HER2-Associated Radioresistance of Breast Cancer Stem Cells Isolated from HER2-Negative Breast Cancer Cells

Nadire Duru1, Ming Fan1, Demet Candas1, Cheikh Menaa1, Hsin-Chen Liu3, Danupon Nantajit1, Yunfei Wen6, Kai Xiao2, Angela Eldridge1,3, Brett A. Chromy3,5, Shiyong Li7, Douglas R. Spitz8, Kit S. Lam2,4, Max S. Wicha9, and Jian Jian Li1,4

Abstract

**Purpose:** To understand the role of HER2-associated signaling network in breast cancer stem cells (BCSC) using radioresistant breast cancer cells and clinical recurrent breast cancers to evaluate HER2-targeted therapy as a tumor eliminating strategy for recurrent HER2+/low breast cancers.

**Experimental Design:** HER2-expressing BCSCs (HER2+/CD44+/CD24−/low) were isolated from radiation-treated breast cancer MCF7 cells and *in vivo* irradiated MCF7 xenograft tumors. Tumor aggressiveness and radioresistance were analyzed by gap filling, Matrigel invasion, tumor-sphere formation, and clonogenic survival assays. The HER2/CD44 feature was analyzed in 40 primary and recurrent breast cancer specimens. Protein expression profiling in HER2+/CD44+/CD24−/low versus HER2+/CD44+/CD24−/low BCSCs was conducted with two-dimensional difference gel electrophoresis (2-D DIGE) and high-performance liquid chromatography tandem mass spectrometry (HPLC/MS-MS) analysis and HER2-mediated signaling network was generated by MetaCore program.

**Results:** Compared with HER2-negative BCSCs, HER2+/CD44+/CD24−/low cells showed elevated aldehyde dehydrogenase (ALDH) activity and aggressiveness tested by Matrigel invasion, tumor sphere formation, and *in vivo* tumorigenesis. The enhanced aggressive phenotype and radioresistance of the HER2+/CD44+/CD24−/low cells were markedly reduced by inhibition of HER2 via siRNA or Herceptin treatments. Clinical breast cancer specimens revealed that cells coexpressing HER2 and CD44 were more frequently detected in recurrent (84.6%) than primary tumors (57.1%). In addition, 2-D DIGE and HPLC/MS-MS of HER2+/CD44+/CD24−/low versus HER2+/CD44+/CD24−/low BCSCs reported a unique HER2-associated protein profile including effectors involved in tumor metastasis, apoptosis, mitochondrial function, and DNA repair. A specific feature of HER2–STAT3 network was identified.

**Conclusion:** This study provides the evidence that HER2-mediated prosurvival signaling network is responsible for the aggressive phenotype of BCSCs that could be targeted to control the therapy-resistant HER2+/low breast cancer. *Clin Cancer Res;* 18(24); 6634–47. ©2012 AACR.

**Authors’ Affiliations:** Departments of 1Radiation Oncology, 2Biochemistry and Molecular Medicine, and 3Pathology and Laboratory Medicine, University of California Davis School of Medicine; 4NCI-Designated Comprehensive Cancer Center, University of California Davis, Sacramento; 5Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, California; 6Department of Gynecologic Oncology, MD Anderson Cancer Center, University of Texas, Houston, Texas; 7Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia; 8Free Radical and Radiation Biology Laboratory, Livermore, California; and 9University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan

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**Current address for N. Duru:** California National Primate Research Center, University of California Davis, Davis, CA 95616.

**Corresponding Author:** Jian Jian Li, Department of Radiation Oncology, University of California Davis School of Medicine, 1138 Oak Park Building, 2700 Stockton Boulevard, Sacramento, CA 95817. Phone: 916-703-5174; Fax: 916-734-4107; E-mail: jian-jian.li@ucdmc.ucdavis.edu
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**Introduction**

Despite advances in early diagnosis and treatment, breast cancer–related death remains significantly high due to resistance of metastatic and recurrent tumors to current anticancer regimens; with as many as 40% relapsing with metastatic disease (1, 2). Accumulating evidence of tumor heterogeneity and the presence of cancer stem cells (CSC) detected in many tumors with the stem cell–like characteristics offer new paradigms to understand and generate effective targets to treat recurrent and metastatic tumors (3, 4). Breast cancer cells that are able to propagate as mammospheres and possess CSC properties are more radioresistant (5) and the population of breast cancer stem cells (BCSC) is increased after chemotherapy (6). Breast cancer cells surviving radiation show enhanced clonogenic survival indicating the enrichment of radioresistant cells (7, 8). HER2 belongs to the HER family of transmembrane glycoproteins, which consists of 4 homologous receptors (9). About 25% of patients with breast cancer are diagnosed...
HER2-Mediated Aggressiveness in Breast Cancer Stem Cells

Translational Relevance

Despite a trend toward overall improvement in early detection and therapy outcomes, breast cancer mortality remains unacceptably high due to the ineffectiveness of the control over the recurrent and metastatic lesions, especially for HER2-negative breast cancer. Herein, we identified a population of breast cancer stem cells (BCSC) expressing HER2 (HER2+/CD44+/CD24−/low) isolated from radioresistant HER2-negative breast cancer cells, and the HER2+/CD44+/CD24−/low was more frequently detected in the recurrent tumors as compared with the primary breast cancer. A HER2-linked protein network, such as HER2–STAT3, was detected in the HER2-positive BCSCs by proteomics analysis. Thus, the HER2-associated signaling network may be an effective target to treat resistant breast tumors with HER2+/low status.

suggest that HER2-initiated proliferative network is responsible for the resistant phenotype of BCSCs that are enriched in the therapy-resistant breast cancer.

Materials and Methods

Cell culture

Human breast cancer MCF7 cells (American Type Culture Collection), radioresistant MCF7/C6 cells (20), and MCF7 cells transfected with HER2, MCF7/HER2 (7) were maintained as described earlier (20) in Eagle’s minimum essential medium (EMEM), supplemented with 10% FBS (HyClone), 5% sodium pyruvate, 5% nonessential amino acid (NEAA), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C incubator (5% CO₂).

Irradiation of xenograft tumor and cells

A standard cell inoculation was used to generate mouse breast cancer xenograft tumors using MCF7 cells as described (20) following the protocol approved by the Institutional Animal Care and Use Committee of University of California Davis (IACUC No. 15315, Davis, CA). Eight-weeks old female athymic nude mice (Jackson Laboratory) were pretreated for 5 days with estrogen pellets (Innovative Research of America) followed by inoculation with 5 × 10⁶ MCF7 cells. Three weeks after cell inoculation, the average tumor size was 2,000 to 2,300 mm³. The mice were divided into two groups; one received sham radiation and the other exposed to local tumor radiation with fractionated doses (5 × 2 Gy) delivered by GR-12 irradiator of 60Co γ-rays, under anesthetic agents using ketamine. Twenty hours after the last radiation treatment, mice were sacrificed and tumor cells suspension was prepared for fluorescence-activated cell sorting (FACS). Radiation was delivered to cultured cells using a Cabinet X-rays System Faxitron Series (dose rate: 0.997 Gy/min; 130 kVp; Hewlett Packard). Cells sheltered from radiation were included as the sham-IR control.

Western blotting

Total cell lysates (20 μg) were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membrane. The membrane was incubated with specific primary antibody overnight at 4°C, followed by the horseradish peroxidase (HRP)-conjugated secondary antibody, and visualized by the ECL Western blotting detection system (Amersham). STAT3 antibody was purchased from Santa Cruz (SC-8019). HER2 (MS-730-P) and β-actin antibodies were purchased from Lab Vision Corporation and Sigma, respectively.

Clonogenic survival assay

Standard radiation clonogenic survival assays were conducted as previously described (7) following either 0 Gy (sham) or 5 Gy radiation in combination with or without HER2 inhibition by siRNA or Herceptin treatment (10 μg/mL for 5 days with refreshed Herceptin every 48 hours). For siRNA treatment, cells were seeded to achieve 30% to 50% confluence on the day of transfection and siRNA (20 nmol/L) was transfected using Lipofectamine RNAiMAX reagent (Invitrogen) in antibiotic-free medium.
for 24 hours. Scrambled RNA Duplex (Ambion) and multiple HER2 siRNA were tested and served as negative controls. All transfectants were maintained in antibiotic-free complete medium and replaced with fresh medium before radiation. The colonies were fixed and stained with Coomassie blue and colonies containing more than 50 cells were counted as surviving clones and normalized to the plating efficiency of cells without radiation.

**Invasion assay**

Aliquots (0.4 mL) of Matrigel (BD Biosciences) were diluted in serum-free minimum essential medium (MEM; Life Technologies) to the final concentration of 3 mg/mL. Matrigel was then loaded into the upper chamber of 24-well transwell (Costar) and incubated at 37°C for 1 hour before cells (10⁵/mL) were added. The lower chamber was filled with 600 μL of MEM media containing 5 μg/mL fibronectin as an adhesive substrate (Santa Cruz Biotechnology). The transwell with differently treated cells was incubated for varied times and stained with Diff-Quick Stain (Fisher Scientific), and the invasive cells were counted using light microscopy.

**Gap-filling rate assay**

Gap-filling capacity was conducted as described (21) with 3 x 10⁵ cells grown in each of 6-well plates to 100% confluence followed by culture in medium without serum for 48 hours for cell starvation. The gap was created by scraping the cells diagonally with a sterile pipette’s tip and the filling capacity was monitored at different times, as specified in results.

**Tumor sphere formation**

Tumor sphere assay was conducted as described (22). Cells were sieved with 40 μm-cell strainers (Fisher) and single-cell suspensions were seeded into ultra low-attachment 60-mm Petri dishes at a density of 1,000 cells/mL. The cells were grown in serum-free mammary epithelial basal medium (MEBM, Lonza), supplemented with B27 (Life Technology), 20 ng/mL EGF (Biovision), 20 ng/mL basic-fibroblast growth factor (FGF), and 4 μg/mL heparin (VWR). Cells were cultured for 10 days and tumor spheres were counted under light microscopy.

**Orthotopic mammary fat tumor formation**

Tumor-initiating test was conducted following the described methods (23, 24), and the protocol was reviewed and approved by the IACUC at the University of California Davis (IACUC No. 15315). Eight-weeks old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory) were pretreated for 5 days with estrogen pellets (Innovative Research of America), and freshly sorted BCSCs were resuspended and diluted in serum-free PBS/Matrigel mixture (1:1 v/v) and inoculated into the mammary pads side by side of the animal using 27G needle (500 cells/injection). Tumorigenesis was assessed twice a week with palpation. Tumor sizes were measured using a caliper and experiments were terminated when tumors reached approximately 1.2 cm in the largest diameter.

**Mitochondrial membrane potential (Δψm) and apoptosis**

Irradiated cells were incubated with 2 μg/mL of JC-1 for 30 minutes. The fluorescence intensity of the red precipitate (JC-1 red), and green monomer (JC-1 green) were determined by a plate reader (Spectra Max M², Molecular Devices Co.) at 485 nm/595 nm or 485 nm/525 nm (excitation/emission). The ratio of JC-1 red (595)/JC-1 green (525) was calculated as ΔΨm. For apoptotic analysis, cells were harvested 24 hours postirradiation and were incubated with Annexin-V fluorescein isothiocyanate (FITC; Biosource) and propidium iodide (PI; Sigma) for 15 minutes before analysis with FACS-Canto (Becton Dickinson).

**BCSC sorting**

Following described procedures (3), cell suspension was rinsed with PBS containing 2% FBS, suspended in PBS containing 0.5% FBS and PI (0.5 mg/mL), and sorted using the Cytopeia inFlux Cell Sorter (BD Biosciences). The antibodies applied for sorting were anti-HER2/neu conjugated to allophycocyanin (APC; BD Biosciences), anti-human CD44 conjugated to FITC and human CD24 conjugated to phycoerythrin (PE; Invitrogen). Cell viability was assessed by 7-AAD staining during cell sorting, and by Trypan blue exclusion after sorting.

**ALDH activity**

Cells were suspended (10⁶ cells/mL) in ALDEFLUOR assay buffer containing activated ALDH substrate BAAA (borondipyrromethene-aminoacetaldehyde) with or without 50 mmol/L DEAB (diethylaminobenzaldehyde, a specific ALDH inhibitor) and incubated at 37°C for 40 minutes and analyzed by FACS. The gates were normalized to cells treated with ALDEFLUOR-DEAB.

**siRNA-mediated target gene inhibition**

The siRNA targeting HER2 was synthesized using the Silencer siRNA Construction Kit (Ambion) with 2 targeting sequences: 5’ AACCTGGAACCTCAGAGATTG 3’ and 5’ AAG-CCCTAGAGATCTTGAA 3’. Cell transfection was conducted at 30% to 50% cell confluence using Lipofectamine RNAiMAX reagent (Invitrogen). Scrambled RNA Duplex (Ambion) was used for specificity as a negative control.

**FISH and IHC of breast cancer specimens**

The pathology database was searched for invasive breast cancer, and cases with clinical follow-up were retrieved following Institutional Review Board’s approval (Emory University, Atlanta, GA, IRB#: 901-2004). Total 40 clinical samples were grouped according to pathologic classification. Fluorescence in situ hybridization (FISH) was conducted using the PathVysion kit (Vysis, Inc.). A centromere 17:HER2 probe signal ratio of more than 2.2 obtained from 30 interphase nuclei was considered positive for HER2 gene amplification. Immunohistochemical staining (IHC) was measured using a caliper and experiments were terminated.
conducted using UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen staining kit (Thermo Scientific) after heat-induced epitope retrieval. The slides were incubated with anti-HER2 (Sigma, Cat. E2777), anti-CD44 (R&D Systems, Cat. BBA10), and anti-CD24 (Santa Cruz, Cat. Sc-11406) primary antibodies for overnight at 4°C, followed by 3 washes, and then incubation with the secondary antibody for 1 hour at room temperature. The slides were then incubated with 4′,6-diamidino-2-phenylindole (DAPI; 300 nmol/L in PBS) for 5 minutes at room temperature, followed by 2 washes and then visualized with a Nikon microscope (Eclipse, E1000M).

Nikon microscope (Eclipse, E1000M) and graded as HER2+ (IHC 2+ and 3+) or amplified (FISH, HER2: centromere 17 signal ratio >2.2); HER2− (IHC 0) or nonamplified (FISH, HER2: centromere 17 signal ratio <1.8); and HER2 equivocal (IHC 1+ and FISH, HER2: centromere 17 signal ratio = 1.8–2.2).

Two-dimensional difference gel electrophoresis

Total cellular protein (50 μg) was minimally labeled with Cy3 and Cy5 following the reported protocol (25, 26). Labeled samples were mixed together and diluted to 450 μL in rehydration buffer, applied to a 24 cm pH 3 to 10 NL IPG strips (GE Life Sciences) and isoelectric focusing conducted until 62,500 Vh was reached. The second dimension separation was then conducted using 12.5% Tris–Glycine precast gel for 16 hours at 2 W per gel. CyDye protein spots were imaged on a Typhoon 9410 and differential protein expression analyzed using DeCyder 6.5 software. Differential In-gel Analysis (DIA) module was used to conduct spot detection, quantitation and Cy3/Cy5 volume ratio calculations, fold changes greater than 1.5 were considered significant.

High-performance liquid chromatography tandem mass spectrometry

For large scale analysis, 1 mg proteins, for each 3 biologic replicate samples, were precipitated and processed with strong cation exchange (SCX) column as described (27) followed by a 120-minute reverse-phase gradient to separate peptides based on their hydrophobicity. The LTQ Orbitrap XL (Thermo Scientific) coupled to reverse phase high-performance liquid chromatography (RP-HPLC) system with a low flow ADVANCED Michrom MS source, was set to scan the precursors with a top 4 data-dependent MS/MS method in the profile mode on the precursors and centroid mode on the MS/MS spectra. Tandem mass spectra were analyzed using Mascot database search engine (MatrixScience) with mass tolerances set to 1 Da for parent ions and 0.3 Da for MS/MS ions and allowance of up to 1 missed cleavages. All searches were done against the International Protein Index human protein database. The relative abundance of each identified protein in different samples were analyzed by QTools and Prolucid search algorithm and DTASelect using IP2 (Integrated Proteomics Pipeline) server and Amazon Cloud Computing. Comparative peptide abundance was evaluated by IP2-Census, a Label-free analysis for automated differential peptide/protein spectral counting analysis. Using MetaCore Version 6.9, a cluster of proteins and effectors were extracted from the data and identified to interact with both STAT3 and HER2.

Statistical analysis

The significance of the data was analyzed using an analysis of variance single-factor test and the 2-tailed Student t test. A difference was considered significant when P < 0.05.

Results

Enhanced aggressiveness of radioresistant MCF7 cells due to HER2 induction

To identify the key factors responsible for tumor resistance, we first assessed the question of whether HER2 expression is enhanced in the radiation-treated HER2-low breast cancer cells MDA-MB231 and a radiosensitive cell line (MCF7/C6) that survived a treatment course using fractionated doses of ionizing radiation (8, 20). Consistent with previous observations (7, 20), substantial amount of HER2 proteins was detected in the irradiated MDA-MB231 and the radiosensitive cell line MCF7/C6 (Fig. 1A; refs. 20, 28). To further elucidate the mechanism of radiation-induced tumor radioresistance, we studied HER2 expression in MCF7/C6 cell line with MCF7/HER2 and MCF7 wt cells as positive and negative controls, respectively. The induced HER2 protein expression in the MCF7/C6 cells could even reach to the level of HER2 expression in MCF7 cells stably overexpressing HER2 gene (MCF7/HER2). In addition, cell invasiveness of MCF7/C6 cells in Matrigel analysis (Fig. 1B and Supplementary Fig. S1C) was also significantly enhanced as compared with wt MCF7 cells. Interestingly, the gap-filling capacity remained in a similar rate in all cells except a tendency of increased gap-filling rate of MCF7/C6 and MCF7/HER2 cells at 96 hours (Fig. 1B and Supplementary Fig. S1A). Using tumor sphere formation assay (22), we found that, in addition to increased sizes of tumor spheres, the number of tumor spheres was increased 3.48- and 3.79-folds in MCF7/C6 and MCF7/HER2 cells, respectively, as compared with wt MCF7 cells (Fig. 1B and Supplementary Fig. S1D). The clonogenicity was increased 2.85- and 2.62-folds in the MCF7/C6 and MCF7/HER2, respectively (Fig. 1B). These results raise the possibility that the expression of HER2 that was silenced in many HER2-low breast cancers could be induced in therapy-resistant fraction of cancer cells leading to the overall aggressiveness of recurrent tumors.

HER2+/CD44+ cells enriched in irradiated MCF7 cells with increased radioresistance

Significant reduction in cell sensitivity to IR-induced apoptosis (Fig. 1C) and increased clonogenic survival
Figure 1. HER2 protein expression causes the aggressive growth in breast cancer cells with HER2low status. A, HER2 protein expression was dose-dependently induced by IR in MDA-MB-231 cells and in radioresistant MCF7/C6 (C6) cells (8, 20). MDA-MB-231 were exposed to increased doses of irradiation (0–4 Gy). Twenty-four hours postirradiation, cells were collected and tested for the expression of HER2. For MCF7/C6 cells, parental MCF7 cells (wt) and MCF7 cells stably transsected with HER2 gene (HER2) were used as negative and positive controls, respectively. B, evaluation of cell aggressiveness. Matrigel invasion assay: cells were cultured in Matrigel and cells migrating through the transwell were counted to reflect cell’s aggressiveness (mean ± SE; n = 6; *, P < 0.05; **, P < 0.01; depicted images of invaded cells are illustrated in Supplementary Fig. S1C). The gap-filling capacity was calculated as the ratio of filled gaps at 96 hours postscraping compared with the filled gap at 0 hour (additional data are shown in Supplementary Fig. S1A). The tumor sphere formation was evaluated as the number of tumor spheres formed per dish (mean ± SE; n = 6; **, P < 0.01; images of tumor sphere formation are illustrated in Supplementary Fig. S1D). Clonogenic survival was evaluated as the percentage of cells seeded that were able to form colonies 14 days after irradiation (mean ± SE; n = 3; **, P < 0.01). C, the radioresistant phenotype of MCF7/C6 (C6) cells was compared with MCF7/HER2 (HER2) and the parental MCF7 cells after treatment with 5 Gy IR by measuring apoptosis, clonogenic survival, and gap-filling rates (mean ± SE; n = 3; **, P < 0.01). D, quantification of BCSCs with the feature of HER2+ /CD44+ /CD24low in the surviving fraction of MCF7 cells (left) or xenograft tumors (right) treated with 5 × 2 Gy IR. Cell suspensions from control (sham) or irradiated cells or tumors were sorted by FACS with conjugated antibodies (APC for HER2, PE for CD44, and FITC for CD24; n = 3 from separate sorting, *, P < 0.05; **, P < 0.01; additional FACS data are shown in Supplementary Fig. S3 and Supplementary Table S1).
Inhibition of HER2 resulted in reduction of $$\Delta Y_{\text{os}}$$ and HER2 inhibition followed by radiation further reduced $$\Delta Y_{\text{os}}$$ in both MCF7/C6 and MCF7/HER2 cells (Supplementary Fig. S2). Interestingly, contrary to a similar gap-filling rate in all 3 cell lines without IR (Fig. 1B and Supplementary Fig. S1A), the gap-filling rates were markedly reduced in wt MCF7 cells but remained a relative high level in the MCF7/C6 and MCF7/HER2 cells after radiation (Fig. 1C and Supplementary Fig. S1B). To verify that the HER2-expressing BCSCs were enriched by multiple IR, we analyzed the expression of HER2 and BCSC markers in irradiated MCF7 cells and xenograft tumors (Fig. 1D, Supplementary Fig. S3, and Supplementary Table S2). IR was delivered by 5 fractionated doses with 2 Gy each to cells or locally to tumor xenografts in mice. In both cases, CD44$$^+/\text{CD24}^-$$, HER2$$^+/\text{CD44}^+$$, and HER2$$^{\text{hi}}/\text{CD24}^{\text{lo}}$$ populations were markedly increased. It should be noticed that about 1% of non-irradiated tumor cells were HER2$$^+/\text{CD44}^-$$, but this population was increased about 30-fold after irradiation in xenograft tumors (Fig. 1D, right). The HER2$$^{\text{hi}}/\text{CD44}^{\text{lo}}$$ population was also significantly increased (95.8%) in the radioresistant MDA-MB231 cells (Supplementary Fig. S4; the FIR Clone 4 was derived from the triple-negative wild-type MDA-MB231 cells after long-term irradiation; ref. 20). Similar to MCF7/C6, the increase in the HER2$$^{\text{hi}}/\text{CD44}^{\text{lo}}$$ subpopulation was due to the induction of HER2 expression in response to irradiation. However, the CD44$$^+/\text{CD24}^-$$ cells per se did not significantly change compared with the wt cells, probably due to the fact that MDA-MB-231 cells were found to hold naturally a high level of BCSCs (29, 30). The high level of BCSCs may explain the inherited aggressiveness phenotype of MDA-MB-231 compared with MCF7 cells. However, we believe that the overexpression of HER2 will further increase their aggressiveness and resistance to radiation.

Furthermore, we found that the increased population of HER2$$^+/\text{CD44}^-$$ cells was further enhanced in the irradiated xenograft tumors (Fig. 1D, right) compared with irradiated cells in culture (Fig. 1D, left), highlighting the possible role of other factors in the development of radioresistant BCSCs in vivo. Taken together, these data suggest that the tumor microenvironment under therapeutic stress conditions promotes the survival of HER2-expressing BCSCs. These results reinforce our hypothesis that HER2 expression in BCSC is a predominant feature of radioresistance in HER2$$^{\text{hi}}$$ cancer.

Identification of HER2$$^{\text{hi}}/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ cells

To investigate whether HER2 expression is a unique marker of radioresistant BCSCs in HER2$$^{\text{hi}}$$ breast cancer cells, CD44$$^+/\text{CD24}^{\text{lo}}$$ population was further sorted according to the level of HER2 expression (Fig. 2A and B and Supplementary Table S1). Of the total of $$2.4 \times 10^6$$ MCF7/C6 cells, $$3.7 \times 10^4$$ cells were identified as CD44$$^+/\text{CD24}^{\text{hi}}$$, CD44$$^+/\text{CD24}^{\text{lo}}$$, and CD44$$^-/\text{CD24}^{\text{low}}$$ (1.51%). Interestingly, only 9.84% of the CD44$$^+/\text{CD24}^{\text{lo}}$$ population was detected to express a high level of HER2 (termed as HER2$$^+/\text{CD44}^{\text{lo}}$$) and 6.85% showed low expression (termed as HER2$$^-/\text{CD44}^{\text{lo}}$$) population (Fig. 2B and Supplementary Table S1). The difference in HER2 expression between these 2 populations was confirmed by immunoblotting (Fig. 2B, right). ALDH is established to play a major role in cell-death resistance and is associated with BCSCs’ aggressiveness (31). We found that the ALDH activity was about 3-fold elevated in HER2$$^+/\text{CD44}^{\text{lo}}$$ compared with their counterpart HER2$$^-/\text{CD44}^{\text{lo}}$$ population (Fig. 2C and D). These results provide the evidence indicating that a fraction of HER2-expressing CD44$$^+/\text{CD24}^{\text{lo}}$$ BCSCs is present in the surviving breast cancer cells with status of HER2$$^{\text{hi}}$$.

Enhanced aggressiveness of HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ BCSCs

The HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ BCSCs were more aggressive in migration compared with the HER2$$^+/\text{CD44}^{\text{lo}}$$ BCSCs under the condition of sham or 5 Gy IR (Fig. 3C and Supplementary Fig S4), indicating that HER2-expressing BCSCs are more resistant to genotoxic stress. The increased radioresistance of HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ BCSCs was further supported by elevated $$\Delta Y_{\text{os}}$$, reduced apoptosis, and increased clonogenic survival (Fig. 3A and B). In addition, the invasiveness and tumor sphere formation (Fig. 3C) were increased approximately 5- and 4-fold, respectively, in HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ cells as compared with the HER2$$^-/\text{CD24}^{-}\text{low}$$ cells. To elucidate whether HER2 expression endows BCSC a higher tumorigenic capacity, we measured tumor formation of HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ versus HER2$$^-/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ cells using NOD scid gamma/SCID mouse xenograft model. HER2 status, indeed, affects the tumor-initiating rate of BCSCs. All the sites inoculated with HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ cells (500 cells/injection, HER2$$^+$$) developed tumors (6/6) with an average volume of 92 mm$^3$ at day 21; whereas no detectable tumors were recorded in all sites (0/6) with the same number of HER2$$^-/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ cells (HER2$$^-$$) up to day 45 (Fig. 3C). To verify the direct function of HER2 expression in radioresistance, siRNA-mediated HER2 inhibition was applied and the results showed a reduction in cell invasiveness by approximately 70% that was further reduced by HER2 inhibition combined with 5 Gy IR (Fig. 4A). Noticeably, the gap-filling rates were also severely reduced by HER2 inhibition with or without combination with IR (Fig. 4B and Supplementary Fig. S5). In agreement, treatment with HER2 siRNA (Fig. 4C) or Herceptin (Fig. 4D) reduced the clonogenicity of HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ BCSCs, which was further decreased when combined with IR treatment.

Coexpression of HER2 and CD44 in recurrent breast cancer

To investigate whether the feature of HER2$$^+/\text{CD44}^{\text{lo}}/\text{CD24}^{-}\text{low}$$ is related to the aggressiveness of metastatic

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breast cancers, we analyzed the expression of HER2, CD44, and CD24 in 40 randomly selected pathologic specimens that are clinically diagnosed as primary, recurrent, or unknown breast tumors by the pathology department at the Emory University School of Medicine. In each tumor, we correlated the degree of HER2 gene amplification assessed by FISH with the HER2 protein expression detected by immunohistochemistry. By FISH analysis, 12 of 40 tumors were determined as HER2-amplified (30%), whereas HER2 protein was expressed in 33 of 40 tumors by IHC (82.5%; Fig. 5A), suggesting that mechanisms other than gene amplification are responsible for the enhanced expression of HER2 protein. Interestingly, cells positively stained with HER2 and CD44 antibody by IHC were significantly increased (11/13, 85%) in the recurrent tumor samples compared with the level of the samples of primary tumors (12/21, 57%; Fig. 5B). Subsequently, 85% of the recurrent tumors were CD24− contrasted with 48% in primary tumors (Supplementary Fig. S6C). A representative picture of the tumors costained with HER2 and CD44 without CD24 expression are shown in Supplementary Fig. S6D. Taken together, these results support that HER2 expressing BCSC are enriched in recurrent breast tumors.

Proteomics of HER2+/CD44+/CD24−/low versus HER2−/CD44+/CD24−/low cells

To identify the signaling network causing the aggressive growth of HER2-expressing BCSCs, we conducted proteomics experiments using two-dimensional difference gel electrophoresis (2-D DIGE; Supplementary Fig. S7) and
RP-HPLC tandem mass spectrometry (MS-MS; Supplementary Fig. S7 and Supplementary Tables S3 and S4) that revealed a specific protein profile differentially regulated in HER2+/CD44+/CD24−/low cells as compared with HER2−/CD44+/CD24−/low BCSCs. 2-D DIGE results highlighted differences between these 2 samples and additional proteomics using RP-HPLC/MS-MS to separate trypsin-digested peptides identified 6,537 peptides differentially expressed in HER2+/CD44+/CD24−/low versus HER2−/CD44−/CD24+/low BCSCs. Using peptide counts determining differential proteins, 499 and 182 proteins were upregulated respectively in HER2+/CD44+/CD24−/low and HER2−/CD44+/CD24+/low BCSCs.

Figure 3. Enhanced radioresistance and aggressiveness in HER2+/CD44+/CD24−/low BCSCs. A, mitochondrial membrane potential (left) and apoptosis (right) were measured in HER2+/CD44+/CD24−/low and HER2−/CD44−/CD24+/low BCSCs treated with or without 5 Gy IR for 24 hours. Data represent mean ± SE of 3 independent experiments and statistical significance was evaluated as *, P < 0.05; **, P < 0.01. B, enhanced radioresistance in HER2+/CD44+/CD24−/low cells treated with sham (0 Gy) or 5 Gy of IR is tested by gap-filling and clonogenic survival assays. The gap-filling rate (left) was calculated as the ratio of the filled gap at 96 hours compared with 0 hour. The clonogenic survival (right) of HER2−/CD44−/CD24+/low BCSCs was evaluated by counting the number of colony formation (more than 50 cells) 14th day after cells were treated with 5 Gy of IR (n = 6; **, P < 0.01; additional data are shown in Supplementary Fig. S5). C, the aggressiveness of HER2+/CD44+/CD24−/low (HER2−) and HER2−/CD44−/CD24+/low (HER2+) BCSCs was further analyzed by Matrigel invasion assay (left), tumor sphere formation (middle, n = 6; **, P < 0.01; scale bar, 50 μm), and in vivo tumorigenesis (right). Tumor numbers and volumes were measured in NOD/SCID mice 2 weeks after the inoculation of 500 cells from each BCSC population in 2 opposite sites (HER+, left and HER−, right) of the animal as depicted by arrows.
In the context of BCSCs, CD44+/CD24⁻/low cells (cutoff-fold change ≥ 2). These peptides and proteins were further grouped according to their cellular functions and a representative list was presented according to functions of tumor metastasis, mTOR signaling, apoptosis, mitochondrial function, and redox balancing (Supplementary Table S3).
Cross-talk between HER2 and STAT3 in HER2⁺/CD44⁺/CD24⁻/low BCSCs

Among the key signaling elements and effectors involved in tumor proliferation and metastasis found in our protein expression profiling (Supplementary Table S3), the expressions of STAT3 and Src were detected in HER2⁺/CD44⁺/CD24⁻/low but not in the HER2⁻/CD44⁺/CD24⁻/low BCSCs (Supplementary Table S3). Because HER2 is known to activate STAT3 through both JAK2- and Src-dependent manners (32), resulting in tumor aggressiveness, we confirmed the coexpression of HER2 and STAT3 in HER2⁺/CD44⁺/CD24⁻/low but not in the HER2⁻/CD44⁺/CD24⁻/low BCSCs by Western blotting (Fig. 6A), and immunofluorescence and IHC analyses (Fig. 6C and D). Reporter gene analysis showed increased activity of STAT3 promoter only in HER2 expressing BCSCs (Fig. 6B). As shown in Fig. 6D, the overexpression of HER2 (green membrane) in radiosensitive cancer cells is strongly associated with high levels of STAT3 (red cytoplasmic); and STAT3 level is low in cells that are not expressing HER2 protein. Interestingly HER2⁺-positive human specimen showed similar pattern of coexpression of HER2 and STAT3. Taken together, these data suggest a possible strong connection between HER2 and STAT3 signaling pathways. Furthermore, using the MetaCore Version 6.9 program, we created the HER2–STAT3 signaling network consisting of all effectors that are directly linked to both HER2 and STAT3. This network revealed c-Src kinase that was expressed in HER2⁻/CD44⁺/CD24⁻/low BCSCs (Supplementary Table S3) as one of the mediators in the HER2–STAT3 connection (Fig. 6E). Interestingly, recently Korkaya and colleagues reported that STAT3 activation might account for Herceptin resistance in HER+ breast cancer cells due to the expression and secretion of IL-6 (33). Thus, interleukin-6 (IL-6) could be one of the factors mediating the radioresistance of CSCs in HER⁻/low breast cancers. Further investigation of the HER2–STAT3 signaling network and other HER2-linked effectors and pathways may generate additional mechanistic insights to understand the aggressive phenotype of CSCs.

Discussion

Increasing evidence supports the concept that the tumor bulk is consisted of heterogeneous population and potentially contains tumor-initiating cells or CSCs (34, 35). Here, we identified HER2-expressing CD44⁺/CD24⁺/low BCSCs from both in vivo and in vitro irradiated HER2⁻/low breast cancer cells and tumor samples from patients with breast cancer. Compared with the HER2-negative BCSCs, HER2⁺/CD44⁺/CD24⁻/low cells showed a more aggressive phenotype and in vivo tumorigenesis with the enhanced resistance to radiation. Cells coexpressing HER2 and CD44 were more frequently detected in recurrent than the primary tumors in patients with breast cancer. Our results further provide a unique group of proteins in HER2⁺/CD44⁺/CD24⁻/low cells governing therapy outcomes including metastasis, mTOR signaling, apoptosis, mitochondrial function, redox balancing, DNA repair, and HER2–STAT3 prosurvival network. These results suggest that HER2-expressing BCSCs may be effective targets to treat recurrent tumors.

Although CSCs isolated from many tumors show an increased tumor-initiating ability and invasiveness, not all CSCs are able to reside and proliferate at sites of metastasis (36). These results raise an important question about whether or not CSCs are themselves a heterogenic population. Using patient-derived xenograft tumors, different radiation sensitivities were detected in the CSC population (37), supporting that not all BCSCs are radiosensitive. HER2-positive breast cancer are resistant to an array of therapies with a high risk of local relapse and recurrence (38) and blocking HER2 expression or using specific agents targeting HER2 signaling pathway (trastuzumab, lapatinib, and others) improve the cancer control (16). However, this benefit is not only reported for the HER2⁺ tumors, but also for patients with HER2⁻/low status (39, 40), suggesting that acquired resistance due to HER2 induction could develop under anticancer treatments. In fact, we have shown that HER2 expression is inducible in HER2⁻/low breast cancer cells via NF-kB–mediated HER2 promoter transactivation,
HER2 and STAT3 cross-talk in HER2⁺/CD44⁺/CD24⁻/CD105⁻ BCSCs. A, HER2 and STAT3 protein expression levels were further determined by Western blot analysis in the HER2⁺/CD44⁺/CD24⁻/CD105⁻ and HER2⁻/CD44⁺/CD24⁻/CD105⁻ BCSCs. B, increased STAT3 transcriptional activity in HER2⁺/CD44⁺/CD24⁻/CD105⁻ compared with HER2⁻/CD44⁺/CD24⁻/CD105⁻ BCSCs. STAT3 luciferase reporters (52) were transfected in HER2⁺/CD44⁺/CD24⁻/CD105⁻ and HER2⁻/CD44⁺/CD24⁻/CD105⁻ cells and luciferase activity was measured 24 hours after transfection (n = 3; *, P < 0.01). C, colocalization of HER2 and STAT3 expression in radioresistant cancer cells and (D) HER2⁺ human breast specimen. E, a putative HER2–STAT3 cross-talk signaling network for the aggressiveness of HER2⁺/CD44⁺/CD24⁻/CD105⁻ BCSCs. For the interaction between HER2 and STAT3 signals, a cluster of factors searched from the database of MetaCore Version 6.9 was identified to interact with both STAT3 and HER2. This network causing a cross-talk between HER2 and STAT3 may play a critical role for the overall aggressiveness of the HER2 expressing BCSCs in breast tumors with HER2⁻ status.
and the induced HER2 activation is responsible for the resistance to radiation (20). Our present data suggest that HER2-expressing BCSCs grow more aggressively than HER2-negative BCSCs under genotoxic conditions. Thus, tumor cells surviving a course of anticancer radiotherapy are likely enriched with more aggressive cancer cells expressing HER2.

A dynamic repopulation process may be initiated in tumors under chemo- and/or radiotherapy (5, 41, 42). In agreement with reported BCSC-resistant phenotype, BCSCs were increased in the surviving fraction of HER2+/low MCF7 cells and xenograft tumors after exposure to multiple doses of irradiation (Fig. 1D). Together with an increased frequency of HER2+/CD44+ in the recurrent tumors (Fig. 5A and B), these results raised the possibility that breast tumors originally diagnosed as HER2+/low status (mainly by HER2 transcript enhancements), may be induced to express a high level of HER2 protein. Thus, the radioresistant fraction within the CD44+/CD24+/low population could be enriched because of HER2 expression, an oncogene well-recognized in tumor resistance, metastasis, and tumor relapse (10, 11, 43). In this regard, HER2 protein expression could be more enhanced, whereas HER2 gene copies may remain constant. These results may explain the additional curative benefit gained with trastuzumab as a coadjuvant for patients initially classified as HER2− candidates (39, 40). It is highly possible that genotoxic agents that activate NF-κB, may result in a selection of BCSCs with the feature of HER2+/CD44+/CD24+/low in the HER2− breast cancer.

In agreement with the low population of CSCs (44), we found that 1.5% to 2.0% of the radioresistant MCF7/C6 cells contain the feature of CD44+/CD24−/low in which, 9.8% was detected as HER2+/CD44+/CD24−/low cells (Fig. 2). The HER2+/CD44+/CD24+/low BCSCs showed 4- to 5-fold increase in cell invasion, tumor sphere formation with an enhanced in vivo tumorigenesis (Fig. 3C). Although the tumor-initiating capacity of CD44+/CD24−/low cells isolated from both primary patient tumors and immortalized cell lines is well established, our data suggested that CD44+/CD24−/low cells fail to grow tumors when HER2 is not expressed (HER2+/CD44+/CD24−/low vs. HER2−/CD44+/CD24−/low cells, Fig. 3C; ref. 18). Importantly, tumors with HER2 protein expression were obviously increased in the recurrent breast cancer tissues as compared with the primary tumors (Fig. 5). Therefore, it is highly possible that a small fraction of BCSCs with elevated de novo HER2 expression in HER2− breast cancer has the survival advantage under genotoxic condition, such as ionizing radiation or anticancer chemotherapy, and thus will allow repopulation after a course of treatment. However, we could not exclude the possibility that HER2 transactivation is preferably induced in a specific fraction of BCSC population under genotoxic stress that is responsible for tumor repopulation.

It has been reported that CD44 and HER2 interaction promotes human ovarian tumor (45). Clinically, although CD44+/CD24−/low BCSC phenotype has been linked to basal-type breast cancer tumors, particularly in BRCA1-inherited cancers, this phenotype does not correlate with the clinical outcome (46), suggesting that, besides the feature of CD44+/CD24+/low, specific prosurvival networks are associated with CSC resistance. ALDH, a key BCSC marker, also present in only about 30% of tumors, further divide the CD44+/CD24−/low population into highly tumorigenic ALDH+/CD44+/CD24−/low that is capable of generating tumors as low as 20 cells; whereas ALDH−/CD44+/CD24+/low show reduced tumorigenesis (31). Unlike CD44+/CD24−/low, the ALDH phenotype correlates with clinical outcome, tumor grade, and the status of HER2 and Ki67. Korkaya and colleagues reported that HER2 over-expression enhances the ALDH activity (18) and Diehn and colleagues found that ALDH+/HER2 cells are more tumorigenic than ALDH− cells (6), further confirming the link between ALDH activity, HER2 expression and CSCs. In this study, we found that ALDH activity is elevated in the HER2-expressing CD44+/CD24+/low cells (Fig. 2C and D), suggesting that this radioresistant subpopulation of BCSCs is able to activate both HER2 and ALDH. Thus, ALDH+/HER2 and their potential downstream effectors may provide more specific sets of targets to treat metastatic and recurrent breast cancer, which needs to be further investigated.

To identify key factors causing the resistant phenotype of HER2+/CD44+/CD24−/low cells, our proteomics data from LC/MS-MS revealed a comprehensive protein profile in HER2+/CD44+/CD24+/low versus HER2−/CD44+/CD24−/low BCSCs (Supplementary Table S3). The strong prosurvival and antiapoptotic factors are believed to be the major mechanism the resistance of CSCs to radio- and chemotherapy (47, 48). Although many of these proteins are already known for cell survival, such as DNA repair (Supplementary Table S3; refs. 47, 49), angiogenesis (41), cell adhesion and proliferation (50), elements involved in metastasis, mTOR signaling, mitochondrial ATP generation, and redox balancing seem to be required for the enhanced aggressiveness in HER2-expressing BCSCs. A lowered rate of apoptosis in CSCs (50) with enhanced mitochondrial function and DNA repair ability contributes to the BCSC survival. Among key proteins and effectors detected in the in the HER2+/CD44+/CD24+/low versus HER2−/CD44+/CD24−/low BCSCs profile, we found that STAT3 was expressed in HER2+/CD44+/CD24+/low but not in HER2−/CD44+/CD24−/low BCSCs (Supplementary Table S3). Because HER2 is known to activate STAT3 through both JAK2- and Src-dependent manners (32) and STAT3 promotes the microRNA expression and chemoresistance in CD44-activated head and neck cancer cells (51), our data on the coactivation of HER2 and STAT3 further illustrate a HER2−/STAT3 signaling network related to the aggressiveness of HER2-expressing BCSCs (Fig. 6D). Further investigation of the HER2−/STAT3 network and other HER2-linked pathways may generate additional mechanistic insights to understand the development of aggressive CSCs.

In summary, these results have important implications as they suggest that HER2-overexpressing BCSCs are causally
associated with the aggressive phenotype and radioresistance of HER2–/low breast cancer cells. The ability of HER2+/CD44+/CD24−/low to display an enhanced aggressiveness suggests that the coexpression of HER2 and CD44 is a unique feature of BCSCs in HER2–/low breast cancer. The HER2-associated prosurvival signaling network including the STAT3 pathway could be enhanced because of a substantial HER2 gene transactivation during or after anticancer therapy. Therefore, further characterization of HER2-initiated pathways in the therapy-resistant CSCs, especially the HER2–STAT3 cross-talk (Fig. 6E) identified in this study, may generate new targets for the control of recurrent and metastatic tumors.

Disclosure of Potential Conflicts of Interest
M.S. Wicha has ownership interest (including patents) in Oncomed Pharmaceuticals and is a consultant/advisory board member of Oncomed Pharmaceuticals and Verastem. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: N. Duru, D. Candas, B.A. Chromy, D.R. Spitz, J.J. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Duru, D. Nantajit, Y. Wen, K. Xiao, A. Eldridge, B.A. Chromy, S. Li, D.R. Spitz
Analysis and interpretation of data (e.g., statistical analysis, biostatistical methods): N. Duru, D. Candas, C. Menaa, H.-C. Liu, Y. Wen, A. Eldridge, B.A. Chromy, S. Li, D.R. Spitz, M.S. Wicha, J.J. Li
Writing, review, and/or revision of the manuscript: N. Duru, M. Fan, D. Candas, C. Menaa, D. Nantajit, Y. Wen, A. Eldridge, B.A. Chromy, S. Li, D. R. Spitz, K.S. Lam, J.J. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Fan, D. Candas, A. Eldridge, B.A. Chromy, S. Li, D.R. Spitz, J.J. Li
Study supervision: M. Fan, D. Candas, B.A. Chromy, D.R. Spitz, J.J. Li

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Nadire Duru, Ming Fan, Demet Candas, et al.


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