

Title: Inhibition of HER2 Increases Jagged1-dependent Breast Cancer Stem Cells: Role for Membrane Jagged1

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Running Title: Anti-HER2 Therapy Promotes Jagged1-High Stem Cells

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ABSTRACT

Purpose: Human Epidermal Growth Factor Receptor 2 (HER2) positive breast cancer is driven by cells possessing stem-like properties of self-renewal and differentiation, referred to as Cancer Stem Cells (CSCs). CSCs are implicated in radiotherapy, chemotherapy resistance, and tumor recurrence. Notch promotes breast CSCs survival and self-renewal, and overexpression of Notch1 and the Notch ligand Jagged1 predict poor outcome. Resistance to anti-HER2 therapy in HER2+ breast cancer requires Notch1, and that combination of trastuzumab and a Gamma Secretase Inhibitor (GSI) prevents tumor relapse in xenograft models.

Experimental Design: The current study investigates mechanisms by which HER2 tyrosine kinase activity regulates Notch-dependent CSC survival and tumor initiation.

Results: Lapatinib-mediated HER2 inhibition shifts the population of HER2+ breast cancer cells from low membrane Jagged1 expressing to higher levels, independent of sensitivity to anti-HER2 treatment within the bulk cell population. This increase in membrane Jagged1 is associated with higher Notch receptor expression, activation, and enrichment of CSCs *in vitro* and *in vivo*. Importantly, lapatinib treatment results in growth arrest and cell death of Jagged1 low-expressing cells while the Jagged1 high-expressing cells continue to cycle. High membrane Jagged1 protein expression predicts poor overall cumulative survival in women with HER2+ breast cancer. **Conclusions:**

These results indicate that higher membrane Jagged1 expression may be used to either predict response to anti-HER2 therapy or for detection of Notch sensitive CSCs post therapy. Sequential blockade of HER2 followed by Jagged1 or Notch could be more effective than simultaneous blockade to prevent drug resistance and tumor progression.

TRANSLATIONAL RELEVANCE

Critical concerns for women with HER2+ breast cancer are drug resistance, tumor recurrence, and disease progression. The current study describes a novel role for membrane Jagged1 in enrichment of breast CSCs and resistance to anti-HER2 therapy. The clinical significance of this work is that higher membrane Jagged1 is a biomarker for higher Notch activation, and it predicts poor overall cumulative survival. Sequential blockade of HER2 followed by Jagged1 or Notch could be more effective than simultaneous blockade to prevent drug resistance and tumor progression.

INTRODUCTION:

Breast cancer remains the most common form of cancer among women worldwide. By 2026, it is estimated that there will be approximately 4,571,210 breast cancer survivors in the United States. Gene expression profiling has revealed distinct intrinsic subtypes of breast cancer. There are four major classes – Luminal A, Luminal B, human epidermal growth factor receptor 2 enriched (HER2+) and basal-like (1-3). Approximately, 15-20% of breast cancers are HER2+ (4, 5). These cancers contain amplification for a section of chromosome 17q containing the ERBB2 gene locus and neighboring genes GRB7 and MIEN1, accompanied by protein overexpression of HER2 detectable as 3+ staining by immunohistochemistry (6). Treatment options include the use of biologics, trastuzumab and pertuzumab, along with taxane-based chemotherapy, use of the antibody drug conjugate TDM1 (trastuzumab emtansine) or dual epidermal growth factor receptor (EGFR)/ HER2 tyrosine kinase inhibitor lapatinib (6).

Despite improved prognosis for women with HER2+ breast cancer, drug resistance, disease progression, and tumor recurrence remain major challenges preventing a cure (6). It is estimated that less than 35% of the patients initially respond to trastuzumab. This could be deemed as primary or intrinsic resistance (7, 8). Out of those who respond, about 70% exhibit metastatic progression within one year, suggesting secondary or acquired drug resistance (9). Drug resistance could be due to multiple factors including survival of resistant cells referred to as cancer stem cells (CSCs). The CSC hypothesis suggests that a tumor is comprised of a heterogeneous population of cells including small subsets or clones with stem-like properties. These stem-like properties can be intrinsic to cell clones or acquired through epigenetic remodeling by

hypoxia, EMT or treatment-induced cell stress. These small subsets of stem-like cells (CSCs) are thought to be selected by treatment which kills the majority of non-stem-like cells, while the surviving CSCs are responsible for drug-resistant recurrences (10). Notch signaling has been implicated in mammary stem cell self-renewal as well as in survival and self-renewal of breast CSCs (11, 12).

Notch signaling is a developmentally conserved signaling pathway mediating communications between cells. Typically, the ligand is expressed on the signal sending cell and the receptor is expressed on the signal receiving cell. Mammals express five Notch ligands (Jagged1, Jagged-2, Delta-like 1, 3, and 4), and four Notch receptors (Notch1-4). Delta-like 3 is thought to be an inhibitory ligand, while Delta-like 1 and 4 and Jagged1 and 2 are stimulatory ligands. The relative affinity of Notch receptors for different ligand classes is modulated by glycosylation of the extracellular subunits of Notch receptors mediated by Fringe family N-acetyl-glucosaminidyltransferases, POFUT fucosyltransferase, and Rumi glucosyl transferase (13). When a ligand engages a receptor, it is ubiquitinated by an E₃ ligase, Neuralized or Mindbomb (14). This allows the ligand to be endocytosed into ligand expressing cells with the extracellular subunit of the Notch receptor, separating the latter from the transmembrane subunit. The receptor transmembrane subunit then undergoes subsequent cleavages by ADAM10 and finally by γ -secretase, to release the Notch intracellular domain (NICD). NICD trans-locates into the nucleus of the signal receiving cell to initiate the transcription of Notch target genes (15). Overexpression of intracellular domains of Notch1, Notch3, and Notch4 in the mammary gland of mice leads to the formation of metastatic and aggressive breast tumors (16, 17). Notch1, Notch3, and Notch4

receptors are required for survival and self-renewal of breast CSCs (18-20). High co-expression of Notch1 and Jagged1 transcripts are associated with poor overall survival of women with breast cancer (21). High levels of Jagged1 transcripts are associated with increased stem cell self-renewal in the Luminal subtypes of breast cancer (22). Further, increased Jagged1 expression has recently been implicated in promoting stemness and angiogenesis (23). Recent reports showed that a Jagged1-Notch-4 axis is critical for mediating endocrine resistance and survival of breast CSCs (24). We have shown previously that Notch1 or Jagged1 is necessary for mediating trastuzumab resistance (25-27). Further, previous results demonstrated that Notch signaling is necessary for dormancy and tumor recurrence following anti-HER2 therapy (28, 29).

Here, we show that overexpression and importantly, hyperactivity of HER2 restricts Jagged1 cell surface expression. When HER2 is inactivated by lapatinib, there is an increase in cell surface Jagged1, Notch target gene expression, % of cells in the S-phase of the cell cycle, and increased survival of breast CSCs. In HER2+ breast cancer tissue, higher Jagged1 membrane staining, but not cytoplasmic or perinuclear Jagged1 expression, predicts poor overall survival for women with primary, invasive HER2+ breast cancer. Overall, results of the study demonstrate that HER2 expression and activity status specifically restricts high membrane Jagged-1-enriched breast CSCs. The consequence of HER2 inactivation is a shift from low Jagged1 membrane expression to high Jagged-1 membrane expression responsible for breast CSCs that are resistant to lapatinib.

MATERIALS AND METHODS:

Cell Culture

HCC1954, MDA-MB-453 and MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA). MCF-7 cells stably overexpressing HER2, designated as MCF-7-HER2, were generously provided by Dr. Mien-Chie Hung from The University of Texas, M.D. 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 media was supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Sacramento, CA), 1% L-glutamine (2 mM, Thermo Fischer Scientific, Waltham, MA) and 1% non-essential amino acids (100 μ M, Invitrogen, Carlsbad, CA). Cell lines were authenticated using short tandem repeat allelic profiling (DCC Medical) in 2015 and maintained at low passage numbers (below 20). All cell lines were maintained by incubating at 37°C with 95% humidity and 5% CO₂.

Drugs and Chemicals:

A γ -secretase inhibitor (GSI), MRK-003 was provided by Merck Oncology & Co. (Whitehouse Station, NJ). For *in vitro* studies, 5 mM MRK-003 GSI stock solution was prepared by dissolving in dimethyl sulfoxide (DMSO). The working concentration was 5 μ M and the prepared drug was stored at -80°C for future use. Lapatinib, a dual HER2-EGFR tyrosine kinase inhibitor was purchased from Selleck Chemicals, Houston, TX. For *in vitro* studies, 4 mM stock concentration of lapatinib was prepared by dissolving it

in dimethyl sulfoxide (DMSO). The working concentration was 2 μ M and the prepared drug was stored at -80°C for future use.

RNA Interference and Transfection Reagents:

Jagged1 stealth small interfering RNA (siRNA) having two different sequences was purchased from Thermo Fisher Scientific, Waltham, MA (Cat. HSS176254 and HSS176255). The sequences were Jagged1 A (GATAACTGTGCGAACATCACATTTA) and Jagged1 B (CGCGACGAGTGTGACACATACTTCA). HER2 siRNA (rGrCrCrArArCrArArArGrArArArUrCrUrUrArGrArCrGrAAG) was purchased from Origene, Rockville, MD (Cat. SR301443). 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. The siRNAs were reconstituted with RNase free water to yield a stock concentration of 10 μ M. The final working concentration of the siRNA was 10 nM. For Jagged1 siRNA transfection, 17 μ L siRNA and 17 μ L of lipofectamine 300 or equal volume of siRNA to RNAiMax was used in a 60mm plate. For HER2 siRNA transfection, 20 μ L of siRNA and 20 μ L RNAimax was used in a 60mm plate. The transfection was performed according to the manufacturer's protocol.

Flow Cytometry:

HCC1954 (250,000 cells/well – 6 well plate), MDA-MB-453 (350,000 cells/well – 6 well plate for treatment and 700,000 cells/well – 60 mm plate for transfection), MCF-7 (40,000 cells/well – 6 well plate), or MCF-7-HER2 (40,000 cells/well – 6 well plate) were treated for four days with DMSO or 2 μ M lapatinib (Cat. S1028, Selleck). For 4 days of lapatinib treatment, cells were trypsinized and replated after 2 days at a similar density. This was done to avoid over-confluency and to maintain similar density. The impact of pharmacologic inhibition of HER2 on the surface expression of Jagged1 was assessed using flow cytometry. The cultured cells were harvested using gentle trypsinization or Cellstripper (Cat.25–056-CI, Corning Cellgro, Manassas, VA). The harvested cells were neutralized using DMEM media 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 (Cat. FC001, R&D Systems, Minneapolis, MN) 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 in the presence of 100 μ g/mL DNase I to limit cell clumping. After the second wash, the excess staining buffer was aspirated, leaving about 250 μ l of buffer and the cell pellet. The cells were then stained by using biotinylated human Jagged1 primary antibody (Cat. BAF1277, R&D Systems, Minneapolis, MN). About 3-6 μ l of primary antibody was added to each tube containing 1– 2 million cells. The cell suspension/antibody mixture was then incubated for 45 minutes at room temperature. Subsequently, the cells were washed twice in 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 μ l. To this cell suspension, APC conjugated secondary antibody (Cat.

405207, BioLegend, San Diego, CA) 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 2-8 μ l of the antibody was added to each tube containing 100,000 – 1 million cells. The tubes were then incubated at room temperature for 45 minutes. After the incubation, tubes were washed twice with flow cytometry staining buffer as described before. The cell pellet was finally resuspended in 250 μ l flow cytometry staining buffer for analysis by using BD FACS Canto II (BD BioSciences, San Jose, CA). Data was captured using BD FACSDiva software. Data analysis was performed using FlowJo software. Specificity of Jagged1 expression by flow cytometry was confirmed using a Jagged1 siRNA. For CD44 High/CD24 low assay, HCC1954 cells were harvested and stained per the protocol described above. The cells were stained with APC conjugated CD44 (Cat. 103012, BioLegend, San Diego, CA)/ FITC conjugated CD44 (Cat. 103021 BioLegend, San Diego, CA) and PE conjugated CD24 (Cat. 311106, BioLegend, San Diego, CA), and were analyzed as mentioned above.

Cell Sorting:

HCC1954 or MCF-7-HER2 cells were treated with DMSO or 2 μ M lapatinib for 4 days, and were stained with biotinylated Jagged1 (3-8 μ L/1-2 million cells) and streptavidin-APC antibodies (5 μ L undiluted) as described previously. To sort sufficient cells (HCC1954 and MCF-7-HER2), 2 T150 flasks (4 million cells/flask) were used for DMSO treatment and 3 T150 flasks (4.5 million cells/flask) were used for lapatinib treatment. After staining, cells were sorted based on Jagged1 surface expression (Jagged Low and Jagged High) by a BD FACSAria cell sorter (BD BioSciences, San Jose, CA). The

cells were sorted into 24 well plates containing mammosphere medium (35,000 cells/well) supplemented with DMSO/5 μ M GSI for mammosphere assays. The cells were sorted into FACS tubes to perform Aldefluor assays, CD44 high/CD24 low assessment, and to detect the expression of Notch target genes. Subsequently, cell suspensions were centrifuged at 1300 rpm for 3 minutes, and cells were then used for performing these assays. The detailed protocols for mammosphere assays, Aldefluor assays, CD44 high/CD24 low assessment and detection of Notch target genes using real-time PCR are described below. Control experiments were performed whereby HCC1954 cells (3-T150 flasks) were stained for Jagged1 surface expression as described above, and Jagged1-high and Jagged1-low cells were sorted (25,000 cells/well) into 24 well plates containing mammosphere media supplemented with DMSO/ 5 μ M GSI. Mammosphere formation was assessed as described previously (30).

Cell Proliferation Assays:

Cells HCC1954, MCF-7, or MCF-7-HER2) were plated at a known density as described previously and then subsequently treated every day with vehicle (DMSO) or 2 μ M lapatinib up to 4 days. Total numbers of viable cells were counted using the Countess Cell Counter. Cell cycle analysis was conducted using propidium iodide according to the manufacturer's instructions (Cell Signaling Technology).

Aldefluor Assay:

Aldehyde Dehydrogenase ALDH1A1 is one of the 19 ALDH isoforms expressed in humans, and is believed to be responsible for the ALDH activity of CSCs. ALDH is a detoxifying enzyme, and is responsible for the oxidation of intracellular aldehydes. An Aldefluor kit (Cat. 01700, STEMCELL Technologies, Vancouver, Canada) was used to detect the cancer stem cell (CSC) population based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1A1). Two tubes containing 200k sorted/ unsorted HCC1954 cells suspended into 1mL of Aldefluor assay buffer were prepared – a test tube that received 5 µl ALDH1A1 substrate, and a control tube that received 10 µL aldehyde dehydrogenase inhibitor N, N-diethylaminobenzaldehyde (DEAB) and 5 µl substrate. The tubes were incubated at 37°C for 45 minutes in the dark, put on ice and 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 ice and were analyzed using flow cytometry as mentioned before. Live cells were gated based on propidium iodide staining and forward and side scatter. Percent Aldefluor positive cells were determined in the DEAB negative sample compared to the DEAB positive sample based on ALDH- cell gating assignments. Three gating assignments were assessed: ALDH-high, moderate, and low.

Mammosphere Forming Assay:

This protocol is a modified version of the Shaw et al assay (30). Preparation of Methyl Cellulose based Mammosphere Medium – 196 mL of DMEM-F12 medium (Cat. 11039021, Gibco) was warmed at 60°C and added to a bottle containing 2g of methyl cellulose. The contents were then allowed to stir at 60°C for 2.5 hr until methyl cellulose

was uniformly mixed. The DMEM-F12 methyl cellulose medium-mixture was then stirred overnight at 4°C. The following day, 4 mL B-27 supplement and 4 µL recombinant hEGF (Cat. E-9644, Sigma Aldrich) was added to the medium. The medium was then stirred at 4°C for 30 mins and then transferred into centrifuge tubes. The tubes were then centrifuged at 9500 rpm in a Beckman rotor at 4°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°C for 2-3 hours prior to use. Excess mammosphere medium was stored at -20°C for future use. To perform a mammosphere forming assay, cells were harvested using trypsin or FACS-sorted. The single cell suspension was prepared and 100,000 cells/well (6 well ultra-low attachment plates) or 35,000 cells/well (24 well ultra-low attachment plates) were added into the mammosphere medium containing vehicle control DMSO or 5 µM MRK-003 GSI.

In order to study the effect of Jagged1 knockdown, 600,000 HCC1954 or MCF-7-HER2 cells were plated into 60mm plates. 3 plates each were used for transfection of non-targeting siRNA or Jagged1 siRNA. The transfection was performed as described previously. After transfection, cells were harvested and re-plated for DMSO or lapatinib treatment for 4 days. After treatment, 100,000 cells in a single cell suspension were used for mammosphere forming assays, and the remaining cells were used for Western blots. After the addition or sorting of the cells, mammosphere plates were rocked back and forth several times to ensure that the cell suspension was evenly distributed. The mammosphere plates were then incubated at 37°C and 5% CO₂ for 7-10 days, after which mammospheres were harvested and counted. In order to harvest the

mammospheres, 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. The plate was observed under the microscope to ensure that all the mammospheres were harvested. 15 mL conical tubes containing mammosphere suspensions were centrifuged at 1500 rpm for 5 minutes. 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 centrifuged at 5000 rpm for 5 mins. One more wash with 2 ml PBS was performed and then the mammosphere pellet was again re-suspended into 2mL PBS (2 ml PBS was used as resuspension volume if 100k cells were plated. For 35,000 sorted cells, 500 μ L PBS was used for resuspension). After resuspension, 50 μ L was transferred into a well of a 96 well plate. 350 μ L PBS was then added to the well. Mammosphere-containing wells were photographed under a microscope and five fields were taken at 4X magnification. The photographs contained a measurement scale. Taking five fields ensured that the photographs captured all the mammospheres in the well. The photographs 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 μ m i.e. all the spheres \geq 50 μ m were counted as mammospheres. Based on resuspension volume, the dilution factor was used to count the total mammospheres. Percentage mammosphere forming efficiency was defined as $100 \times (\text{Number of mammospheres} / \text{number of cells plated})$.

Tumor Initiating Potential:

HCC1954 cells were treated with either DMSO or 2 μ M lapatinib for 4 days and were sorted by flow cytometry on the basis of Jagged1 surface expression. For pilot experiments, 10,000 sorted cells were injected into mammary fat pads of 5 female, ovariectomized FoxN1 nu/nu athymic nude mice (Harlan Sprague-Dawley, Madison, WI). During the course of the study, each mouse and its tumor was tracked using an ear tag. Tumor take was assessed for up to 8 weeks and the tumorigenic potential of the two subpopulations was calculated. In order to perform a limiting dilution assay to detect the frequency of CSC formation, mice were randomized into following groups – 100/ 1000/ 10,000 DMSO Jagged Low cells/site (n=8-10 per group) or 100/ 1000/ 10,000 Lapatinib Jagged High cells/site (n=8-10 per group). Beginning one week after injection, 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 a tumor ≥ 40 mm² was determined and the tumor initiating potential was calculated by using the Extreme Limiting Dilution Analysis (ELDA) software. The protocol for animal study was approved by Loyola University's Institutional Animal Care and Use Committee (IACUC).

Immunohistochemical staining of human HER2+ breast tumors:

The Nottingham Tenovus Primary Breast Carcinoma Series were used. These are a series of 1,944 cases of primary operable invasive breast carcinoma from woman diagnosed from 1986–1998 (31). Primary, invasive stage II-III HER2+ breast tumor tissues were adhered by placing the tissue microarray (TMA) sections in an oven at 58°C to 60°C. Xylene was used for de-paraffinizing the tissue followed by rehydration

using ethanol, and washed with PBS 1X. Reveal treatment in a Decloaking Chamber (Biocare Medical) for antigen retrieval. Once the TMA sections were rinsed by PBS for 15 minutes, sections were treated using 3% H₂O₂ in PBS for 20 minutes to quench endogenous peroxidase activity. Sections were incubated for one hour in 3% normal rabbit serum (Vector Laboratories) in PBS at room temperature to block non-specific binding. Sections were then incubated with primary antibody (anti-Jagged1) prepared in PBS containing 1.5% normal rabbit serum and were incubated for one hour in a hydrated chamber at room temperature. TMA sections incubated with 1 µg/ml normal goat IgG (Santa Cruz Biotechnologies, CA) were used as negative controls. Following multiple 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 ImmPact™ DAB peroxidase substrate kit (Vector Laboratories, CA). Sections were then counterstained in 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 (Drs. Alexandra Filipovic 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. All patients enrolled in this study provided a written consent. This study was approved by

the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer' and the study was carried out according to recognized United Kingdom ethical guidelines.

Western Blot Analysis:

HCC1954, MCF-7, and MCF-7-HER2 cells were transfected with siRNA and/or treated with DMSO or 2 μ M lapatinib for 4 days. The cells were maintained in the incubator at 37⁰C with 95% air and 5% CO₂. After transfection and/or treatment, the medium was aspirated and cells were washed twice using ice-cold phosphate-buffered saline (PBS). Depending upon the cell confluency and the size of the plate, 150 μ L-300 μ L 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 ethylene-diaminetetraacetic 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 a cell scraper, and the lysate was collected into Eppendorf tubes. The lysate was allowed to incubate on ice for 10 minutes. The cells were sonicated 3 times for 10 seconds each using the Sonic Dismembrator (Model 100, Thermo-Fischer Scientific, Waltham, MA). Protein concentration was determined using Pierce bicinchoninic acid (BCA) Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, Cat. 23225). Based on the protein concentration, protein samples were prepared (20 μ g – 50 μ g) 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 samples were boiled at 95⁰C for 5 minutes. The denatured protein

samples were then run on 7% tris-acetate PAGE 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. Separated proteins were then transferred onto Polyvinylidene difluoride (PVDF) membrane. After 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 blocking, the membrane was incubated overnight with primary antibody at 4⁰C with constant shaking. The primary antibody was diluted at the recommended concentration in the diluent that was used for blocking. The following day, the membrane was washed 3 times using TBST. Each wash was for 10 minutes and the blots were shaken vigorously at room temperature. After the 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. After the final wash, proteins were detected 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. Protein bands

were visualized by exposing the membrane to X-ray film in the dark room. To reprobe membranes, they were washed in TBST at room temperature for 10 minutes and then stripped using Restore Plus Western Blot Stripping buffer (Thermo Fischer Scientific, Waltham, MA). The membrane was covered with stripping buffer and incubated for 20 minutes at 37⁰C. The membrane was washed three times using TBST as mentioned before, blocked and probed with the primary antibody of interest.

Real-time PCR:

HCC1954 cells were treated for four days with DMSO or 2 μ M lapatinib. Cells were sorted by flow cytometry on the basis of Jagged1 surface expression as described previously. For unsorted cells, total RNA was extracted according to the manufacturer's protocol using RiboPure RNA Purification kit (Ambion, Austin, TX, Cat. AM1924). For cells 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 numbers of cells. After extraction, the RNA yield was determined by using the NanoDrop Spectrophotometer (Thermo Fischer Scientific, Waltham, MA). The cDNA was synthesized through reverse transcription by using 1 μ g RNA in 100 μ L volume containing 1X RT buffer, 5.5 mM MgCl₂, 500 μ M dNTPs, 2.5 μ M random hexamers, 0.4 U/ μ l RNase inhibitors, and 1.25 U/ μ l RT enzyme (MultiScribe™ Reverse Transcriptase Kit, Applied Biosystems, Foster City, CA, Cat. N8080234). For reverse transcription, parameters were as follows: 10 minutes at 25⁰C, 30 minutes at 48⁰C, 5 minutes at 95⁰C, 60 minutes at 25⁰C, and at 4⁰C 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 μ L of prepared cDNA was added to 22.5 μ L of a mastermix-containing 2X SYBR Green Universal Master Mix, and 50 μ M forward and reverse primers for the intended target. The assay was performed in triplicate wells of 96-well plate. The plate was then sealed to prevent evaporation and quantitative Real Time PCR was performed using following parameters: the initial denaturation was for 10 minutes at 95⁰C; PCR cycling for 40 cycles was carried out for 10 seconds at 95⁰C and 45 seconds at 60⁰C. A melt curve was added after completion of the 40 cycles set by the StepOnePlus thermocycler manufacturer (Applied Biosystems, Foster City, CA). Relative expression of various gene transcripts in different samples was calculated based on the C_t value. C_t value indicates the number of cycles that were required to detect the initiation of amplification of the cDNA amplicon. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene transcripts were used as an 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^(- $\Delta\Delta C_t$) method – ΔC_t sample = [C_t value of gene of interest (sample) - C_t value of HPRT(sample)] ΔC_t control = [C_t value of gene of interest (control) - C_t value of HPRT(control)] $\Delta\Delta C_t$ = (ΔC_t sample - ΔC_t control) Fold change in gene expression = 2^(- $\Delta\Delta C_t$) The primer sequences that were used for detecting the gene transcript expression are listed in Supplemental Table 1.

Statistical Analysis:

The experiments were performed at least three independent times and results were reported as Mean \pm standard deviation (S.D.). For data involving two comparisons,

Microsoft Excel was used to perform an unpaired, two-tailed Student's *T*-test. Multiple comparisons were done using ANOVA (Tukey's test for multiple comparison) and GraphPad Prism 6 was used for the analysis. All the graphs were generated using GraphPad Prism 6. Extreme Limiting Dilution Analysis (ELDA) software (<http://bioinf.wehi.edu.au/software/elda/>) was used to calculate CSC frequency for the limiting dilution *in vivo* study. Kaplan-Meier curves were generated for the overall survival of women with HER2+ breast cancer, and for % tumor free mice. Statistical significance was calculated using the Log rank, Mantel-Cox test.

RESULTS:

Pharmacologic inhibition of HER2 increases survival of CSCs

Since Notch activation was required for HER2+ tumor recurrence post-anti-HER2 therapy (28, 29), we tested if Notch activation is necessary for the formation of CSCs derived from HER2+ breast cancer cells in response to anti-HER2 therapy. Lapatinib was chosen as a pharmacologic inhibitor of HER2 signaling due to its high efficacy in cell culture models and its use as second/third line therapy in trastuzumab-refractory disease (32). Results showed that lapatinib increased mammosphere forming efficiency (MFE), a surrogate assay for CSC survival (30), in HER2+ breast cancer cells, HCC1954 (Figure 1A) and MCF-7-HER2 cells (Figure 1B), whereas it did not significantly affect mammosphere formation in HER2 non-overexpressing MCF-7 cells (Figure 1C). Furthermore, the MRK-003 GSI inhibited mammosphere formation in these cell lines (Figure 1A-C), confirming previous reports that Notch activation is required for breast cancer stem survival (11, 18, 20, 33). To test whether the increase in %MFE in response to lapatinib was due to growth inhibition of bulk cells, bulk cell proliferation assays were conducted. Proliferation of bulk HER2+ HCC1954 cells is inhibited by lapatinib, GSI or the combination (Suppl Figure 1A). In contrast, MCF-7-HER2 (Suppl Figure 1B) or MCF-7 (Suppl Figure 2C) cells are resistant to lapatinib with moderate

sensitivity to GSI. These results suggest that the lapatinib-mediated increase in CSCs is most likely independent of sensitivity of bulk cells to lapatinib or GSI.

As lapatinib is a dual EGFR and HER2 tyrosine kinase inhibitor, expression levels of phosphorylated HER2 and EGFR in response to lapatinib were measured. HER2+ cells (HCC1954 and MCF-7-HER2) and HER2- wild type cells (MCF-7) express detectable levels of EGFR (Figure 1A-C, lower panels). Lapatinib treatment inhibited tyrosine phosphorylation of both EGFR and HER2 in HER2+ cells (Figure 1A and B, lower panels) but only inhibited EGFR in HER2- wild type MCF-7 cells (Figure 1C, lower panel). These results suggest that the lapatinib-mediated increase in CSC survival from HER2+ breast cancer cells is most likely dependent on HER2 expression, not EGFR, and potentially independent of bulk cell sensitivity to lapatinib.

Lapatinib Promotes Jagged1 Surface Expression

Previous reports demonstrated that Jagged1 expression at both RNA and protein levels predict poor outcome and higher metastatic potential in women with breast cancer (21, 34, 35). We previously published that HER2+ breast cancer cells express Jagged1 protein but it is limited to intracellular compartments. Upon HER2 blockade using genetic or pharmacologic methods, Jagged1 localizes to the cell membrane and is required for Notch activation and resistance to anti-HER2 therapy (26). Based on these previous findings, we investigated whether lapatinib enriched for Jagged1-high membrane expressing cells with CSC-like features and whether these cells were resistant to lapatinib. Results showed that lapatinib treatment increased cell surface

Jagged1 expression as assessed by flow cytometry in HER2+ breast cancer cells, HCC1954 (Figure 2A), MCF-7-HER2 (Figure 2B), and MDA-MB-453 (Figure 2C), but not in MCF-7 cells that do not overexpress HER2 (Figure 2D). Control studies were conducted to assess specificity of the flow cytometry Jagged1 antibody. Controls showed that lapatinib treatment had little effect on the APC fluorophore channel in unstained cells (Suppl Figure 2A). Unstained cells were used as controls for all of the studies as they were identical to cells stained with the secondary antibody-conjugated to APC (Suppl Figure 2B). The lapatinib-induced increase in Jagged-1 cell surface staining was specific as RNAi-mediated knockdown of Jagged-1 almost completely prevented the increase (Suppl Figure 2C-E).

Interestingly, Jagged1 transcripts were increased in response to lapatinib (Suppl Figure 3A), but this did not correlate with total protein expression (Suppl Figure 3B), suggesting that while HER2 tyrosine kinase activity inhibits Jagged1 RNA expression, protein expression on the cell surface is regulated at a post-translational level.

Lapatinib-mediated Jagged1-high expressing cells are enriched for CSCs and resistant to lapatinib

Since HER2 inhibition in response to lapatinib resulted in an increase in MFE and an increase in Jagged1 surface expression, we tested whether lapatinib-induced Jagged1-high-expressing cells have CSC-like features. HCC1954 cells were treated with vehicle (DMSO) or 2 μ M lapatinib for 4 days. The live cell populations based on propidium iodide exclusion were sorted based on gating for Jagged1-low (APC-low) and Jagged-1-high

(APC-high) after vehicle and lapatinib treatments and MFE was assessed (Figure 2E). The initial study showed that vehicle-treated Jagged-1 negative/low and the Jagged-1 moderately higher sorted cells had little detectable MFE. Cells sorted after lapatinib treatment based on low versus high Jagged-1 staining had strikingly different phenotypes. The lapatinib Jagged-1-low sorted cells had little detectable MFE while the Jagged-1-high cells were the only cells with MFE (Figure 2E). Similar results were observed using the MCF-7-HER2 breast cancer cells (Suppl Figure 4).

To assess the proliferative capacity of sorted cells based on Jagged1 surface expression, cell cycle analysis was performed after sorting. DMSO-treated HCC1954 Jagged1-low expressing cells had lower % of cells in the S-phase (20%) compared to Jagged-1-high expressing cells (35%) (Suppl Figure 5). Upon lapatinib treatment, the majority of Jagged-1-low expressing cells were arrested in the G1-phase with 10% undergoing cell death. In contrast, nearly 20% of the lapatinib-induced Jagged-1-high expressing cells were in the S-phase (Suppl Figure 5). These results suggest that Jagged-1-low expressing cells are sensitive to lapatinib while the Jagged1-high expressing cells are resistant.

Based on initial results, subsequent studies focused on sorted cells that expressed the lowest levels of Jagged-1 (vehicle treated) and the highest levels of Jagged-1 (lapatinib treated) on the cell surface. The vehicle-treated Jagged1-low population and lapatinib-treated Jagged1-high population of HCC1954 or MCF-7-HER2 cells were sorted and assessed for CSC survival using MFE. The lapatinib-induced Jagged1-high population showed significantly higher MFE than the vehicle-treated, Jagged1-low population (Figure 3A). Inhibition of the γ -secretase complex using the MRK-003 GSI completely

prevented mammosphere formation, suggesting that the enrichment of Jagged1-high breast CSCs by lapatinib was dependent on Notch activation (Figure 3A). Similar MFE results were observed for MCF-7-HER2 cells sorted based on vehicle treated Jagged-1-low cells compared to lapatinib treated Jagged-1-high cells (Figure 3B).

Lapatinib inhibits proliferation of bulk HCC1954 cells as assessed by fold decrease of viable cell numbers compared to DMSO after 4 days of treatment (Suppl Figure 1A). In contrast, lapatinib treatment increases Jagged-1 expression on the cell surface and these high Jagged-1 expressing cells have higher CSC survival and appear to be resistant to lapatinib.

Lapatinib-induced Jagged1-high cells express higher Notch receptors and gene targets

Since HER2 inhibition resulted in an increase in cell surface Jagged1 expression, we tested the status of Notch expression and activation in the subset of cells that expressed low compared to high Jagged1. HCC1954 cells were treated with lapatinib and then sorted based on Jagged1 cell surface expression. Notch receptors and Notch target gene transcripts were measured using real-time PCR. The lapatinib-treated Jagged1-high cells showed a significant increase in transcript levels of Notch1 and Notch3 compared to vehicle-treated Jagged1-low expressing cells (Figure 4A). Notch gene targets (HEY2, HES4, CCND1, and C-MYC) were also significantly increased in the Jagged-1-high expressing cells (Figure 4B). Notch signaling functions in a cell-cell

contact dependent manner. Surprisingly, the ligand expressing cells showed higher Notch activity. Notch receptors are typically activated in *trans* by ligands on a signal sending cell. However, if the Notch receptor and the Notch ligand are present on the same cell, this can lead to inhibition of Notch signaling, known as *cis* inhibition (36). To address this possibility, we performed flow cytometry to detect the surface co-expression of Notch receptors and Jagged1. Results showed that approximately 50-80% of vehicle treated HCC1954 cells express Notch1, Notch3, and Notch4 proteins on the cell surface with only 5-17% of cells co-expressing a Notch receptor and Jagged1 (Figure 4C). Upon lapatinib treatment, the entire cell population shifts to higher expression of Jagged-1 while Notch receptor expression is maintained or decreased (Figure 4C). This suggests that HER2 inhibition enriches for Jagged1-Notch1/Notch3/Notch4 high co-expressing cells allowing for ligand-receptor engagement to activate Notch signaling and increase survival of CSCs.

Lapatinib-induced Jagged1-high expressing cells have higher Aldefluor activity.

Expression of Aldehyde dehydrogenase1 (ALDH1A1) (37) and CD44/CD24 (38) levels have been reported to be markers of CSCs. Recently, ALDH1A1 activity has been shown to be high in epithelial/luminal CSCs, while CD44-high/CD24-low expressing cells have been shown to be associated with mesenchymal/basal CSCs (39). The lapatinib-induced Jagged1-high-expressing sorted cells had significantly higher Aldefluor activity as compared to control Jagged1-low sorted cells (Suppl Figure 6). Aldefluor+ cell populations were assessed based on three distinct gatings: the entire Aldefluor+ population (Suppl Figure 6A), moderate (Suppl Figure 6B), and high (Suppl

Figure 6C). This result suggests that upon HER2 inhibition, higher Jagged1 expression on the cell surface is necessary for the enrichment of Aldefluor+ CSCs.

HCC1954 cells contain a very small fraction of CD44-high/CD24-low cells (Suppl Figure 7A). Lapatinib treatment divided the entire population into two subsets: CD44-high/CD24-high and CD44-low/CD24-high. When cells were sorted based on Jagged-1 surface expression, the vehicle Jagged-1-low expressing cells expressed similar levels of CD44/CD24 (i.e. CD44-high/CD24-moderate) compared to unsorted cells (Suppl Figure 7B). The lapatinib-induced Jagged-1-high expressing population were similar to unsorted cells (i.e. CD44-High/CD24-High and CD44-low/CD24-High) (Suppl Figure 7B). MDA-MB-231 and MDA-MB-468 cells were used as positive and negative controls for CD44-high/CD24-low expressing cells, respectively (Suppl Figure 7C). These results suggest that HER2 inhibition selects for an epithelial/luminal population of CSCs that have high Jagged1 on the surface.

Jagged1 is required for Lapatinib-mediated enrichment of CSCs *in vitro*

Lapatinib-mediated HER2 inhibition resulted in an increase in MFE. Specifically, the lapatinib-induced Jagged1-high-expressing cells have increased MFE. To test whether the lapatinib-induced MFE was dependent on Jagged1, Jagged-1 was knockdown using two distinct siRNAs and MFE assessed. Jagged1 knockdown significantly reduced lapatinib-induced MFE (Figure 5A). Both siRNAs were efficient at reducing Jagged1 protein levels (Figure 5B). These results indicate that Jagged1 is required for the increase in CSC survival in response to lapatinib.

The Lapatinib-induced Jagged1-high-expressing cells have high tumor initiating potential

The *in vitro* results suggest that the Jagged1-high population in response to lapatinib treatment is enriched for CSCs and that Jagged1 is necessary for the formation of these CSCs. To assess the tumor initiating potential of the Jagged1-high population, a limiting dilution tumorigenesis experiment was performed *in vivo*. HCC1954 cells were sorted based on cell surface expression of Jagged1 by flow cytometry. Following the sort, 10,000 vehicle-treated Jagged1-low cells or lapatinib-treated Jagged1-high cells were injected into the mammary fat pad of female, athymic nude mice and tumor incidence was assessed for 8 weeks. One hundred percent of the mice injected with the lapatinib-treated Jagged1-high-expressing cells developed tumors, whereas only 34% of the mice injected with the vehicle-treated Jagged1-low-expressing cells developed tumors at 8 weeks (Figure 5C). Based on results from the pilot experiment, an extreme limiting dilution assay (ELDA) was performed using 100, 1000, and 10,000 sorted cells and tumor incidence was assessed for 8 weeks. Figures 5D-F show representative photographs of tumors and mice-bearing tumors formed using 10,000 sorted cells. Depending on tumor incidence at each dilution, the CSC frequency was calculated using Extreme Limiting Dilution Analysis (ELDA) software. The results from ELDA revealed that the lapatinib-induced Jagged1-high-expressing population had significantly higher CSC frequency (1/2180, p value = 0.000413) as compared to the vehicle treated Jagged1-low-expressing population (1/12638) (Figure 5F). The *in vivo* results demonstrate that the consequence of lapatinib treatment is increased levels of

Jagged-1-high expressing cells with higher tumor initiating potential and CSC frequency.

Higher Jagged1 membrane expression correlates with poorer cumulative overall survival in women with HER2+ breast cancer

The clinical significance of membrane Jagged1 expression with outcome was assessed using the well characterized Nottingham breast cancer cohort, prepared as tissue microarray. Primary, stage II-III invasive HER2+ breast cancer tissues (N=145) were stained for Jagged1 by immunohistochemistry. Jagged1 protein expression was detected at the membrane, in the cytoplasm, or near the perinucleus. Based on the staining intensity, samples were divided into different groups, high Jagged1 (membrane/cytoplasmic/perinuclear: staining intensity 2-3) or low Jagged1 (membrane/cytoplasmic/perinuclear: staining intensity 0-1). The cumulative overall survival of each patient was assessed. The cumulative survival was defined as the time from the onset of surgery to breast cancer-related death. Supplementary Table 2 shows the case processing summary for membrane Jagged1, perinuclear Jagged1, and cytoplasmic Jagged1. The results showed there was no significant correlation between cytoplasmic or perinuclear Jagged1 protein expression and overall cumulative survival (log-rank $P = 0.186$ for perinuclear Jagged1 and log-rank $P = 0.914$ for cytoplasmic Jagged1). However, there was a significant inverse correlation between membrane Jagged1 protein staining and overall cumulative survival. Positive or higher membrane Jagged1 expression predicted poorer overall survival in women with HER2+ breast cancer compared to low or negative Jagged1 (log-rank $P = 0.01$) (Figure 6A).

Based on results, the current working model is that HER2 inhibition results in an increase in membrane expression of Jagged1, which then can interact with Notch receptors in *trans* to activate Notch signaling and enhance CSC survival (Figure 6B). Jagged1 membrane staining could be a viable pathologic biomarker of Notch activity, resistance, and/or tumor relapse. These findings support a future clinical trial assessing sequential therapy strategies of anti-HER2 followed by anti-Jagged1/Notch to eliminate bulk cancer cells as well as CSCs to prevent drug resistance and tumor progression.

Discussion:

HER2+ breast cancer accounts for 15-20% of all breast cancer cases. Despite improved prognosis due to the availability of anti-HER2 agents, a significant number of patients show intrinsic resistance or develop acquired resistance. CSCs comprise a small subset of cells that are able to repopulate the entire tumor and have the ability to undergo differentiation and self-renewal. CSCs are implicated in drug resistance, tumor recurrence, and disease progression. It is critical to not only effectively target HER2, but also eliminate drug resistant CSCs in order to achieve complete tumor regression.

Magnifico et al. 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 (40). In contrast to this, Diessner et al. have shown that the CD44 high/ CD24 low CSC subpopulation expresses lower levels of HER2 on the cell surface due to autophagy-mediated internalization of HER2. Trastuzumab was not able to effectively target the CD44 high/ CD24 low tumorigenic subpopulation, as Natural Killer cells might not be able to target HER2 to promote antibody-dependent cell-mediated cytotoxicity (41). Ineffective

targeting of this subpopulation by trastuzumab would explain why the response rate of single-agent trastuzumab was only 26% (42). Another report showed that the HER2 low subpopulation-sorted from Luminal A MCF-7-derived mammospheres had higher levels of CSC markers and CSC properties. According to these authors, the HER2 high subpopulation was targeted by trastuzumab. In contrast, the CSC-containing HER2 low subpopulation was not targeted by trastuzumab (43).

In our current study, we used lapatinib to target HER2 tyrosine kinase activity as lapatinib is used in the metastatic setting when trastuzumab or TDM-1 fails. Results from the current work reveal that lapatinib-mediated inhibition of HER2 significantly shifts the entire population of cells from Jagged1-low membrane to high membrane expression resulting in higher CSCs, resistance to lapatinib, and higher tumor initiating potential. Further, the increase in Jagged1 membrane expression is dependent upon overexpression of HER2 and its kinase activity. Lapatinib inhibited the tyrosine kinase activity of HER2 but increased levels of total HER2 by stabilizing HER2 on the surface (32). The increase in Jagged1 surface expression is most likely dependent on lapatinib specifically targeting the receptor tyrosine kinase activity of HER2. The data presented herein support this hypothesis as HER2-overexpressing HCC1954 and MCF-7-HER2 cells, but not MCF-7 cells that express low to moderate levels of HER2, showed an increase in Jagged1 membrane expression and mammosphere forming efficiency upon lapatinib treatment. In addition, the inherently Jagged1-high cells did not show an increase in mammosphere forming efficiency in the absence of HER2 inhibition. These results suggest that the HER2 tyrosine kinase activity is necessary to limit Jagged1-enriched CSCs. This is in contrast to the report by Farnie *et al.*, where they propose that

combined inhibition of HER2 and Notch receptors effectively targets breast ductal carcinoma in situ stem/progenitor cells independent of HER2 status (44). The discrepancy observed could be due to differences in gene expression profiling between ductal carcinoma in situ and invasive carcinoma.

The lapatinib-mediated increase in Jagged1-high expressing cells with CSC features is in agreement with the work reported by Bednarz-Knoll *et al.* who showed that Jagged1 expression in breast tumor samples correlated with higher tumor grade, high Ki-67 and ALDH positivity (21). The Jagged1-high expressing cells after HER2 inhibition also had higher Notch activity as assessed by expression of Notch target genes. Almost all the Jagged1-high cells were found to express Notch1, Notch3, and Notch4. The fact that the receptor and ligand are possibly on the same cell would suggest that the cell could possibly act as a signal sending cell as well as signal receiving cell depending on the ratio of ligand to receptor expression within each. A recent finding by Marcelo *et al.* supports a hybrid phenotype where the cell could send as well as receive Notch signals (45).

What is unclear is the mechanism by which targeting HER2 activity shifts the population of cells from Jagged1 low surface expression to higher levels. One possibility is that HER2 signaling either directly or indirectly regulates vesicular trafficking of Jagged1 in endosomes. For example, when HER2 is overexpressed and hyperactive, Jagged1 could be rapidly endocytosed and the net surface expression is low. HER2 may compete for endocytic components and thus they are not available for Jagged1 or other proteins. On the other hand, it is possible that increased expression of HER2 prevents transport of Jagged1 from Golgi vesicles to the membrane. Multiple

mechanisms have been implicated in cellular protein trafficking of Notch and ligands (46-50).

Upon HER2 inhibition, the Jagged1-high expressing cells had higher tumorigenic potential and CSC frequency. It was interesting that only membrane expression of Jagged1 correlated with poor cumulative overall survival, and that cytoplasmic or perinuclear expression of Jagged1 had no significant impact on the overall survival of women with HER2+ breast cancer. Our findings indicate that sequential targeting of HER2 followed by Jagged1 could eliminate both HER2-driven cells as well as anti-HER2-induced Jagged-1-enriched CSCs. This could be a strategy to prevent resistance and achieve tumor regression. Future studies will focus on understanding the mechanism by which HER2 inhibition shifts the population of cells from low Jagged1 surface expression to higher levels with CSC features and resistance to anti-HER2 therapy.

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Figure Legends:

Figure 1. Lapatinib-mediated HER2 inhibition increases CSC survival. **A**, HCC1954 **B**, MCF-7-HER2 or **C**, MCF-7 cells were treated with 2 μ M Lapatinib or vehicle (DMSO) for 4 days. Cells were harvested and 100,000 cells/well were plated into 6 well ultra-low attachment plates containing mammosphere forming medium-supplemented with 5 μ M MRK-003 GSI or vehicle (DMSO). After 7 days of plating, the mammospheres were collected and counted in order to determine the mammosphere forming efficiency (MFE). Scale bars = 100 μ m. Bar graphs show mean %MFE \pm S.D. from at least three independent experiments. Statistical significance was calculated using ANOVA with a Tukey post-hoc test for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$. Lysates from each treated cell lines were harvested for total protein

and subjected to Western blotting to detect phosphorylated forms of HER2 and EGFR. Actin was detected as a loading control.

Figure 2. Lapatinib induces Jagged1-high expressing cells with mammosphere forming potential. HER2+ HCC1954 (A), MCF-7-HER2 (B), MDA-MB-453 (C), and HER2 wild type expressing MCF-7 (D) cells were treated for four days with 2 μ M Lapatinib or vehicle (DMSO). Cells were harvested and stained for Jagged1 followed by flow cytometry. E. HCC1954 cells were stained for Jagged1 and then sorted by flow cytometry on the basis of Jagged1 surface expression. The Jagged1 -/low or high population was sorted from both vehicle and lapatinib treated cells. The schematic shows the gating for the cells that were sorted. 35,000 HCC1954 cells were sorted into a well of a 24 well ultra-low attachment plate containing mammosphere forming medium. After 7 days, the mammospheres were harvested and %MFE was determined. Scale bars = 100 μ m.

Figure 3. Survival of lapatinib-induced Jagged1-high expressing CSCs is dependent on the γ -secretase complex. HCC1954 (A) or MCF-7-HER2 (B) cells were treated with DMSO (Vehicle) or lapatinib for 4 days followed by flow cytometry to detect cell surface expression of Jagged1. Cells were sorted for low versus high Jagged1 expressing cells and plated onto low attachment plates-containing mammosphere forming medium-supplemented with DMSO (Control) or 5 μ M MRK-003 GSI. %MFE was assessed at day 7. Scale bar = 100 μ m. *Denotes statistical significance of $P < 0.05$; **,

$P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$. One way ANOVA was performed followed by a Tukey post-hoc test.

Figure 4. Lapatinib-induced Jagged1-high cells express higher Notch receptors and gene targets. HCC1954 vehicle treated Jagged1-low cells and lapatinib treated Jagged1-high cells at a density of 400,000 cells were sorted into a collection tube. After the sort, RNA was extracted from sorted cells followed by cDNA synthesis using reverse transcription and real-time PCR to detect transcript levels of Notch receptors (**A**) and target genes (**B**) in the two populations. Bar graphs show mean values of relative transcript expression normalized to HPRT and compared to Vehicle Jagged1-low \pm S.D. from three independent experiments using the $2^{-\Delta\Delta Ct}$ calculation. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$. Non-paired Student *T*-test was performed on ΔCt values after initial normalization to HPRT. **C.** To assess co-expression Notch receptor and Jagged1 proteins within the two populations, HCC1954 cells were treated with DMSO or lapatinib for 4 days followed by dual protein staining for Notch1/Jagged1, Notch3/Jagged1, and Notch4/Jagged1 by flow cytometry. The unstained cells served as negative controls and were used to set the negative gating areas.

Figure 5. Jagged1 is required for lapatinib-mediated increase in MFE and high expressing cells are tumor initiating with higher stem cell frequency. A. HCC1954 cells were transfected with a scrambled control siRNA or two distinct Jagged1 siRNAs for 48 hours using RNAiMax. The cells were then treated with DMSO or lapatinib for 2

followed by re-transfection and then treatment was continued for 2 additional days. Post treatment, cells were plated and %MFE assessed after 7 days. **B.** Cells were harvested post-transfection and assessed for Jagged1 protein expression by Western blotting. Actin protein was detected as a loading control. **C.** Vehicle treated Jagged1-low cells and lapatinib treated Jagged1-high cells were sorted, as described previously. 10,000 sorted cells were resuspended into a Matrigel solution (1:1 Matrigel:PBS) and were then injected into mammary fat pads of 5 female athymic nude mice per group, and tumor incidence was assessed for up to 10 weeks. Based on the tumor incidence, a Kaplan-Meier curve was plotted and statistics were performed using the Log-rank (Mantel-Cox) test. **D.** and **E.** Vehicle treated Jagged1-low expressing and lapatinib treated Jagged1-high expressing cells were sorted followed by 10,000, 1,000 or 100 cells resuspended into a Matrigel solution (1:1 Matrigel:PBS) and injected into mammary fat pad of 8-10 athymic nude mice per group. Tumor incidence was assessed for each dilution of cells for 8 weeks and CSC frequency was calculated using Extreme Limiting Dilution Analysis (ELDA) software (**F**). Panels **D** and **E** show the presentative images of tumors and mice from the groups that were injected with 10,000 cells. Panel **F** shows the CSC frequency estimates and P values calculated using the ELDA software.

Figure 6. Jagged1 membrane expression predicts poor overall survival in women with HER2+ breast cancer. **A,** Tissue microarray (TMA) was performed on the Nottingham cohort of 145 primary, invasive stage II-III HER2+ breast cancer tissues. Jagged1 staining was performed by immunohistochemistry and Jagged1 was assessed to be localized near the perinucleus, cytoplasm or cell membrane. Jagged1 staining was

scored 0.0 for low/negative staining and 1.0 for high/positive staining. A Kaplan-Meier curve was plotted for overall survival and statistics was performed using the log-rank (Mantel-Cox) test. The panels are representative of negative staining, strong membrane + cytoplasmic, cytoplasmic, or perinuclear + cytoplasmic staining. The upper panels are representative images at low magnification with enlarged regions (lower panels) expanded from boxed areas. **B.** Working model of the effects of anti-HER2 therapy on bulk breast cancer cells to enrich for Jagged1-high expressing CSCs, possibly responsible for resistance and tumor recurrence. A combination approach of anti-HER2 and anti-Jagged1 could potentially prevent this enrichment and drug resistance.

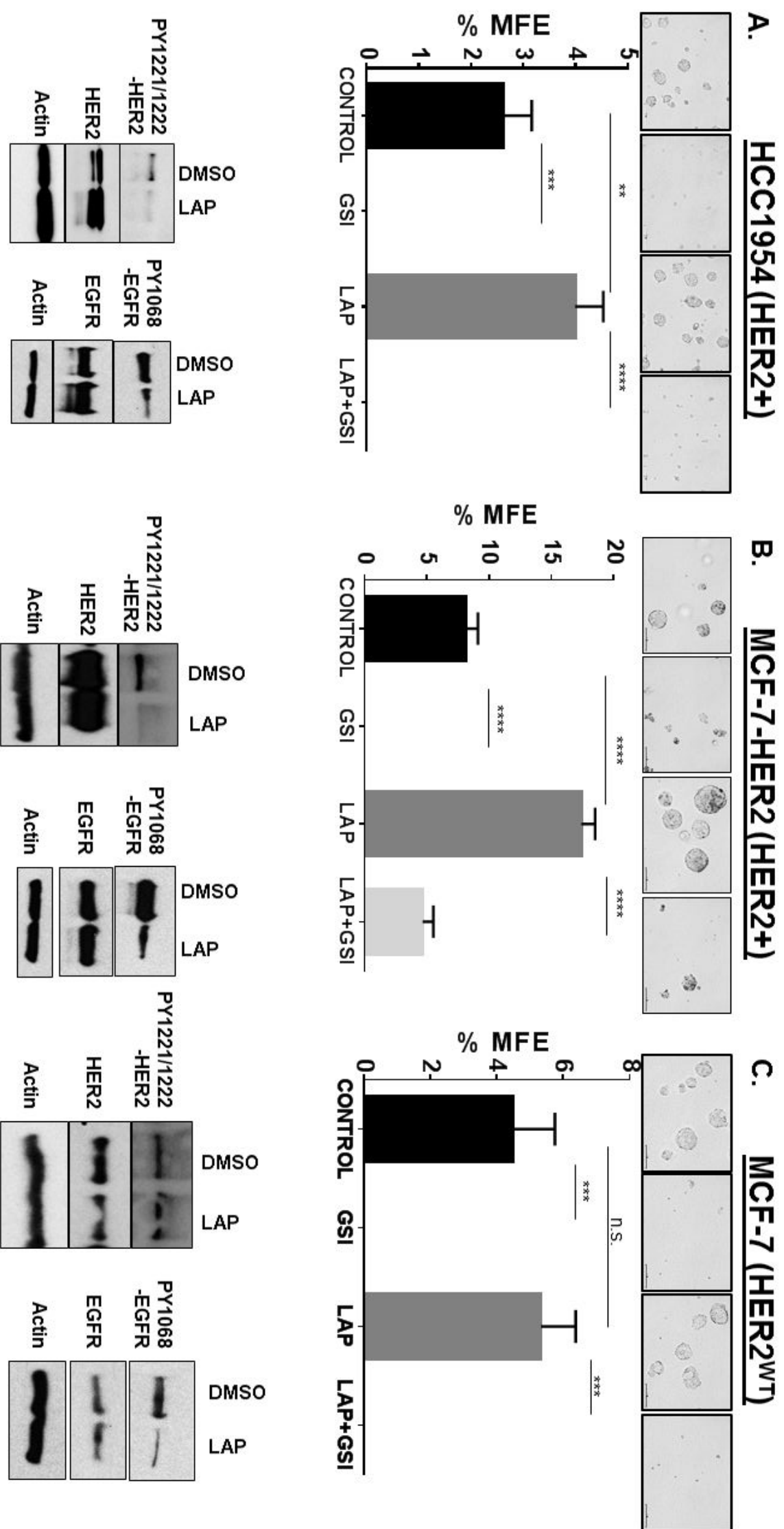


FIGURE 1

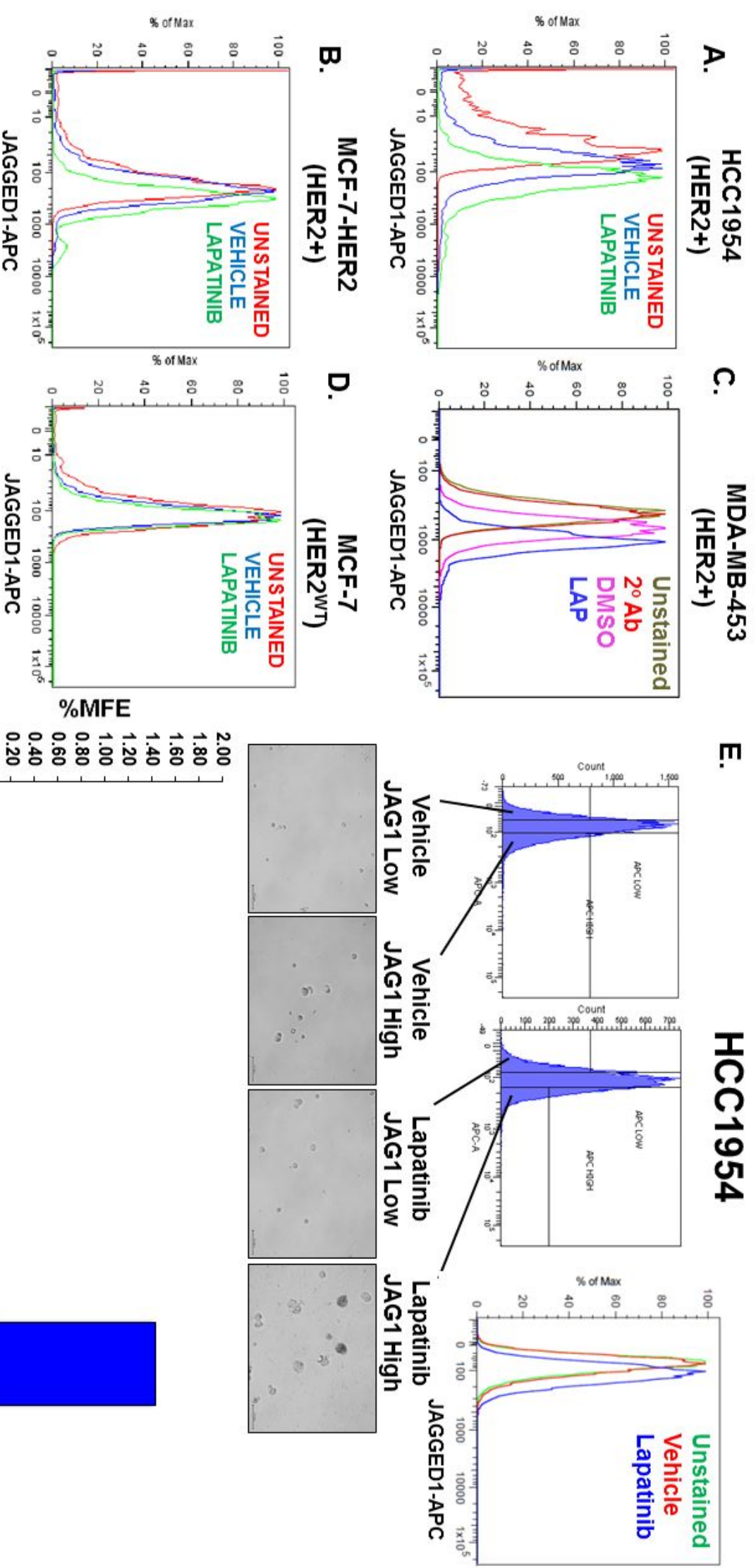
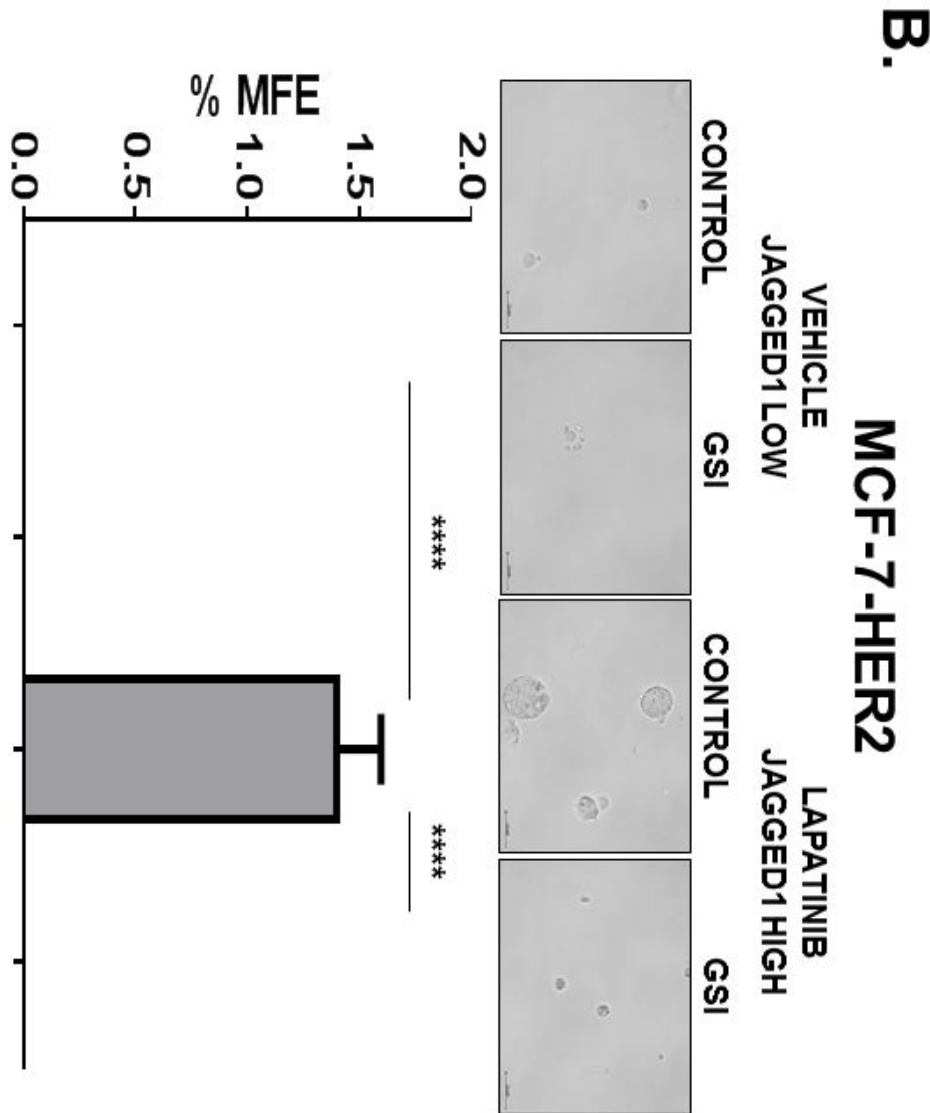
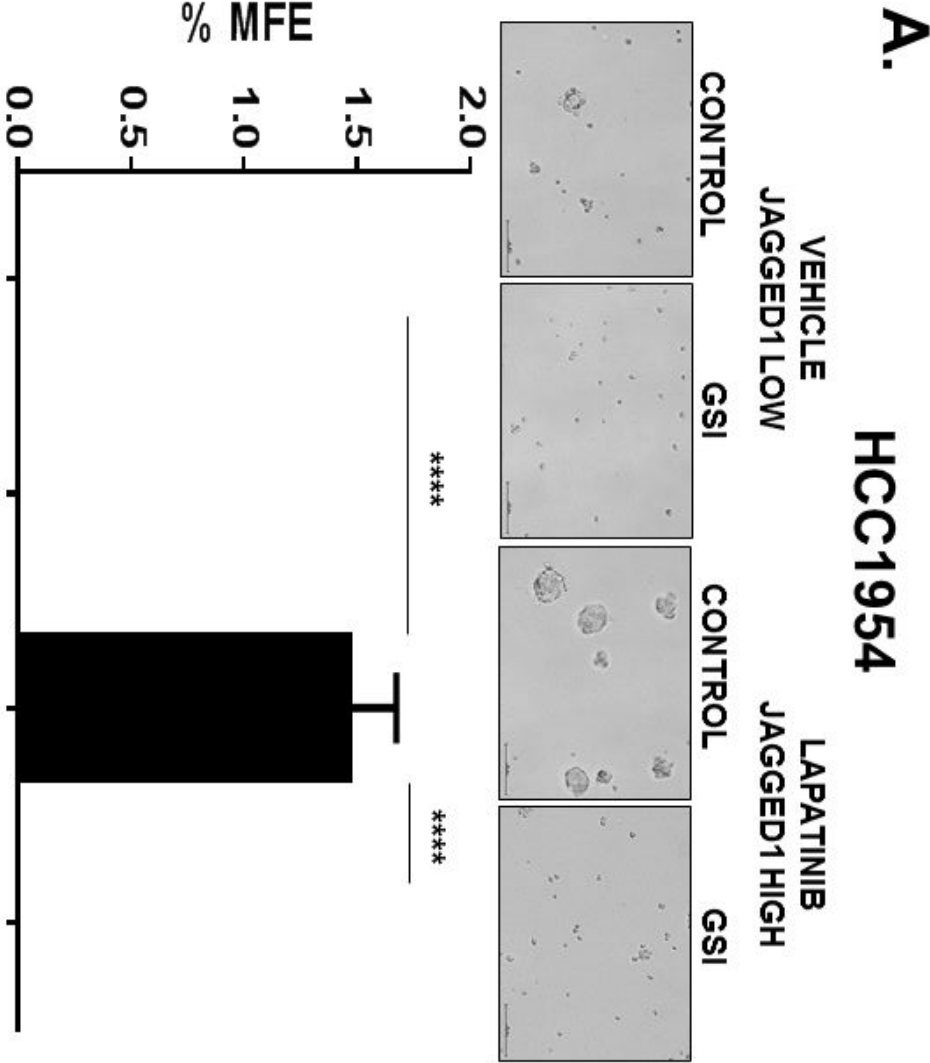
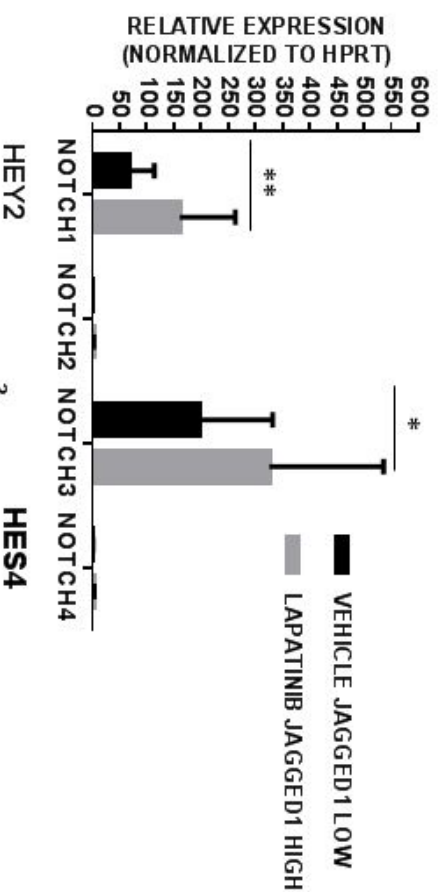


FIGURE 2

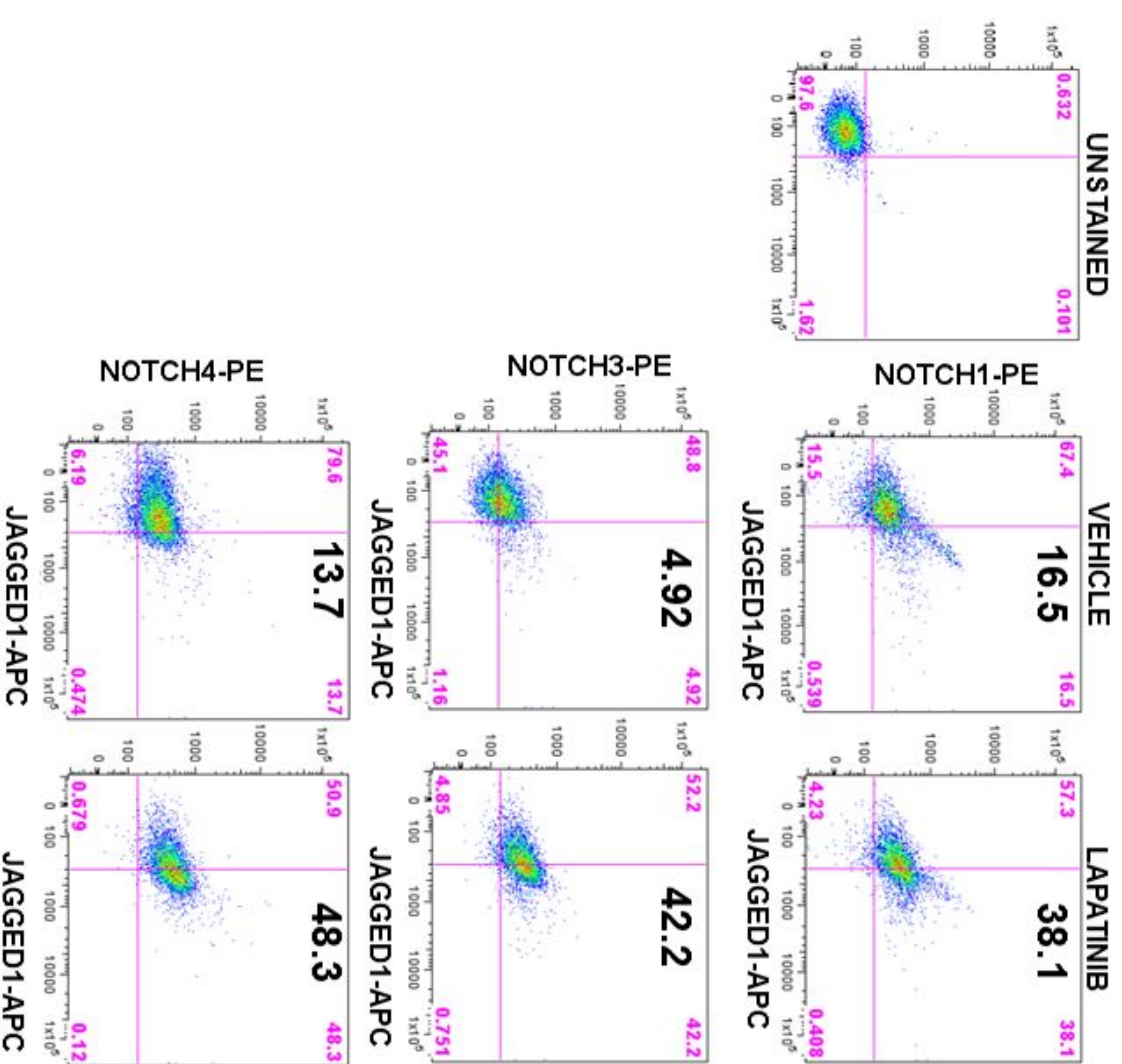
FIGURE 3



A. Notch RNA Expression



C. Notch Protein Expression



B.

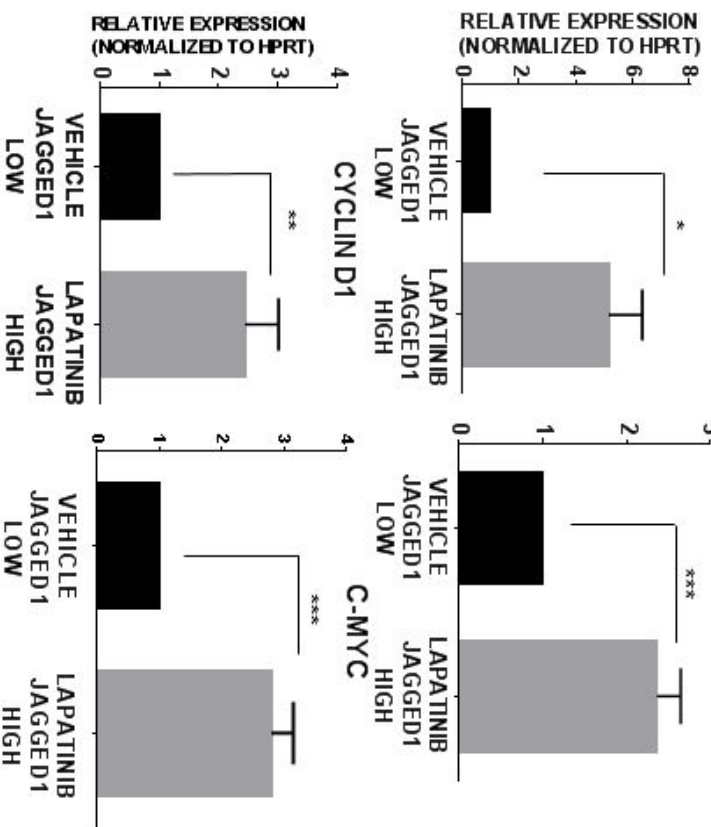
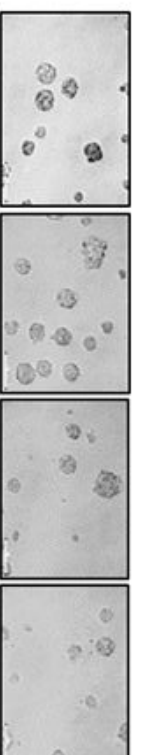
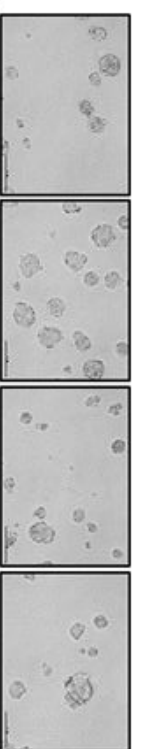
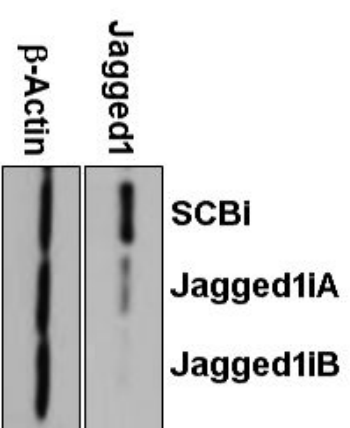
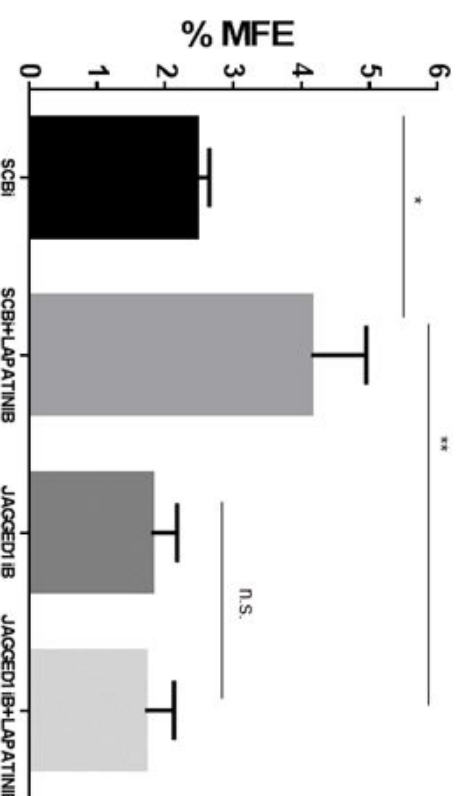
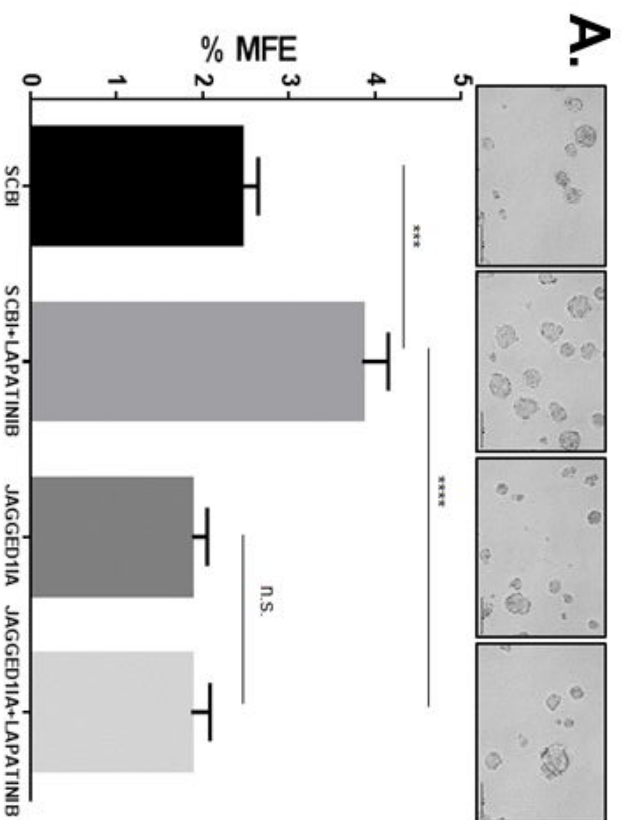
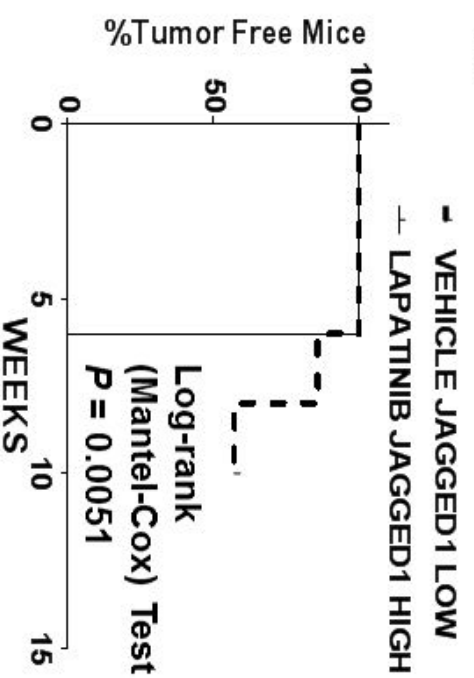
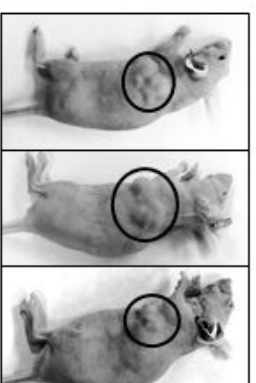
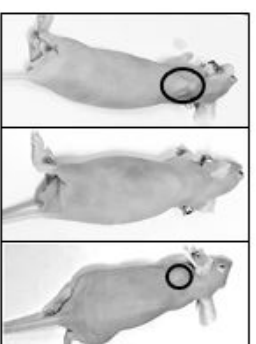


FIGURE 4

A.**B.****FIGURE 5****C.****D.**

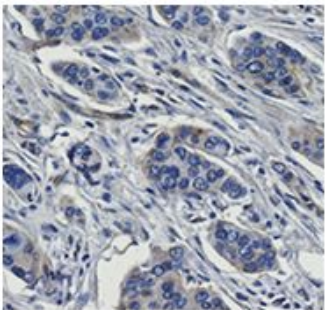
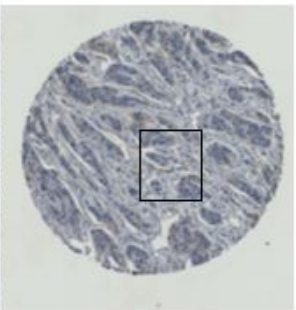
VEHICLE JAGGED1 LOW (10,000 CELLS) LAPATINIB JAGGED1 HIGH (10,000 CELLS)

**F.**

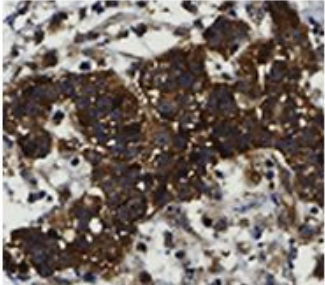
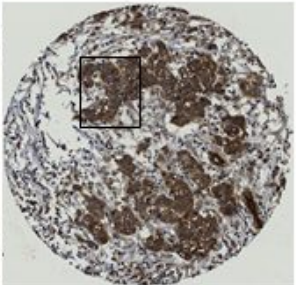
Group	Limiting Dilution			CSC Frequency
	100	1000	10,000	
VEHICLE JAGGED1 LOW	1/7	3/8	2/8	1/12638 (1/5174-1/30870)
LAPATINIB JAGGED1 HIGH	3/10	5/10	7/8	1/2180 (1/966-1/4921)

$P=0.000413$

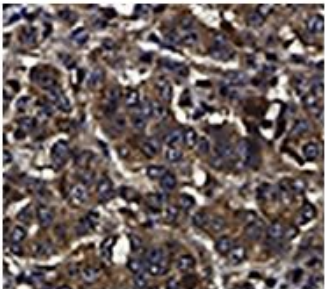
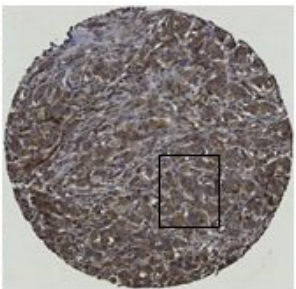
A. No staining



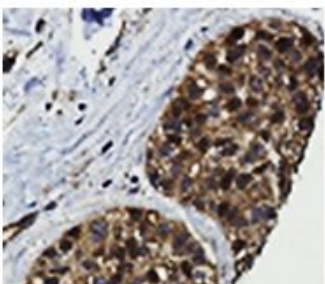
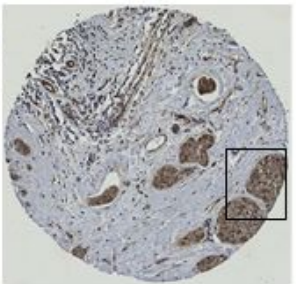
**Strong Membrane +
Cytoplasmic**



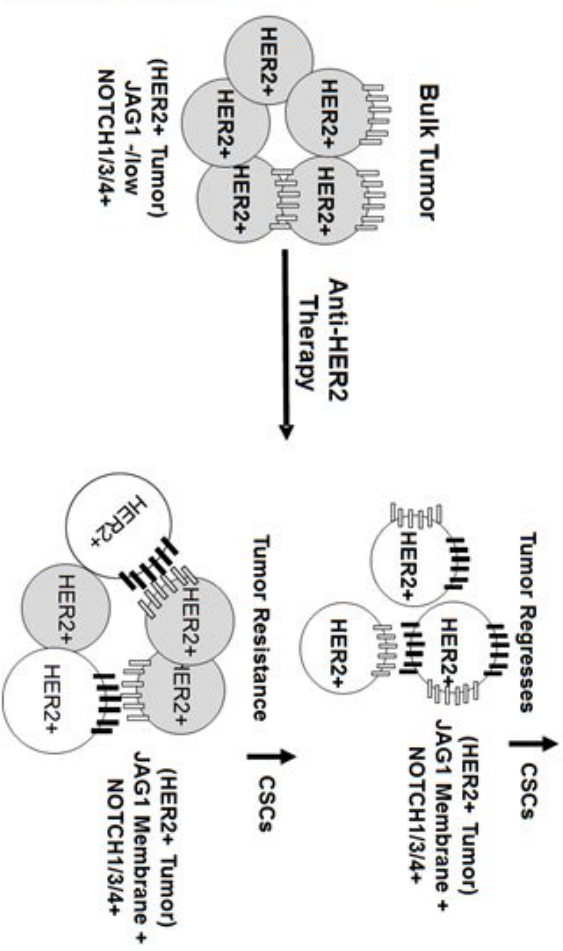
Cytoplasmic



**Perinuclear +
Cytoplasmic**

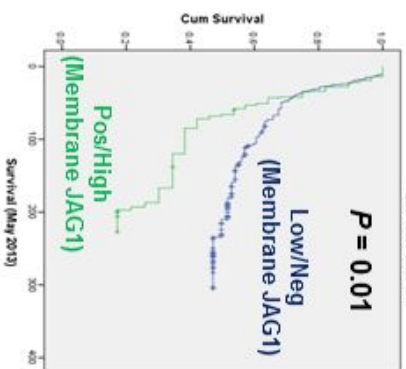


B.



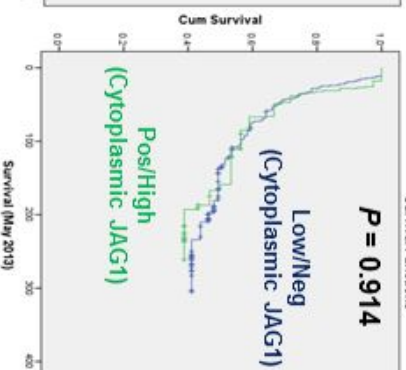
**Strong Membrane +
Cytoplasmic**

Survival Functions



Cytoplasmic

Survival Functions



**Perinuclear +
Cytoplasmic**

Survival Functions

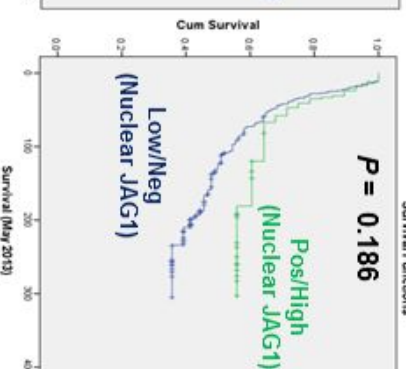


FIGURE 6