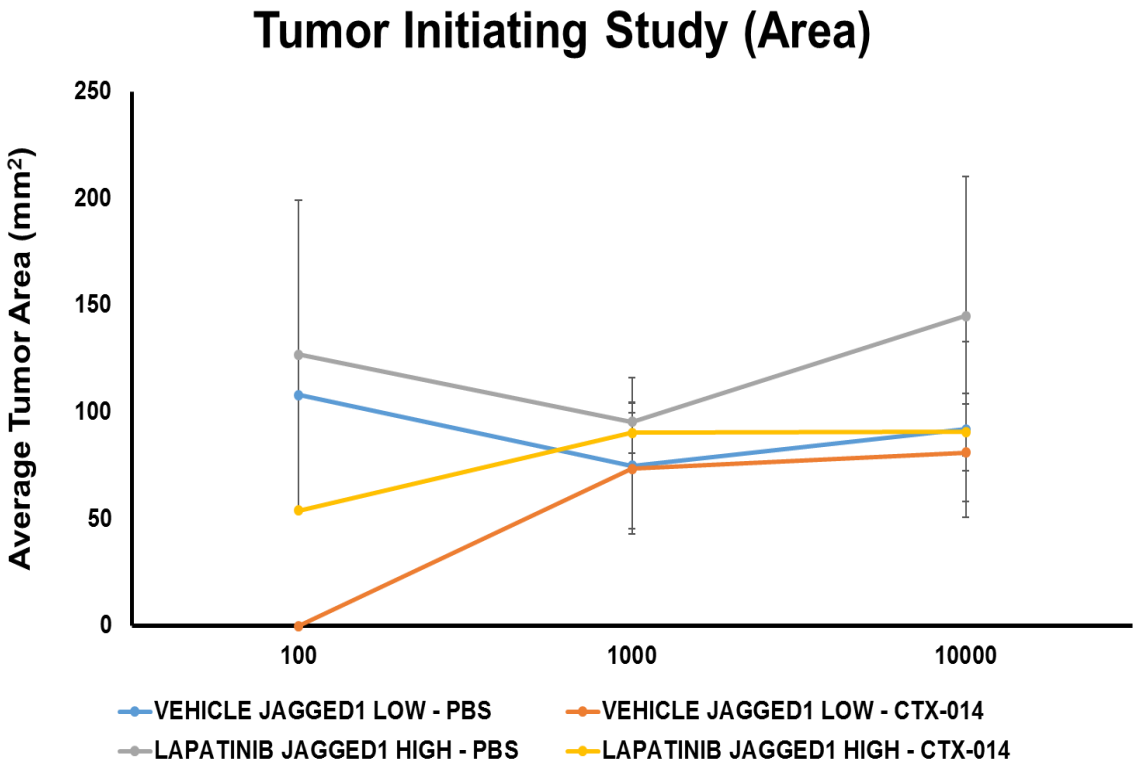


Figure 27: Effect of Jagged1 Neutralizing Antibody (CTX-014) on the Average Tumor Area of the Jagged1 Subpopulations at Different Dilutions.

To assess the true CSC potential of the Jagged1-high subpopulation, vehicle Jagged1-low subpopulation and lapatinib treated Jagged1-high subpopulation of cells were sorted, as described before. Followed by the sort, 10,000, 1,000 or 100 cells were resuspended into matrigel (1:1 matrigel:PBS) and injected into mammary fat pad of athymic nude mice. The tumor take was assessed for each dilution of cells for 8 weeks and tumors ≥ 40 mm^2 were considered for the CSC frequency estimate. To determine the effect of Jagged1 on CSC frequency, the mice were randomized for control or Jagged1 neutralizing antibody (CTX-014) treatment. The treatment was started after one week of injecting the cells. There were four groups of each dilution of 100, 1000 and 10,000 cells- i Vehicle Jagged1-low – control, ii Vehicle Jagged1-low-CTX-014, iii Lapatinib Jagged1-high-control, iv Lapatinib Jagged1-high-CTX-014. The average tumor area was calculated after 8 weeks for each group at different dilutions and the graph of average tumor area versus number of cells injected was plotted. Error bars represent S.D. and the statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. The graph was created using MS-Excel.

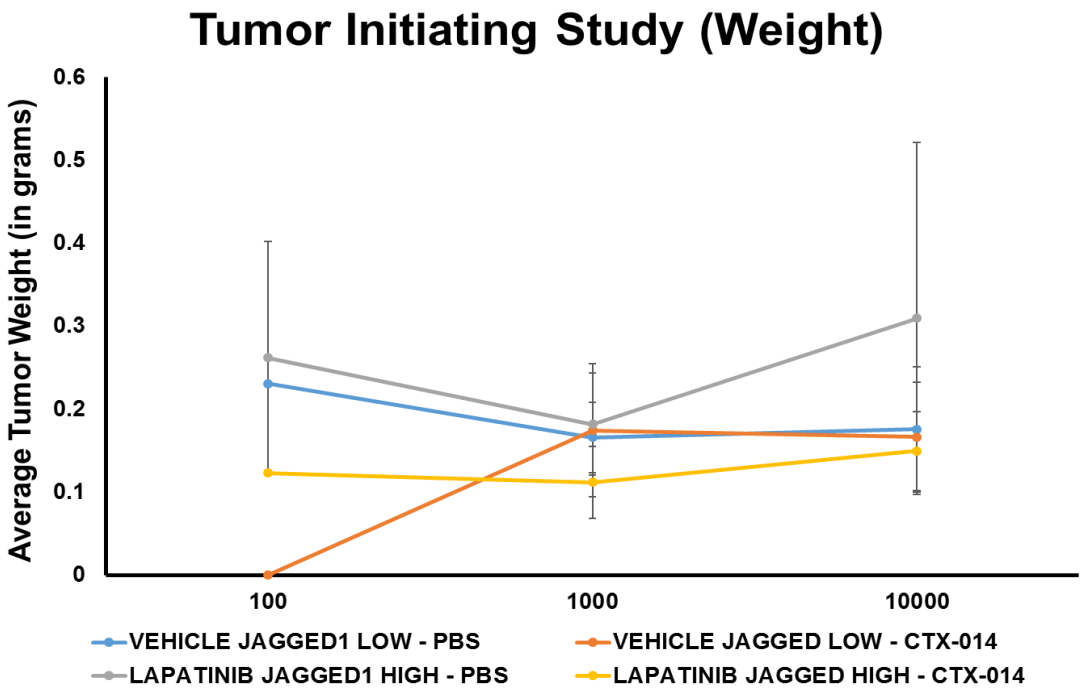


Figure 28: Effect of Jagged1 Neutralizing Antibody (CTX-014) on the Average Tumor Weight of the Jagged1 Subpopulations at Different Dilutions.

To assess the true CSC potential of the Jagged1-high subpopulation, vehicle Jagged1-low subpopulation and lapatinib treated Jagged1-high subpopulation of cells were sorted, as described before. Followed by the sort, 10,000, 1,000 or 100 cells were resuspended into matrigel (1:1 matrigel:PBS) and injected into mammary fat pad of athymic nude mice. The tumor take was assessed for each dilution of cells for 8 weeks and tumors ≥ 40 mm^2 were considered for the CSC frequency estimate. To determine the effect of Jagged1 on CSC frequency, the mice were randomized for control or Jagged1 neutralizing antibody (CTX-014) treatment. The treatment was started after one week of injecting the cells. There were four groups of each dilution of 100, 1000 and 10,000 cells- i Vehicle Jagged1-low-control, ii Vehicle Jagged1-low-CTX-014, iii Lapatinib Jagged1-high-control, iv Lapatinib Jagged1-high-CTX-014. At the end of 8 weeks, average tumor weight was calculated for each group at different dilutions and the graph of average tumor weight versus number of cells injected was plotted. Error bars represent S.D. and the statistical significance was calculated using ANOVA for multiple comparisons. GraphPad Prism was used for the analysis. The graph was created using MS-Excel.

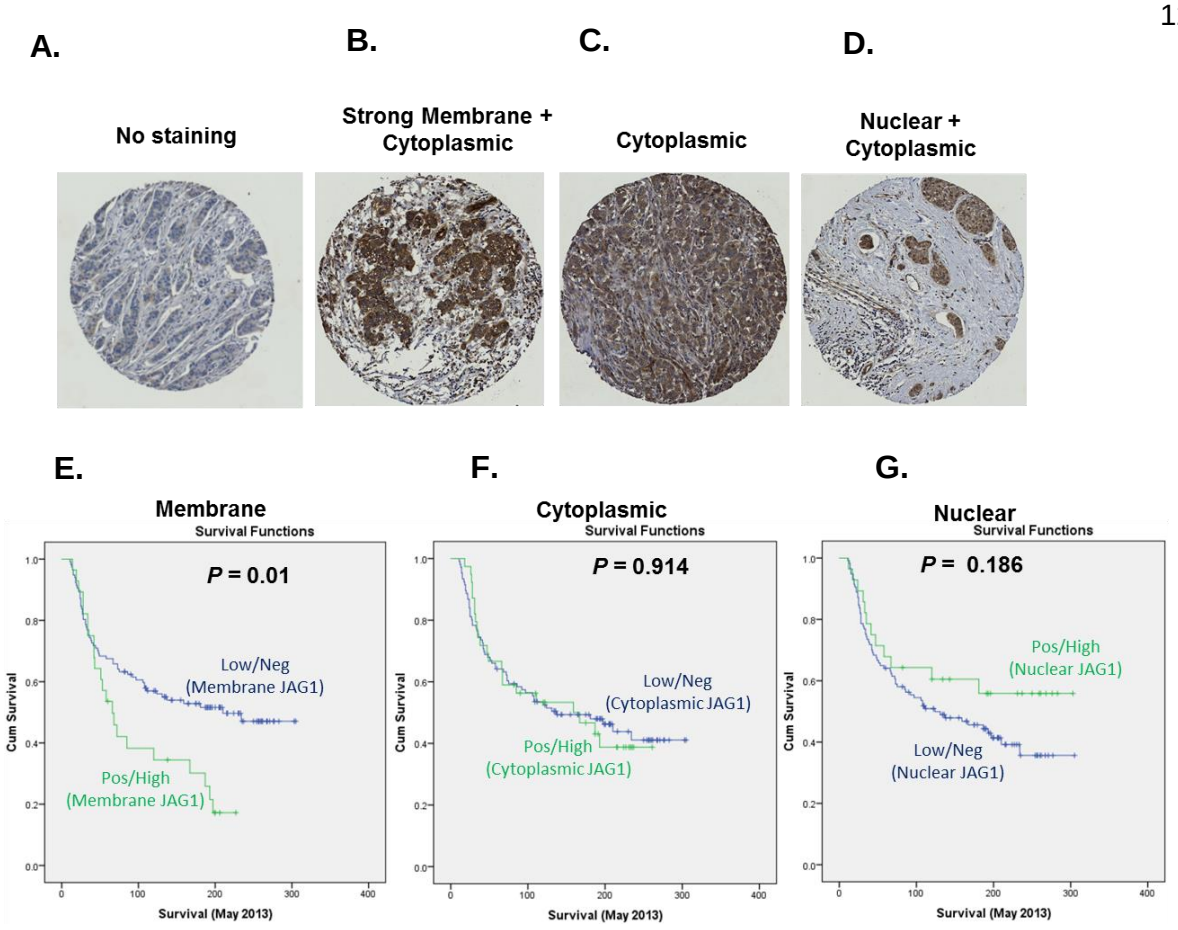


Figure 29: Jagged1 Membrane Expression Predicts Poor Overall Survival in Women with HER2+ Breast Cancer.

TMA was performed on Nottingham cohort of 145 HER2+ breast cancer tissues and Jagged1 staining was performed. Jagged1 was found to be localized in the nucleus, cytoplasm and cell membrane.

The panels are representative of negative staining (A), membrane staining (B), cytoplasmic staining (B,C, and D), and nuclear staining (D).

The tissues were scored 0.0 for low/negative staining and 1.0 for high/positive staining. Kaplan-Meier curve was plotted for overall survival and statistics was performed for membrane Jagged1 (E), cytoplasmic Jagged1 (F), and nuclear Jagged1 (G) using log-rank (Mantel-Cox) test.

A.

JAGGED1 MEMBRANE

JAGGED1_memb	Total N	N of Events	Censored	
			N	Percent
.00	117	57	60	51.3%
1.00	28	22	6	21.4%
Overall	145	79	66	45.5%

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	6.607	1	.010

Test of equality of survival distributions for the different levels of JAGGED1_memb.

B.

JAGGED1 NUCLEAR

Case Processing Summary

JAGGED1_Nuc	Total N	N of Events	Censored	
			N	Percent
.00	117	67	50	42.7%
1.00	28	12	16	57.1%
Overall	145	79	66	45.5%

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	1.749	1	.186

Test of equality of survival distributions for the different levels of JAGGED1_Nuc.

C.

JAGGED1 CYTOPLASMIC

Case Processing Summary

JAGGED1_cyto	Total N	N of Events	Censored	
			N	Percent
.00	106	57	49	46.2%
1.00	39	22	17	43.6%
Overall	145	79	66	45.5%

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.012	1	.914

Test of equality of survival distributions for the different levels of JAGGED1_cyto.

Table 7: Case Processing Summary and Overall Comparisons for the TMA Analysis.

The study was performed on 145 HER2+ breast cancer tissues. The table depicts the case processing summary for membrane Jagged1 (A), nuclear Jagged1 (B), and cytoplasmic Jagged1 (C). Statistics was performed by using log rank (Mantel-Cox) test.

CHAPTER VI

DISCUSSION

HER2+ breast cancer accounts for about 20% of all breast cancer cases. Current therapeutic regimen involves the use of anti-HER2 agents such as trastuzumab, pertuzumab, T-DM1, or lapatinib in combination with chemotherapy. The first line therapy for advanced, metastatic HER2+ breast cancer involves the combination of trastuzumab and pertuzumab along with docetaxel. This combination therapy showed less than 30% progression free survival and 57.6% overall survival at the end of four years. The median duration of response was only 20.2 months (Swain, Baselga et al. 2015). Thus, there is a dire need for better therapeutic strategies for the treatment of HER2+ breast cancer. One possible explanation for low response rate and drug resistance could be the existence of low lying subpopulation of CSCs. Due to tumor heterogeneity, the therapeutic regimen would be ineffective in targeting the CSCs which would then repopulate the tumor and promote drug resistance.

In the context of HER2+ breast cancer, Magnifico et al. have reported that HER2 is overexpressed in CSCs as compared to the bulk cells and that trastuzumab could be effectively used to target CSCs. Moreover, they showed that HER2 levels were upregulated by Notch1 signaling (Magnifico, Albano et al. 2009). In contrast with Magnifico's work, another study has shown that CD44 high/ CD24 low CSC subpopulation express lower levels of HER2 on the cell surface due to autophagy mediated internalization of HER2 and so trastuzumab is not able to effectively target the

CD44 high/ CD24 low tumorigenic subpopulation, as Natural Killer cells are unable to target HER2 to cause ADCC (Diessner, Bruttel et al. 2014). Moreover, HER2 low subpopulation sorted from mammospheres formed from the Luminal A subtype MCF7 cells had higher levels of CSC markers and CSC properties. The investigators found that HER2 high subpopulation was affected by trastuzumab, however, CSC containing, HER2 low subpopulation was not targeted by trastuzumab (Oak, Kopp et al. 2012). Lack of targeting of the CSC subpopulation by trastuzumab would explain why the response rate of single agent trastuzumab was only 26% (Vogel, Cobleigh et al. 2002).

Numerous signaling pathways have been implicated in the formation of CSCs. In HER2+ breast cancer, we have shown before that HER2 restricts Notch1 activation and that targeting HER2 results in the activation of Notch1 (Osipo, Patel et al. 2008). In an *in vivo* model, our group has shown that combining a Notch inhibitor with anti-HER2 agents is effective in the prevention of tumor recurrence and partial reversal of drug resistance (Pandya, Meeke et al. 2011). A recent study combining a mouse model of recurrent tumorigenesis and bioinformatic analyses of patient samples showed that Notch signaling is upregulated following HER2 inhibition, that Notch signaling remains activated in the subset of dormant tumor cells that survive after HER2 downregulation, that activation of Notch signaling accelerates tumor recurrence, and pharmacological and genetic inhibition of Notch signaling is critical for the prevention of tumor recurrence (Abravanel, Belka et al. 2015). This data suggest that the activation of Notch signaling in the probable CSCs promotes breast tumor recurrence. Notch1 and Notch4 receptors have been previously implicated in the formation of CSCs in HER2+ breast cancer

(Magnifico, Albano et al. 2009, Harrison, Farnie et al. 2010). Higher expression of Jagged1 and Notch1 mRNA is associated with overall survival of breast cancer patients (Reedijk, Odorcic et al. 2005). We have previously shown that Jagged1 is necessary for trastuzumab mediated Notch activation, that dual inhibition of HER2 and Jagged1 inhibits cell proliferation in vitro, and drug resistance in vivo (Pandya, Wyatt et al. 2016). In the current study, we sought to investigate the role of Jagged1 in the formation of CSCs in HER2+ breast cancer.

Preliminary results showed that HER2 restricts Jagged1 surface expression and that pharmacologic or genetic inhibition of HER2 results in an increase in Jagged1 on the cell surface (Figure 8 and Figure 9). Also, the increase in Jagged-1 membrane expression is dependent on the amount of active HER2 and not total HER2. Lapatinib would inhibit the tyrosine kinase activity but would increase the levels of total HER2 by stabilizing HER2 on the surface (Scaltriti, Verma et al. 2009). The increase in Jagged-1 upon lapatinib treatment is related to reduced levels of active HER2. Moreover, lapatinib mediated HER2 inhibition resulted in an increase in mammosphere formation suggesting an increase in survival of CSCs. The mammosphere formation was dependent on Notch activation as a GSI prevented or significantly reduced the mammosphere formation. Since lapatinib is a dual HER2-EGFR tyrosine kinase inhibitor, we wanted to confirm whether the effect that we observed was indeed due to HER2 inhibition. To address this, MCF7 cells and MCF7 cells stably overexpressing HER2 (MCF7-HER2) were treated with lapatinib. EGFR protein level is high in MCF7 cells, and they have very little HER2. On the other hand, MCF7-HER2 overexpress

HER2. The results showed an increase in the Jagged1 surface expression and a significant increase in the mammosphere formation upon lapatinib treatment in the MCF7-HER2 cells, but not in MCF7 cells. These results suggest that lapatinib mediated inhibition of HER2 tyrosine kinase activity is responsible for the increase in the Jagged1 surface expression and enrichment of mammospheres (Figure 6). Based on this information, Aim 1 investigated whether HER2 inhibition resulted in a subpopulation that had high Jagged1 on the cell surface and caused the formation of CSCs.

Results from Aim 1 showed that Jagged1-high subpopulation upon HER2 inhibition increased the formation of mammospheres and thus CSCs that is dependent on Notch activation (Figure 11). Besides mammospheres, the Jagged1-high subpopulation upon HER2 inhibition also had enhanced levels of aldehyde dehydrogenase (which is another marker for CSCs) as compared to Jagged1-low subpopulation in the absence of HER2 inhibition (Figure 13). The levels of CD44 high/ CD24 low (another marker for CSCs) did not change significantly between the two subpopulations (Figure 15). Per the recent review from Dr. Max Wicha's group, aldefluor assay is a better indicator of epithelial-like CSCs and CD44 high/CD24 low is a better indicator of mesenchymal-like CSCs. Our results would indicate that we have enriched a subpopulation with high epithelial-like CSCs (Liu, Cong et al. 2014). The results of our study are consistent with an investigation where the evaluation of 228 breast tumor tissues revealed that Jagged1 expression was correlated with higher tumor grade, overexpression of HER2, higher Ki-67 expression and aldehyde dehydrogenase positivity (Bednarz-Knoll, Efstathiou et al. 2016). Interestingly, MMTV-Neu mouse

mammary CSCs were enriched in the CD24+/JAG1- pool. The investigators found CSCs in the CD24+/JAG1+ fraction as well, however, the frequency was lower (Liu, Voisin et al. 2012). This could be due to Notch mediated differential effects observed within mouse microenvironment.

Importantly, the Jagged1-high subpopulation in the absence of HER2 inhibition did not cause the formation of CSCs suggesting that having high Jagged1 on the cell surface is not sufficient for the formation of CSCs and that inhibition of HER2 is necessary (Figure 12). In contrast to this, there has been a report which states that combined inhibition of HER2 and Notch receptors effectively targets Ductal Carcinoma In Situ (DCIS) stem/progenitor cell activity regardless of the HER2 status (Farnie, Willan et al. 2013). HCC1954 cell line that we used for our investigation was derived from a primary stage IIA, grade 3 invasive ductal carcinoma with no lymph node metastases. The discrepancy observed could be due to the difference in gene signature between ductal and invasive carcinoma.

Since HER2 inhibition resulted in an increase in the surface expression of Jagged1, we tested whether Jagged1 transcript and Jagged1 protein were upregulated as well. We did see an increase in the Jagged1 mRNA, however, Jagged1 protein level was decreased (Figure 10). Since the total protein level of Jagged1 is decreased but Jagged1 surface level is increased, it is very likely that HER2 inhibition prevents the endocytosis of Jagged1 or enhances the recycling of Jagged1 from the endosomes onto the cell surface. Previous work from our lab showed that Jagged1 colocalizes with early endosome in the presence of HER2 and that HER2 inhibition by trastuzumab

causes upregulation of Jagged1 on the cell surface (Pandya, Wyatt et al. 2016). This suggests that possibly trafficking of Jagged1 is altered during HER2 inhibition and that causes an upregulation of Jagged1 on the cell surface. The other possibility for decreased Jagged1 protein upon HER2 inhibition could be the post-transcriptional or post-translational regulation of the protein resulting in decreased synthesis or rapid degradation of the protein.

Since the Jagged1-high subpopulation upon HER2 inhibition resulted in an increase in CSCs, we wanted to confirm whether the effect was indeed dependent on Jagged1. To address this, Jagged1 was knocked down using two different siRNAs targeting Jagged1. Knocking down of Jagged1 prevented lapatinib mediated increase in mammosphere formation suggesting that Jagged1 was indeed necessary for lapatinib mediated enhancement of mammospheres and thus CSCs (Figure 17).

As Notch signaling has been implicated in the formation of CSCs and GSI completely prevented the formation of mammospheres from the lapatinib Jagged1-high subpopulation, the status of Notch activation in the Jagged1-high subpopulation upon HER2 inhibition (lapatinib treatment) and the Jagged1-low subpopulation in the absence of HER2 inhibition (vehicle treatment) was determined by real-time PCR. The Jagged1-high subpopulation indeed had higher Notch activation (Figure 18). It was counter intuitive that the ligand expressing i.e. signal sending cell had higher Notch activation. Based on what we know about Notch signaling, it would be the Notch receptor expressing i.e. signal receiving cell would be the one with the higher Notch activation. To address this, we treated cells with or without lapatinib and then co-stained the cells

with Jagged1 and Notch1, Jagged1 and Notch3, and Jagged1 and Notch4. Notch2 was not used for this study as it was the least expressed amongst all the Notch receptors and was barely detected by real-time PCR. Upon performing flow cytometry, it was found that all the cells that express Jagged1 also expressed Notch1, Notch3, and Notch4 (Figure 20 and Figure 21). In the context of Notch signaling, when the ligand and the receptor are expressed on the same cell, it leads to *cis* inhibition, and when they are expressed on adjacent cells, it leads to *trans* activation. This would raise the question: Do we see more Notch activation upon HER2 inhibition due to increased *trans* activation or due to reduced *cis* inhibition? To address this, a co-culture assay was previously performed in the lab by Kinnari Pandya. HER2+ MDA-MB-453 cells, when co-cultured in the presence of mouse fibroblasts overexpressing Jagged1 (LTK – Jagged1), showed a significant increase in Notch activation, suggesting that HER2 is limiting *trans* activation of Notch. Also, if HER2 is promoting *cis* inhibition of Notch through Jagged1, then knocking down Jagged1 should relieve the inhibition and result in an increase in Notch activation. However, when Jagged1 was knocked down in HER2+ MDA-MB-453 or luminal B BT474 cells, it did not result in an increase in Notch activation. These results would confirm that HER2 is not promoting *cis* inhibition (Pandya, Wyatt et al. 2016). During normal development when the cell expresses both the ligand and the receptor, the Notch activity is regulated through lateral inhibition. Notch-Delta signaling is critical in determining the distinct fates of the neighboring cells, whereas Jagged mediated signaling drives the cell to maintain a similar fate to that of its neighbor (Boareto, Jolly et al. 2015). In the context of solid tumors, a recent study

reported the involvement of Jagged1 in the lateral inhibition in glioblastoma and pancreatic cancer model. The researchers demonstrated that when Jagged1 overexpressing cells were co-cultured with Jagged1-low cells, the Jagged1-high cells had lower Notch activation. They found that overexpression of Jagged1 causes its cleavage resulting in Jagged1- intracellular domain (ICD) which reduces Notch signaling potentially through nuclear localization (Lim, Brandt et al. 2015).

Through Aim1, we demonstrated that HER2 inhibition results in the subpopulation that has upregulation of Jagged1 on the cell surface, higher Notch activation, and enhanced ability to form CSCs. For CSC determination, *in vivo* limiting dilution assay is considered as the gold standard in the breast cancer field. The assay involves injecting different dilutions of cells and assessing tumor take. Depending upon the tumor take at each dilution, the CSC frequency is determined. If the population has higher CSC frequency, then very few cells would be required to form the tumor (Hu and Smyth 2009). Our preliminary experiment involved sorting and injecting 10,000 Jagged1-high cells after lapatinib treatment and 10,000 Jagged1-low cells after vehicle treatment. Hundred percent tumor take was observed in the case of mice injected with Jagged1-high cells, as compared to 33.3 percent tumor take in case of mice injected with Jagged1-low cells (Figure 25). The data conclusively demonstrated the higher CSC potential of the Jagged1-high population. To determine the CSC frequency of the two subpopulations and the role of Jagged1 in affecting the CSC frequency, limiting dilution study involved injecting 100, 1,000 and 10,000 sorted Jagged1-high and Jagged1-low cells. The tumor take was assessed until 8 weeks and tumors ≥ 40 mm^2 were

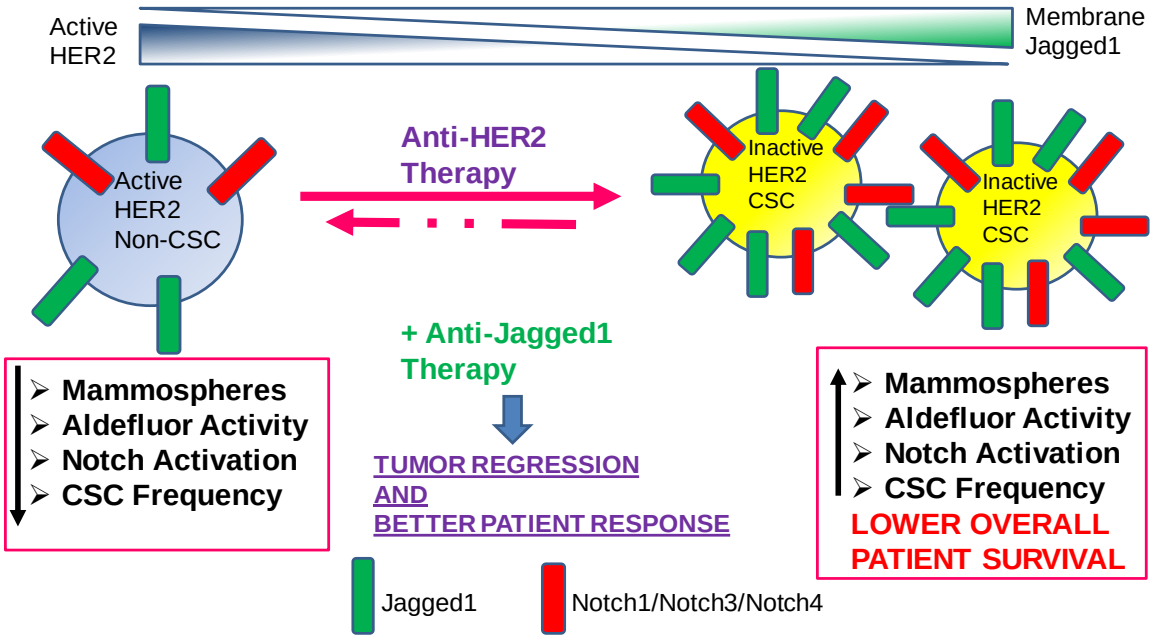
considered. At the end of the first week, the mice in each group were randomized and were treated with either vehicle control or Jagged Neutralizing Antibody (CTX-014). At the end of 8 weeks, the Jagged1-high subpopulation was found to have significantly higher CSC frequency as compared to the Jagged1-low subpopulation. CTX-014 did not significantly affect the CSC frequency of the Jagged1-high subpopulation. However, it did significantly increase the CSC frequency of the Jagged1-low subpopulation (Figure 26 and Table 6). The antibody targets Jagged1 and Jagged2, and is supposed to inhibit the binding of Jagged1 and Jagged2 with the Notch receptors. Since the antibody was ineffective *in vivo,* we determined *in vitro* whether it affected the formation of mammospheres or Jagged1 surface expression, and it didn't. The possible reason for the ineffectiveness of the antibody could be the suboptimal binding affinity of the antibody for Jagged 1. Also, the high affinity of the antibody for the inhibitory Fc receptors such as FcgRIIb, expressed by B-cells, macrophages, dendritic cells, and neutrophils might decrease their overall efficiency (Chames, Van Regenmortel et al. 2009). 20 mg/kg CTX-014 has been previously shown to have the highest efficacy in HER2/neu transgenic model and pancreatic cancer xenograft model. Since we used athymic nude mice in the study, we cannot disregard the possibility of development of anti-therapeutic antibodies against the therapeutic monoclonal antibody due to immunogenicity (Kamath 2016). Rapid clearance of CTX-014 is a possibility. Another reason could be clonal selection of Jagged1-low cells by CTX-014 treatment. The clonal selection might cause Jagged1-low cells to express extremely high levels of surface Jagged1, rendering the antibody ineffective.

To determine whether higher Jagged1 on the cell surface has actual clinical significance, we collaborated with the group in Nottingham, U.K. They stained 145 HER2+ breast tumor tissues for Jagged1 and found that Jagged1 was localized in the membrane, cytoplasm, and nucleus. Based on the staining intensity, the samples were classified into low Jagged1 expression or high Jagged1 expression. They assessed the cumulative survival of each patient i.e. the time from the onset of surgery to breast cancer related death. The results revealed that there was a significant inverse correlation between membrane Jagged1 and overall survival i.e. higher Jagged1 on the membrane significantly predicted poor overall survival in women suffering from HER2+ breast cancer. However, no significant correlation between cytoplasmic and nuclear Jagged1 and overall survival of the HER2+ breast cancer patients was observed (Figure 29 and Table 7). Endocytosis of Jagged1 in the signal sending cell would result in cytoplasmic Jagged1. The other reason for the presence of cytoplasmic Jagged1 and nuclear Jagged1 is that just like the Notch receptors, the Notch ligands could be cleaved by ADAM metalloprotease and gamma secretase complex following ligand-receptor interaction. This would result in the formation of an intracellular fragment of the ligand which then translocates into the nucleus. The Jagged1 intracellular domain has been known to regulate cell proliferation and transformation (Ascano, Beverly et al. 2003, LaVoie and Selkoe 2003, Lim, Brandt et al. 2015). The results from the patient study clearly reveal the importance of targeting membrane Jagged1 for the improvement of overall survival of patients suffering from HER2+ breast cancer.

Conclusion

In the current study, we demonstrated for the first time the role of surface Jagged1 in the enrichment of CSCs in HER2+ breast cancer. We conclusively showed that HER2 inhibition by pharmacological or genetic means enriches membrane Jagged1-high subpopulation that has higher CSC potential and Notch activation. Moreover, higher membrane Jagged1 predicts poor overall survival of women suffering from HER2+ breast cancer. Because of the plasticity between the CSCs and non-CSCs, it is very likely that there is interconversion of CSCs to non-CSCs and vice versa. Hence, it is critical to target both the CSCs and non-CSCs. Using combinatorial approach involving anti-HER2 therapy along with Jagged1 targeted therapy in the neoadjuvant setting could prevent the repopulation of the tumor and provide better patient survival.

Model



HER2 restricts Jagged1 surface expression. Inhibition of HER2 results in an increase in Jagged1 surface expression. Upon HER2 inhibition, the cells that are enriched for Jagged1 on the cell surface possess CSC-like properties and have higher Notch activation. Moreover, higher Jagged1 on the cell surface predicts poor overall survival of women suffering from HER2+ breast cancer. Combining anti-HER2 therapy along with anti-Jagged1 therapy would result in tumor regression and provide a better patient response.

Future Investigations

Our studies demonstrated for the first time that genetic or pharmacological inhibition of HER2 results in a concomitant increase in the surface expression of Jagged1. The increased surface expression is responsible for the enrichment of CSCs and predicts poor overall survival of the patients suffering from HER2+ breast cancer. Even though the surface levels and mRNA of Jagged1 increased upon HER2 inhibition, the level of Jagged1 protein was found to be reduced. Considering the clinical significance of Jagged1 surface expression, the future investigation should entail the mechanism that enhances Jagged1 surface expression upon HER2 inhibition. The role of trafficking could be addressed by studying the interplay between dynamin, Rab4a, Rab11a, and Jagged1. Dynamin is involved in the pinching of vesicles and is important for endocytosis. Rab4 and Rab11a are the GTPases that are involved in the recycling of cargo from the endosomes onto the cell surface (Stenmark 2009). Dominant negative form of dynamin could be used to determine the role of endocytosis. If HER2 enhances endocytosis, then blocking dynamin mediated endocytosis would result in an increase in the surface expression of Jagged1. To understand recycling siRNA against Rab4a or Rab11a could be used. If Rab4a or Rab11a is responsible for recycling of Jagged1 on the cell surface, then knocking down Rab4a or Rab11a would reduce the recycling of Jagged1 onto the cell surface.

Lipopolysaccharide and prostaglandin E2 are known to upregulate the expression of Jagged1 in dendritic cells. Hedgehog causes upregulation of Jagged1 expression in mesenchymal cells and it is important for limb development. TGF-β

upregulates Jagged1 expression in epithelial cells to cause EMT (D'Souza, Miyamoto et al. 2008). Beta-catenin is known to upregulate the transcription of Jagged1 in colorectal cancer and ovarian cancer (Rodilla, Villanueva et al. 2009, Chen, Stoeck et al. 2010). It would be important to determine if any of these effectors are critical for the transcriptional upregulation of Jagged1 upon HER2 inhibition.

To address the possibility of clonal selection versus expansion of Jagged1-high subpopulation of cells, lineage tracing could be performed. A stable HCC1954 cell line that expresses GFP can be created (HCC1954-GFP). HCC1954-GFP cells can be stained with Jagged1 and the cells expressing high Jagged1 on the cell surface can be sorted. Similarly, HCC1954 parental cells can be stained with Jagged1 and the cells expressing low Jagged1 on the cell surface can be sorted. The sorted HCC1954-GFP Jagged1-high cells can then be co-cultured with HCC1954 parental Jagged1-low cells. These co-culture cells can then be treated with 2 μM lapatinib or vehicle control for four days. Followed by the treatment, the cells can again be stained with Jagged1. The Jagged1 and GFP staining of the cells could be examined by flow cytometry. We know that lapatinib treatment increases Jagged1 expressing cells on the surface. If the lapatinib treatment results in surface Jagged1 expressing cells that are only GFP positive, that would indicate clonal expansion of Jagged1-high cells. However, if the surface Jagged1 cells upon lapatinib treatment contains a mix of GFP positive and negative cells, then that would indicate the possible conversion of Jagged1-low cells into Jagged1-high cells.

To understand the plasticity of CSCs, similar lineage tracing approach could be utilized. HCC1954 GFP cells could be treated with lapatinib and stained with Jagged1. The HCC1954 – GFP cells can then be sorted for Jagged1-high surface expression. Similarly, HCC1954 – YFP stable cell line can be created. HCC1954 – YFP cells can be stained with Jagged1 and Jagged1-low cells can be sorted. Like the pilot experiment that we performed for the *in vivo* study, 10k HCC1954-GFP Jagged1-high cells and 10k HCC1954-YFP Jagged1-low cells can be injected into the mice. After the tumor formation, the tumor could be excised and tumor cells could be dissociated. The dissociated tumor cells could then be examined for GFP or YFP expression. If all the cells are not YFP or GFP positive, that would indicate the tumor heterogeneity. Extensive proteome analysis should be performed using the dissociated tumor cells (GFP+, YFP+, and GFP-YFP-). The proteome analysis would give a better idea regarding the differences in the cell signaling networks and potential drivers of the CSCs and non-CSCs.

Jagged1 neutralizing antibody that we used for the *in vivo* study was not able to effectively block Jagged1. In the future, it would be critical to develop therapeutics targeting Jagged1. Small molecule inhibitors targeting the binding of Jagged1 with the Notch receptor that is involved in mediating CSCs would prove to be beneficial. Moreover, newer approaches like exosomes or nanoparticles mediated Jagged1 siRNA delivery could be tried for effectively targeting Jagged1 (Cong-fei Xu 2015).

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VITA

The author, Deep Shah, was born in Ahmedabad, India on September 26, 1988, to Sanjay Shah and Chhaya Shah. He attended Ganpat University, India where he earned a Bachelor's degree in Pharmacy in May 2010. Deep then moved to the United States and got his Master's degree in Pharmacology from Northeastern University, Boston. After completing his M.S. degree in May 2012, Deep matriculated into the Loyola University Chicago Stritch School of Medicine Molecular Pharmacology and Therapeutics Program and began his graduate education under the mentorship of Dr. Clodia Osipo.

While at Loyola, Deep served as a Graduate School Council-Health Sciences Division Representative and as a Graduate Student Advisory Council Representative from 2015-2016.

Deep's dissertation work on the role of Jagged1 in the enrichment of cancer stem cells in HER2+ breast cancer was supported in part by the Arthur J. Schmitt Predoctoral Dissertation Fellowship. After completion of his graduate studies, Deep will pursue a commercial management internship at Regeneron Pharmaceuticals, New York.