Inhibition of HER2 Increases JAGGED1-dependent Breast Cancer Stem Cells: Role for Membrane JAGGED1

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Abstract

Purpose: HER2-positive breast cancer is driven by cells possessing stem-like properties of self-renewal and differentiation, referred to as cancer stem cells (CSC). CSCs are implicated in radiotherapy, chemotherapy resistance, and tumor recurrence. NOTCH promotes breast CSC 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 expression 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-expression 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 posttherapy. Sequential blockade of HER2 followed by JAGGED1 or NOTCH could be more effective than simultaneous blockade to prevent drug resistance and tumor progression. Clin Cancer Res; 24(18): 4566–78. ©2018 AACR.

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, HER2 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 IHC (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 EGFR 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 (CSC). 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 (CSC) are thought to be selected by treatment that kills the majority of nonstem-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 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, JAGGED2, DLL1, 3, and 4), and four NOTCH receptors (NOTCH1–4). Delta-like ligands are 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 ligands is modulated by glycosylation of the extracellular subunits of NOTCH receptors mediated by Fringe family N-acetyl-glucosaminyltransferases, POFUT1 fucosyltransferase, and RUMI glucosyltransferase (13). When a ligand engages a receptor, it is ubiquitinylated 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 translocates 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 expression of NOTCH1 and JAG1 transcripts are associated with poor overall survival of women with breast cancer (21). High levels of JAG1 transcripts are associated with increased stem cell self-renewal in the Luminal subtypes of breast cancer (22). Furthermore, increased JAGGED1 expression has recently been implicated in promoting stemness and angiogenesis (23). Recent reports showed that a JAGGED1–NOTCH4 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). Furthermore, 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, percentage 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 JAGGED1-enriched breast CSCs. The consequence of HER2 inactivation is a shift from low JAGGED1 membrane expression to high JAGGED1 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 ATCC. MCF-7 cells stably overexpressing HER2, designated as MCF-7-HER2, were generously provided by Dr. Mien-Chie Hung from The University of Texas MD Anderson Cancer Center (Houston, TX). HCC1954 and MDA-MB-453 cells were grown in DMEM (Thermo Fisher Scientific), whereas MCF7 and MCF7-HER2 cells were cultured in RPMI1640 (Thermo Fisher Scientific). The cell culture media were supplemented with 10% FBS (Gemini Bio-Products), 1% L-glutamine (2 mmol/L, Thermo Fisher Scientific), and 1% nonessential amino acids (100 μmol/L, Invitrogen).

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. For in vitro studies, 5 μmol/L MRK-003 GSI stock solution was prepared by dissolving in DMSO. The working concentration was 5 μmol/L 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. For in vitro studies, 4 mmol/L stock concentration of lapatinib was prepared by dissolving it in DMSO. The working concentration was 2 μmol/L 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 (catalog no. HSS176254 and HSS176255). The sequences were JAGGED1 A (GATAAAGTTGCAGAATCAGACATTGA) and JAGGED1 B (CGCGCAGACGGACACACTACTCTCA). HER2 siRNA (GtGrCrGrCrArArCrArGrArGrArCrArUrArGrArGr-GaAg) was purchased from Origene (catalog no. SR301443). Nontargeting scrambled control siRNA was purchased from Qiagen (catalog no. 1027281). The transfection reagents Lipofectamine 3000 (catalog no. L3000015) and Lipofectamine RNAiMax (catalog no. 13778150) were purchased from Thermo Fisher Scientific. 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 μmol/L. The final

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.
working concentration of the siRNA was 10 nmol/L. For JAGGED1 siRNA transfection, 17 μL siRNA and 17 μL of Lipofectamine 3000 or equal volume of siRNA to RNAiMax was used in a 60-mm plate. For HER2 siRNA transfection, 20 μL of siRNA and 20 μL RNAiMax was used in a 60-mm plate. The transfection was performed according to the manufacturer's protocol.

Flow cytometry

HCC1954 (2.5 × 10^5 cells/well, 6 well plate), MDA-MB-453 (3.5 × 10^5 cells/well, 6-well plate for treatment and 7 × 10^5 cells/well, 60-mm plate for transfection), MCF-7 (4 × 10^5 cells/well, 6-well plate), or MCF-7-HER2 (4 × 10^5 cells/well, 6-well plate) were treated for four days with DMSO or 2 μM/L lapatinib (catalog no. S1028, Selleck Chemicals). For 4 days of lapatinib treatment, cells were trypsinized and replated after 2 days at a similar density. This was done to avoid overconfluency 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 (catalog no. 25–056-CI, Corning Cellgro). The harvested cells were neutralized using DMEM and the cell suspension was centrifuged at 1,300 rpm for 1.5 minutes. Collected cells were incubated at room temperature for 5 minutes. FITC-conjugated CD44 (catalog no. 103021 BioLegend) and PE–APC-conjugated CD44 (catalog no. 103012, BioLegend)/APC-conjugated secondary antibody (catalog no. BAF1277, R&D Systems) were added to each tube containing 1 × 10^5 cells and the cell pellet. The cells were then stained by using biotinylated human JAGGED1 primary antibody (catalog no. BAF1277, R&D Systems). About 3–6 μL of primary antibody was added to each tube containing 1–2 × 10^6 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 1,300 rpm for 3 minutes. After the second wash, excess staining buffer was aspirated, leaving behind around 250 μL of buffer and the cell pellet. This was done to avoid overconfluency 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 (catalog no. 25–056-CI, Corning Cellgro). The harvested cells were neutralized using DMEM and the cell suspension was centrifuged at 1,300 rpm for 3 minutes in the presence of 100 μg/mL DNase I to limit cell clumping. After the second wash, excess staining buffer was aspirated, leaving behind about 250 μL of buffer and the cell pellet. The excess staining buffer was aspirated, leaving behind about 250 μL of buffer and the cell pellet. The cells were then stained by using biotinylated human JAGGED1 primary antibody (catalog no. BAF1277, R&D Systems). About 3–6 μL of primary antibody was added to each tube containing 1–2 × 10^6 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 1,300 rpm for 3 minutes. After the second wash, excess staining buffer was aspirated, leaving behind around 250 μL of buffer. To this cell suspension, APC-conjugated secondary antibody (catalog no. 405207, BioLegend) 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 μL of the antibody was added to each tube containing 1 × 10^5–1 × 10^6 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 above. The cell pellet was finally resuspended in 250 μL flow cytometry staining buffer for analysis by using BD FACSCanto II (BD Biosciences). Data were 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 CD44high/CD24low assay, HCC1954 cells were harvested and stained per the protocol described above. The cells were stained with APC-conjugated CD44 (catalog no. 103012, BioLegend)/FITC-conjugated CD44 (catalog no. 103021 BioLegend) and PE-conjugated CD24 (catalog no. 311106, BioLegend), and were analyzed as described above.

Cell sorting

HCC1954 or MCF-7-HER2 cells were treated with DMSO or 2 μM/L lapatinib for 4 days, and were stained with biotinylated JAGGED1 (3–8 μL/1–2 × 10^5 cells) and streptavidin–APC antibody (5 μL undiluted) as described previously. To sort sufficient cells, HCC1954 and MCF-7-HER2, 2 T150 flasks (4 × 10^5 cells/flask) were used for DMSO treatment and 3 T150 flasks (4 × 10^5 cells/flask) were used for lapatinib treatment. After staining, cells were sorted based on JAGGED1 surface expression (Jagged low and high ligand) by a BD FACSAria cell sorter (BD Biosciences). The cells were sorted into 24-well plates containing mammosphere medium (3.5 × 10^4 cells/well) supplemented with DMSO/5 μM/L GSI for mammosphere assays. The cells were sorted into FACS tubes to perform Aldefluor assays, CD44high/CD24low assessment, and to detect the expression of NOTCH target genes. 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 μM/L 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 (ALDH) ALDH1A1 is one of the 19 ALDH isozymes 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 (catalog no. 01700, StemCell Technologies) was used to detect the cancer stem cell (CSC) population based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1A1). Two tubes containing 200 k sorted/ unsorted HCC1954 cells suspended into 1 mL of Aldefluor assay buffer were prepared—a test tube that received 5 μL ALDH1A1 substrate, and a control tube that received 10 μL ALDH 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 minutes at 250 × g. 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 on the basis of propidium iodide staining and forward and side scatter. Percent Aldefluor-positive cells were determined in the DEAB-negative sample compared with the DEAB-positive sample based on ALDH-negative 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 and colleagues assay (30). The preparation of methyl cellulose–based mammosphere medium was done as follows: 196 mL of DMEM-F12...
were then incubated at 37°C for 2.5 hours 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 (catalog no. E-9644, Sigma Aldrich) was added to the medium. The medium was then stirred at 4°C for 30 minutes and then transferred into centrifuge tubes. The tubes were then centrifuged at 9,500 rpm in a Beckman rotor at 4°C for 30 minutes. 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 1 × 10^5 cells/well (6-well ultra-low attachment plates) or 3.5 × 10^4 cells/well (24-well ultra-low attachment plates) were added into the mammosphere medium containing vehicle control DMSO or 5 μmol/L MRK-003 GSL.

To study the effect of JAGGED1 knockdown, 6 × 10^4 HCC1954 or MCF-7-HER2 cells were plated into 60-mm plates. Three plates each were used for transfection of nontargeting siRNA or JAGGED1 siRNA. The transfection was performed as described previously. After transfection, cells were harvested and replated for DMSO or lapatinib treatment for 4 days. After treatment, 1 × 10^3 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% CO_2 for 7–10 days, after which mammospheres were harvested and counted. 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. Conical tubes (15 mL) containing mammosphere suspensions were centrifuged at 1,500 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 2-mL tube and was centrifuged at 5,000 rpm for 5 minutes. One more wash with 2-mL PBS was performed and then the mammosphere pellet was again resuspended into 2-mL PBS (2-mL PBS was used as resuspension volume if 1 × 10^5 cells were used for 3.5 × 10^4 sorted cells, 500-μL PBS was used for resuspension). After resuspension, 50 μL was transferred into a well of a 96-well plate. A total of 350-μL PBS was then added to the well. Mammosphere-containing wells were photographed under a microscope and five fields were taken at 4× 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 on the basis of the scale present in the picture. The size cutoff was 50 μm, that is, all the spheres ≥ 50 μm were counted as mammospheres. On the basis of resuspension volume, the dilution factor was used to count the total mammospheres. Percentage mammosphere-forming efficiency was defined as 100 × (number of mammospheres/number of cells plated).

**Tumor-initiating potential**

HCC1954 cells were treated with either DMSO or 2 μmol/L lapatinib for 4 days and were sorted by flow cytometry on the basis of JAGGED1 surface expression. For pilot experiments, 1 × 10^4 sorted cells were injected into mammary fat pads of 5 female, ovariectomized FoxN1 nu/nu athymic nude mice (Harlan Sprague-Dawley). During the course of the study, each mouse and its tumor was tracked using an ear tag. Tumor uptake was assessed for up to 8 weeks and the tumorigenic potential of the two subpopulations was calculated. To perform a limiting dilution assay to detect the frequency of CSC formation, mice were randomized into following groups—100/1,000/10,000 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 × w) was calculated and graphed. At the end of 7 weeks, the number of mice that developed a tumor ≥ 40 mm^2 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).

**IHC staining of human HER2+ breast tumors**

The methodology used to detect JAGGED1 protein by IHC is similar to our previously published work by Pandya and colleagues. The Nottingham Tenvous Primary Breast Carcinoma Series were used. These are a series of 1,944 cases of primary operable invasive breast cancers, which were diagnosed from 1986 to 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 deparaffinizing the tissue followed by rehydration using ethanol, and washed with PBS 1× 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% H_2O_2 in PBS for 20 minutes to quench endogenous peroxidase activity. Sections were incubated for 1 hour in 3% normal rabbit serum (Vector Laboratories) in PBS at room temperature to block nonspecific binding. Sections were then incubated with primary antibody (anti-JAGGED1) prepared in PBS containing 1.5% normal rabbit serum and were incubated for 1 hour in a hydrated chamber at room temperature. TMA sections incubated with 1 μg/mL normal goat IgG (Santa Cruz Biotechnologies) were used as negative controls. Following multiple washing, antigen–antibody complexes were detected using the Vectastain Elite ABC Kit (Vector Laboratories) as per the manufacturer’s protocol. Staining was performed with ImmPACTTM DAB Peroxidase Substrate Kit (Vector Laboratories). Sections were then counterstained in Gill 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 two independent investigators [Drs. Alexandra Filopovic (Faculty of Medicine, Department of Surgery & Cancer at The Imperial College of London) and Lucio Miele (Louisiana...
Western blot analysis

HCC1954, MCF-7, and MCF-7-HER2 cells were transfected with siRNA and/or treated with DMSO or 2 μmol/L lapatinib for 4 days. The cells were maintained in the incubator at 37°C with 95% air and 5% CO2. After transfection and/or treatment, the medium was aspirated and cells were washed twice using ice-cold PBS. Depending upon the cell confluency and the size of the plate, 150–300 μL of Triton X-100 lysis buffer [50 mmol/L L-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% Triton X-100, 150 mmol/L sodium chloride (NaCl), 5 mmol/L ethylene-diaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate, 10 mmol/L 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 Fisher Scientific). Protein concentration was determined using Pierce Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Fisher Scientific, catalog no. 23225).

On the basis of the protein concentration, protein samples were prepared (20 μg–50 μg) using 2 × or 4 × Laemmli buffer (Bio-Rad, catalog no. 1610737 or 1610747) and β-mercaptoethanol (Thermo Fisher Scientific, catalog no. BP-176-100). The samples were boiled at 95°C for 5 minutes. The denatured protein samples were then run on 7% tris-acetate PAGE gels (Thermo Fisher Scientific, catalog no. EA03580BOX) along with HiMark Prestained protein standard (Thermo Fisher Scientific, catalog no. LC56590) at 150V for 60 minutes. 20 μL of prepared cDNA was added to 22.5 μL of Mastermix-containing 2X SYBR Green Universal Master Mix, and 50 μmol/L 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 the StepOnePlus thermocycler manufacturer (Applied Biosystems). Real-time PCR

Real-time PCR

HCC1954 cells were treated for four days with DMSO or 2 μmol/L 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, catalog no. AM1924). For cells sorted on the basis of JAGGED1 surface expression, RNeasy Plus Micro Kit (Qiagen, catalog no. 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 Fisher Scientific). The cDNA was synthesized through reverse transcription by using 1 μg RNA in 100 μL volume containing 1 × RT buffer, 5.5 mmol/L MgCl2, 500 μmol/L dNTPs, 2.5 μmol/L random hexamers, 0.4 μL/μL RNase inhibitors, and 1.25 μL/μL RT enzyme (MultiScribe Reverse Transcriptase Kit, Applied Biosystems, catalog no. N8080234). For reverse transcription, parameters were as follows: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, 60 minutes at 25°C, and at 4°C until use. The cDNA was synthesized, real-time PCR was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad) 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 a Mastermix-containing 2X SYBR Green Universal Master Mix, and 50 μmol/L 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 95°C, PCR cycling for 40 cycles was carried out for 10 seconds at 95°C and 45 seconds at 60°C. A melt curve was added after completion of the 40 cycles set by the StepOnePlus thermocycler manufacturer (Applied Biosystems).

Relative expression of various gene transcripts in different samples was calculated based on the Ct value. Ct 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 Ct values. Relative fold change in the gene expression between different samples was calculated on the basis of $2^{(\Delta\Delta Ct)}$ method – ΔCt sample = [Ct gene value of gene of interest (sample) – Ct gene value of HPRT(sample)] ΔCt control = [Ct value of gene of interest (control) – Ct value of HPRT(control)] ΔCt sample – ΔCt control. Fold change in gene expression = $2^{(\Delta \Delta Ct)}$. The primer sequences that were used for detecting the gene transcript expression are listed in Supplementary Table S1.

Statistical analysis

The experiments were performed at least three independent times and results were reported as Mean ± SD. For data involving...
two comparisons, Microsoft Excel was used to perform an unpaired, two-tailed Student t test. Multiple comparisons were done using ANOVA (Tukey test for multiple comparison) and GraphPad Prism 6 was used for the analysis. All the graphs were generated using GraphPad Prism 6. 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

Because NOTCH activation was required for HER2+ tumor recurrence post-anti-HER2 therapy (28, 29), we tested whether 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 (Fig. 1A) and MCF-7-HER2 cells (Fig. 1B), whereas it did not significantly affect mammosphere formation in HER2 nonoverexpressing MCF-7 cells (Fig. 1C). Furthermore, the MRK-003 GSI inhibited mammosphere formation in these cell lines (Fig. 1A–C), confirming previous reports that NOTCH activation is required for breast CSC 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 (Supplementary Fig. S1A). In contrast, MCF-7-HER2 (Supplementary Fig. S1B) or MCF-7 (Supplementary Fig. S2C) 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 (Fig. 1A–C, bottom). Lapatinib treatment inhibited tyrosine phosphorylation of both EGFR and HER2 in HER2+ cells (Fig. 1A and B, bottom) but only inhibited EGFR in HER2+ wild-type MCF-7 cells (Fig. 1C, bottom). 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). On the basis of 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 (Fig. 2A), MCF-7-HER2 (Fig. 2B), and MDA-MB-453 (Fig. 2C), but not in MCF-7 cells that do not overexpress HER2 (Fig. 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 (Supplementary Fig. S2A). 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 (Supplementary Fig. S2B). The lapatinib-induced increase in JAGGED1 cell surface staining was specific as RNAi-mediated knockdown of JAGGED1 almost completely prevented the increase (Supplementary Fig. S2C–S2E).

Interestingly, JAG1 transcripts were increased in response to lapatinib (Supplementary Fig. S3A), but this did not correlate with total protein expression (Supplementary Fig. S3B), suggesting that while HER2 tyrosine kinase activity inhibits JAG1 RNA expression, protein expression on the cell surface is regulated at a posttranslational level.

Lapatinib-mediated JAGGED1-high expressing cells are enriched for CSCs and resistant to lapatinib

As 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 μmol/L lapatinib for 4 days. The live cell populations based on propidium iodide exclusion were sorted on the basis of gating for JAGGED1-low (APC-low) and JAGGED1-high (APC-high) after vehicle and lapatinib treatments and MFE was assessed (Fig. 2E). The initial study showed that vehicle-treated JAGGED1-negative/low and the JAGGED1 moderately higher sorted cells had little detectable MFE. Cells sorted after lapatinib treatment based on low versus high JAGGED1 staining had strikingly different phenotypes. The lapatinib JAGGED1-low sorted cells had little detectable MFE while the JAGGED1-high cells were the only cells with MFE (Fig. 2E). Similar results were observed using the MCF-7-HER2 breast cancer cells (Supplementary Fig. S4).

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 percentage of cells in the S-phase (20%) compared with JAGGED1-high-expressing cells (35%; Supplementary Fig. S5). Upon lapatinib treatment, the majority of JAGGED1-low-expressing cells were arrested in the G1 phase with 10% undergoing cell death. In contrast, nearly 20% of the lapatinib-induced JAGGED1-high-expressing cells were in the S-phase.

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 μmol/L 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. A total of 3.5 × 10^6 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 μm.
JAGGED1-high
low (Supplementary Fig. S5). These results suggest that JAGGED1-low–expressing cells are sensitive to lapatinib while the JAGGED1-high–expressing cells are resistant.

On the basis of initial results, subsequent studies focused on sorted cells that expressed the lowest levels of JAGGED1 (vehicle treated) and the highest levels of JAGGED1 (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 (Fig. 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 (Fig. 3A). Similar MFE results were observed for MCF-7-HER2 cells sorted on the basis of vehicle-treated JAGGED1-low cells compared with lapatinib-treated JAGGED1-high cells (Fig. 3B).

Lapatinib inhibits proliferation of bulk HCC1954 cells as assessed by fold decrease of viable cell numbers compared with DMSO after 4 days of treatment (Supplementary Fig. S1A). In contrast, lapatinib treatment increases JAGGED1 expression on the cell surface and these high JAGGED1-expressing cells have higher CSC survival and appear to be resistant to lapatinib.

Lapatinib-induced JAGGED1-high–expressing cells express higher NOTCH receptors and gene targets

Because 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 with high JAGGED1. HCC1954 cells were treated with lapatinib and then sorted on the basis of 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 with vehicle-treated JAGGED1-low–expressing cells (Fig. 4A). NOTCH gene targets (HEY2, HES4, CCND1, and CMYC) were also significantly increased in the JAGGED1-high–expressing cells (Fig. 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 coexpression 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 coexpressing a NOTCH receptor and JAGGED1 (Fig. 4C). Upon lapatinib treatment, the entire cell population shifts to higher expression of JAGGED1 while NOTCH receptor expression is maintained or decreased (Fig. 4C). This suggests that HER2 inhibition enriches for JAGGED1-NOTCH1/NOTCH3/NOTCH4–high coexpressing 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 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 CD44high/CD24low–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 with control JAGGED1-low sorted cells (Supplementary Fig. S6). Aldefluor+ cell populations were assessed on the basis of three distinct gateings: the entire Aldefluor+ population (Supplementary Fig. S6A), moderate (Supplementary Fig. S6B), and high (Supplementary Fig. S6C). 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 CD44high/CD24low cells (Supplementary Fig. S7A). Lapatinib treatment divided the entire population into two subsets: CD44high/CD24low and CD44low/CD24high. When cells were sorted on the
basis of JAGGED1 surface expression, the vehicle JAGGED1-low–expressing cells expressed similar levels of CD44/CD24 (i.e., CD44high/CD24moderate) compared with unsorted cells (Supplementary Fig. S7B). The lapatinib-induced JAGGED1-high-expressing population were similar to unsorted cells (i.e., CD44high/CD24high and CD44low/CD24high; Supplementary Fig. S7B). MDA-MB-231 and MDA-MB-468 cells were used as positive and negative controls for CD44high/CD24low–expressing cells, respectively (Supplementary Fig. S7C). 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 population were similar to unsorted cells (i.e., CD44high/CD24high and CD44low/CD24high; Supplementary Fig. S7B). MDA-MB-231 and MDA-MB-468 cells were used as positive and negative controls for CD44high/CD24low–expressing cells, respectively (Supplementary Fig. S7C). These results suggest that HER2 inhibition selects for an epithelial/luminal population of CSCs that have high JAGGED1 on the surface.

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 on the basis of cell surface expression of JAGGED1 by flow cytometry. Following the sort, 1 × 10^6 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 cells developed tumors, whereas only 34% of the mice injected with the vehicle-treated JAGGED1-low–expressing cells developed tumors at 8 weeks (Fig. 5C). On the basis of results from the pilot experiment, an ELDA was performed using 100, 1,000, and 10,000 sorted cells and tumor incidence was assessed for 8 weeks. Figure 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 ELDA software. The results from ELDA revealed that the
lapatinib-induced JAGGED1-high–expressing population had significantly higher CSC frequency (1/2180, \( P = 0.000413 \)) as compared with the vehicle-treated JAGGED1-low–expressing population (1/12,638; Fig. 5F). The in vivo results demonstrate that the consequence of lapatinib treatment is increased levels of JAGGED1-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 IHC. JAGGED1 protein expression was detected at the membrane, in the cytoplasm, or near the perinucleus. On the basis of 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 S2 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 with low or negative JAGGED1 (log-rank \( P = 0.01 \); Fig. 6A).

On the basis of 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 (Fig. 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.
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 to achieve complete tumor regression.

Magnifico and colleagues reported that HER2 is overexpressed in CSCs, as compared with the bulk cells, and that trastuzumab could be effectively used to target CSCs. Moreover, they showed that HER2 levels were upregulated by NOTCH signaling (40). In contrast to this, Dieser and colleagues have shown that the CD44high/CD24low 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 CD44high/CD24low tumorigenic subpopulation, as natural killer cells might not be able to target HER2 to promote antibody-dependent cell-mediated cytoxicity (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. Furthermore, 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 MFE upon lapatinib treatment. In addition, the inherently JAGGED1-high cells did not show an increase in MFE in the

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 IHC 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 images at low magnification with enlarged regions (bottom) 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.
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 and colleagues, 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 and colleagues 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 and colleagues 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 JAGGED1-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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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