Aspirin Suppresses the Acquisition of Chemoresistance in Breast Cancer by Disrupting an NFκB–IL6 Signaling Axis Responsible for the Generation of Cancer Stem Cells

Shilpi Saha¹, Shravanti Mukherjee¹, Poulami Khan¹, Kirti Kajal¹, Minakshi Mazumdar¹, Argha Manna¹, Sanhita Mukherjee², Sunanda De³, Debarshi Jana³, Diptendra K. Sarkar³, and Tanya Das¹

Abstract

Acquired chemoresistance has curtailed cancer survival since the dawn of chemotherapy. Accumulating evidence suggests a major role for cancer stem cells (CSCs) in chemoresistance, although their involvement in acquired resistance is still unknown. The use of aspirin has been associated with reduced cancer risk and recurrence, suggesting that the anti-inflammatory drug may exert effects on CSCs. In this study, we investigated the contribution of CSCs to acquired chemoresistance of breast cancer and the avenues for reversing such effects with aspirin. We observed that the residual risk of recurrence was higher in breast cancer patients who had acquired chemoresistance. Treatment of preexisting CSCs with a genotoxic drug combination (5-fluorodeoxyuracil, doxorubicin, and cyclophosphamide) generated an NFκB–IL6–dependent inflammatory environment that imparted stemness to nonstem cancer cells, induced multidrug resistance, and enhanced the migration potential of CSCs. Treatment with aspirin prior to chemotherapy suppressed the acquisition of chemoresistance by perturbing the nuclear translocation of NFκB in preexisting CSCs. Therefore, disruptions to the NFκB–IL6 feedback loop prevented CSC induction and sensitized preexisting CSCs to chemotherapy. Collectively, our findings suggest that combining aspirin and conventional chemotherapy may offer a new treatment strategy to improve recurrence-free survival of breast cancer patients. Cancer Res; 76(7); 2000–12. ©2016 AACR.

Introduction

Although the advent of highly targeted therapeutics based on small-molecule inhibitors and mAbs has revolutionized the treatment of some cancers, chemotherapy remains the mainstay treatment for most tumors, especially for locally advanced breast cancer (LABC; ref. 1). Although a majority of LABC patients treated with neoadjuvant chemotherapy (NACT) experience a reduction in tumor size (1), a substantial number either exhibits significant intrinsic resistance or acquire characteristics of multidrug resistance during chemotherapy, i.e., acquired resistance (2, 3), thereby limiting the efficacy of chemotherapeutic regimens.

Several hypotheses have been proposed to explain this treatment failure and recurrence (4). In particular, it has been suggested that a small subpopulation of cells within tumors, called cancer stem cells (CSCs), by virtue of their relative resistance to chemotherapy may contribute to tumor recurrence (5, 6). Consistent with these reports, several groups reported enrichment for CSCs when solid cancers were subjected to classical anticancer treatment (7–11). In accumulation, these studies suggest that significant improvement in patient outcome will require drugs that selectively target CSCs in combination with conventional chemotherapeutic regimens that target rapidly proliferating cells.

Epidemiologic and clinical studies indicate that inflammation is correlated with an increased risk of recurrence in breast cancer patients (12), and NFκB plays a causative role in inflammation (13). Recent literature highlights the importance of NFκB-controlled family of proinflammatory cytokines, significantly IL6, in CSC maintenance (14) and in regulating plasticity of neoplastic cells (15), thereby suggesting the possible involvement of NFκB–IL6 pathway in aggressive disease recurrence following NACT.

Interestingly, multiple meta-analyses showed that patients with cardiovascular diseases treated with FDA-approved anti-inflammatory drug, aspirin, have reduced cancer risk (16, 17). Recent epidemiologic studies have found that women who took aspirin after a diagnosis of breast cancer had a better prognosis than women who did not take aspirin, indicating that aspirin was associated with a decreased risk of distant recurrence (18), although the underlying mechanism is still unclear. Considering all this information, we envisaged the role of aspirin in improving the response of breast tumors to chemotherapy regimen to finally improve recurrence-free survival of patients. We therefore investigated whether aspirin could suppress acquisition of
resistance during standard chemotherapy by targeting CSC pool, which would be expected to diminish disease progression and consequently improve overall and disease-free survival (DFS) of LABC patients.

Materials and Methods

Preclinical study design

Retrospective cohort observational study. A total of 203 patients, enrolled for treatment in either Bankura Sammilani Medical College (BSMC; Bankura, West Bengal, India) or Comprehensive Breast Service Clinic in IPGMER and SSKM Hospital, (Kolkata, West Bengal, India) between January 2010 and December 2013, with histologically or cytologically confirmed breast cancer recurrence were retrospectively reviewed for their medical records and radiologic images to determine the DFS period and their response to chemotherapy cycles. Patients having histologically or cytologically documented LABC characteristics, that is, primary tumors > 5 cm, with skin or chest wall involvement or with matted axillary adenopathy at the time of the first presentation of disease and grade III according to American Joint Committee on Cancer (AJCC) criteria (19) and were prescribed neoadjuvant 5-fluoro-uracil 500 mg/m², doxorubicin 50 mg/m², and cyclophosphamide 500 mg/m² per cycle (FAC) chemotherapeutic regimen, were eligible for this study, whereas previous solid tumors, age < 18 years, distant recurrences, and neoadjuvant chemotherapy dropouts were considered exclusion criteria of the study.

Prospective cohort experimental study. Fifty patients with histologically or cytologically documented LABC characteristics, who attended above-mentioned hospitals from January 2009 to December 2010, were assessed for their pathologic (20) and clinical (21) responses toward neoadjuvant FAC treatment. On the basis of their responses during the course of chemotherapy, patients were categorized into chemotherapy-sensitive (CS), acquired chemoresistant (CR), and inherent resistance group. Response of primary cells to treatments was also determined on the basis of their estrogen receptor (ER), progesterone receptor (PR), and HER2 status. Tumor characteristics, that is, clinical (age, tumor, and node), histopathologic (grade, ER, PR, and HER2 status, ductal, lobular, and invasive), and molecular [luminal and basal, by assessing their ER/PR status (22, 23)] characteristics of each patient were collected from the pathology departments of above-mentioned hospitals and tabulated for reference in Supplementary Table S1A. Patients with inherent chemotherapy resistance, previous solid tumors, < 18 years, and breast cancer recurrences or contralateral tumors were excluded from the study. All LABC patients were treated with FAC regimen with a gap of 21 days between two cycles, followed by surgery. After the completion of treatment, patients were followed up monthly for first three months and then once in four months for a total period of three years to determine their "clinical outcome." DFS period was calculated from the date of surgery to the date of recurrence of disease.

Ex vivo study. Patients having histologically or cytologically documented LABC characteristics, and of grade III according to AJCC criteria (19), luminal histotype, i.e., ER/PR+ and either negative (luminal A subtype) or positive (luminal B subtype) HER2 status (Supplementary Fig. S1A), and those not been treated with chemotherapy or radiotherapy were eligible for this study. Selected cases consisted of 30 primary breast cancer patients. Basal histotype breast cancers were excluded from the study as basal subtypes displayed “inherent” resistance to chemotherapy (Supplementary Table S1A; ref. 24). Tumor characteristics (clinical and histopathologic) were available and collected retrospectively from the pathology departments of above-mentioned hospitals. Furthermore, the response of primary cells to in vitro treatments was also determined on the basis of their ER/PR/HER2 status. All this information has been tabulated in Supplementary Table S1B.

All these studies were planned and approved by the Human Research Ethics Committees of BSMC, Bankura, India (approval number: CNMC/ETHI/162/P) and IPGMER and SSKM Hospital (approval number: Inst/IEC/308), Kolkata, and associated research and analyses were done in Bose Institute (Kolkata, India) in accordance with the Institutional Human Ethics Committee (approval number: BIHEC/2010/11/11). Written informed consent was obtained from all patients.

Primary human tissue culture

For prospective cohort experimental study, ultrasound-guided core biopsy samples were obtained at baseline before any chemotherapy, and thereafter, upon completion of treatment, surgically resected breast cancer tissue, tumor marginal tissue, and normal breast tissue samples were obtained. For ex vivo preclinical study, untreated primary human breast cancer tissue samples were procured. All samples were mechanically disaggregated with collagenase (25). Tumor cells were sorted twice after staining with APC-anti-CD44 and PE-anti-CD24. Nontumor cells were depleted with FITC-tagged cocktail of lineage marker antibodies, and the purity was verified flow cytometrically (Supplementary Fig. S1B). Dilutions of antibodies are listed in Supplementary Table S2.

Cell lines and cell culture

Human breast cancer cell lines, MCF-7, T47D, and ZR-75-1 were obtained from National Centre for Cell Science (Pune, India) between 2012 and 2015, and MDA-MB-361 and BT-474, received in 2015, were kind gifts from Dr. A. Mal, Roswell Park Cancer Institute (Buffalo, NY). All cell lines were authenticated by short tandem repeat analysis and each was passaged for fewer than 6 months after resuscitation. Cells were routinely maintained in DMEM as described previously (26).

Antibodies and treatments

Cells were treated with chemotherapeutic regimen FAC (MP Biomedicals) in 1:1:10 ratios and with 1 to 5 mmol/L aspirin (MP Biomedicals) for specific experiments. Because aspirin is sparingly soluble in distilled water (27), the 1 mol/L stock solution was prepared in DMSO. For all in vitro assays with aspirin, DMSO was used as control. In selected experiments, cells were incubated with IL6-neutralizing antibody (1.5 μg/mL), recombinant IL6 (rIL6, 1.2 ng/mL), Brefeldin A (1 μg/mL), PMA (500 ng/mL), ionomycin (500 ng/mL, BD Biosciences), SN50 (18 μmol/L) or MG132 (10 μmol/L; Calbiochem), and mitomycin c (10 μg/mL, MP Biomedicals).

In vitro tumorigenicity assay and mammosphere culture

Different breast cancer cells (2.5 × 10⁴ cells/well) were cultured and propagated in suspension culture as described previously (28). Mammosphere-forming efficiency was determined by
Saha et al.

counting the number of mammospheres (average diameter, 100 μm) under a light microscope at 10× magnification and reported as the number of mammospheres divided by the number of cells seeded and expressed as percentage.

Flow cytometry

Human breast CSC markers, chemoresistance, EMT, and dedifferentiation phenomena were analyzed flow cytometrically (FACSVerse, Becton Dickinson; ref. 29). Percent apoptosis of CSCs/nonstem cancer cells (NSCC) were determined (26). For assaying intracellular IL6, IL6-PE was used (30). Dilutions of antibodies are listed in Supplementary Table S2. Positive staining was considered based on the negativity of an isotype control. A minimum of 10,000 events were recorded for all samples. Most phenotypic data were validated using the same antibodies with alternate labels.

Plasmid transfections

CSCs were transfected separately with 300 pmol of IL6 shRNA (Addgene). The pcDNA3.1 plasmid encoding IκBζ-R-32A/36A cDNA (IκBζ super-repressor, IκBζ-SR; kind gift from Dr. J. Didonato, The Cleveland Clinic (Cleveland, OH) was transfected in sorted CSCs for overexpression studies, and pd2EGFP-N1 vector (Clontech) was used for labeling sorted NSCCs (29).

Confocal microscopy

Confocal microscopy for the visualization of NFκB was done as previously described (31).

Transwell migration assay

Cells (2.5 × 10⁵) were seeded into 24-well cell culture inserts with 8 μm pores (BD Falcon), and migration of cells was recorded after 12 hours (29).

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP Assay Kit (Millipore) according to the manufacturer’s instructions. PCR assay for identification of NFκB-binding region on ABCG2 and ABCCI promoters was performed using specific primer sets. Primers are listed in Supplementary Table S2. The PCR products were analyzed by 2% agarose gel electrophoresis (32, 33).

Statistical analysis

Values are shown as SEMs except otherwise indicated. Comparison of multiple experimental groups was performed by two-way ANOVA test. Kaplan–Meier method was used to plot DFS curves, and log-rank test was used to estimate the statistical difference between groups. Data were analyzed, and when appropriate, significance of the differences between mean values was determined by a Student t test. Results were considered significant at P ≤ 0.05.

Other standard reagents and methods are provided in Supplementary Experimental Procedures.

Results

Residual risk of recurrence was higher in LABC patients with acquired chemoresistance

Analysis of retrospective cohort observational study revealed that the number of chemoresistant patients (n = 153) with recurrence of disease was higher than chemosensitive patients (n = 50; Fig. 1A). Further scrutiny of the analysis revealed a close association between locoregional recurrences (LCR) of disease with acquired resistance to therapy (Supplementary Fig. S2A).

These indications were confirmed by prospective cohort experimental study. According to clinical and pathologic response guidelines (20, 21), 11 cases were categorized in acquired CR-group (CR-primary tumor), whereas 29 cases were categorized in CS-group (CS-primary tumor; Supplementary Table S1A). Inherently, CR patients were excluded from this study. Acquisition of resistance by cells of CR-primary tissue was validated by their higher expression levels of chemoresistant markers, ABCG2 and ABCCI, following NACT when compared with cells of CS-primary tissue (Supplementary Fig. S2B). The analysis of tumor molecular characteristics showed that all CS- and CR-primary tumors were of luminal histotype, that is, ER⁺/PR⁺, whereas inherently CR patients were of basal histotype, i.e., ER⁻/PR⁻ and were excluded from this study. Luminal primary tumors were of two subtypes: (i) ER⁺/PR⁻/HER2⁻ luminal A histotype and (ii) ER⁻/PR⁺/HER2⁺ luminal B histotype. For this study, primary breast cancer tissues/cells of luminal histotype were included as a broad category, although the presence or absence of ER/PR and HER2 was predictive of the response of tumor to chemotherapy (Supplementary Table S1A). Follow-up studies further validated the relation between poor therapeutic response and LCR as confirmed by Kaplan–Meier survival analysis (Fig. 1B). These findings confirmed that the residual risk of breast cancer recurrence was higher in patients who acquire resistance with treatment as compared with CS patients.

Chemotherapy increases CSC pool in LABC patients with acquired chemoresistance

Next, we investigated the effect of chemotherapy on CSC content of mammary epithelial tumors. Higher percent CSC content of CR-tumors was detected as compared with CS-tumors (Fig. 1C). Moreover, increase in percent CSCs with chemotherapy was observed when paired specimens from CR patients, obtained prior to chemotherapy and at surgery following chemotherapy, were compared (Fig. 1D and Supplementary Fig. S2C). Increase in the expression levels of other CSC-related markers, Oct-4, Nanog, Sox-2, and Aldh1 in chemotherapy-treated CR-primary tumor samples when compared with matched untreated samples, supported above-mentioned findings (Fig. 1E and Supplementary Fig. S2D).

In vitro validation of chemotherapy-mediated increase in CSC content

For in vitro validation of above findings, breast cancer cell lines, MCF-7, T47D, ZR-75-1, MDA-MB-361, and BT-474, mimicking the characteristics of the primary cell lines, i.e., luminal histotype, were included in the study. Among these, MCF-7, T47D, and ZR-75-1 were ER⁺/PR⁻/HER2⁻, whereas MDA-MB-361 and BT-474 were ER⁻/PR⁺/HER2⁺. Percent CSCs, expression levels of CSC-related markers, and mammosphere-forming efficiency (Fig. 1F, Supplementary Fig. S2E) increased when these cells were treated with chemotherapeutic regimen FAC as administrated to the patients included in the study (Supplementary Fig. S3A). Furthermore, FAC treatment selectively killed NSCCs as opposed to CSCs (Fig 1G, top). These findings supported our postulation that posttreatment increase in CSC repertoire can be “apparent” due to relative decrease in NSCCs. Revalidating analysis, showing increase in CSC number upon FAC treatment, ruled out the
The possibility that such increase in CSC repertoire was only due to selection of “preexisting” CSCs (Fig. 1G, bottom).

Therapy-generated CSC pool displays enhanced chemoresistance and migration potential

Next, CSCs purified from FAC-treated CR-primary tumors (Fig. 2A, right and Supplementary Fig. S3B) or MCF-7 cells (Supplementary Fig. S3D) demonstrated increase in chemoresistance markers when compared with CSCs from their untreated counterparts. However, CSCs from FAC-treated CS-primary tumors failed to demonstrate such increase in chemoresistant markers (Fig. 2A, left). In addition, relative increase in migration property and associated markers confirmed enhanced migration potential of FAC-treated CSCs of primary CR-tumors (Fig. 2B and Supplementary Fig. S3C) and MCF-7 cells (Supplementary Fig. S3E). Therefore, besides...
increasing CSC proportion, chemotherapy also enhanced their chemoresistance and metastatic potential, thereby promoting the chances of recurrence in CR-patients when compared with CS-patients.

Figure 2. Therapy-generated aggressive CSC pool enhances risk of LCR in CR-breast cancer patients. Relative mean fluorescence intensities of ABCG2 and ABCC1 (A) in CSCs isolated from pre and posttreatment primary tumors of CS- (left) and CR-patients (right) and E-cadherin and vimentin (B) in CSCs isolated from pre and posttreatment primary tumors of CR-patients (left). Graphical representation of migration of these cells in transwell migration assay (right). C, representative flow cytometric density plots showing percent CSCs in tumor surgical margins (left) and normal breast tissue (right) of CS- and CR-patients are represented as dot plots and bar plots (bottom). Middle, illustration depicting tumor surgical margins and normal breast tissue. D, ratio of percent CSCs in normal breast tissue and primary tumor of CS- and CR-patients is represented graphically as dot plot (left) and as bar plot (right). E, relative mean fluorescence intensities (MFI) of ABCG2 and ABCC1 in normal breast tissue of CS- and CR-patients (left). Graphical representation of migration of CSCs purified from normal breast tissue of CS- and CR-patients (right). Data are presented as mean ± SEM or representative of three independent experiments. *, P < 0.05; **, P < 0.01; and ***, P < 0.001. AU, arbitrary units.
enchymal phenotype increases in the blood of patients (34, 35). 

These findings indicate the possibility that aggressive CSCs hiding in distant anatomic sites even after resection of primary tumor might play a role in disease recurrence at the local site in CR-patients (Fig. 1B). 

Therapy induces expression of CSC phenotypic markers in NSCCs 

FAC-induced increase in CSCs might be due to (i) proliferation of preexisting CSC pool or (ii) conversion of NSCCs into a stem-like state. However, proliferation blocker mitomycin C failing to significantly inhibit FAC-induced increase in CSCs and the expression of CSC-specific markers (Fig. 3A and Supplementary Fig. S4A) highlighted the second possibility. Interestingly, irrespective of the presence or absence of mitomycin C, FAC treatment failed to significantly increase the number of purified CSCs (Fig. 3B). These findings confirmed that induction of stemness in NSCCs was a major cause underlying FAC-induced CSC expansion, although the input of CSC proliferation was not completely negated. 

Therapy-mediated induction of CSC phenotype in NSCCs is driven by preexisting CSCs 

In paradox with our previous findings, no significant induction of CSC markers (Fig. 3C), chemoresistance, and migration related markers (Fig. 3D and Supplementary Fig. S4B) was observed in FAC-treated NSCCs from pretreatment CR-primary tumors and MCF-7 cells (Supplementary Fig. S4C–S4E). Treatment-mediated induction of stem-like signature in NSCCs might thus be influenced by preexisting CSCs. To validate our hypothesis, (i) purified NSCCs were GFP-tagged and thereafter cocultured with untreated CSCs (1:1) and (ii) CSCs and NSCCs (1:1) purified from MCF-7 cells were cocultured in contact-independent manner. Appearance of GFP-CSCs in the mixed population postchemotherapy (Fig. 3E) confirmed the contribution of preexisting CSCs in NSCC-to-CSC conversion. Also, several fold increase in FAC-mediated CSC induction was observed in contact-independent NSCCs as compared with that in FAC-treated NSCCs (Fig. 3F). Increase in the expression levels of other CSC-specific markers (Fig. 3F and Supplementary Fig. S5A) only in NSCCs cocultured with CSCs further validated preexisting CSC-mediated induction of CSC phenotype in NSCCs. 

Therapy triggers NfκB-dependent IL6 inflammatory feedback loop in preexisting CSCs 

Aforementioned results raised the possibility of the involvement of CSC-derived soluble factors in NSCC-to-CSC conversion. Recent reports have demonstrated NfκB-dependent IL6 activation (13, 14) and the role of IL6 in inducible transition of NSCCs to CSCs (15). Our results showed FAC-induced significant increase in nuclear NfκB in CR-primary tumors (Fig. 4A) and in different breast cancer cells (Supplementary Fig. S5B). However, FAC-treated CS-primary tumors failed to demonstrate such increase (Fig. 4A). Interestingly, CSCs of MCF-7 cells displayed increased levels of nuclear NfκB (Fig. 4B and Supplementary Fig. SSC) and its downstream inflammatory and antiapoptotic regulators, Cox-2, Bcl-2, and XIAP (Fig. 4C), when compared with their corresponding NSCCs, and FAC treatment further increased the expressions. Posttreatment CR-primary tumors, but not CS-primary tumors, also demonstrated increase in IL6 mRNA when compared with pretreatment ones (Fig. 4D). Time-dependent stimulation of intracellular IL6 was also observed in MCF-7 cells upon FAC treatment (Fig. 4E, left) and also in different breast cancer cells upon 3 hours of FAC treatment (Fig. 4E, right). IL6 secretion was higher from CSCs than NSCCs of MCF-7 cells and increased further with FAC treatment (Fig. 4F). That FAC-mediated increase in CSC-shed IL6 was NfκB-dependent was confirmed when blocking NfκB nuclear activity in CSCs significantly decreased IL6 secretion (Fig. 4G, left). Interestingly, although IL6 inhibition failed to retain NfκB in nucleus in preexisting CSCs even upon FAC treatment, exposing CSCs to rIL6 (1.2 ng/mL) enhanced the same (Fig. 4G, middle and right), confirming that FAC treatment activated an NfκB–IL6–positive inflammatory feedback loop in preexisting CSCs. 

Preexisting CSCs promote NSCC-to-CSC conversion in a paracrine manner 

Next, NSCCs of MCF-7 cells were (i) cocultured with conditioned media (CM) of untreated/FAC-treated purified CSCs, or (ii) treated with rIL6 (Fig. 5A, left). After 72 hours, CM of FAC-treated CSCs or rIL6 was found to induce CD44+/CD24low phenotype in NSCCs of MCF-7 cells, while reversing such conditions resulted in significant inhibition, although not complete, in inducing CSC signature in NSCCs (Fig. 5A, middle). Increase in IL6 in the CM of CSCs also increased ABCG2 level in newly generated CSCs (Fig. 5A, right). Therefore, FAC-triggered robust positive inflammatory feedback loop in preexisting CSCs significantly contributed in the transition of NSCCs-to-CSCs as well as in their attainment of aggressive phenotype, although the involvement of other soluble factors could not be ruled out. 

Aspirin inhibits chemotherapy-mediated de novo generation of aggressive CSC pool 

Next, preexisting CSCs, purified from MCF-7 cells, were treated with plasma achievable dose range (1–5 mmol/L; ref. 36) of aspirin. Results of Fig. 5B and C and Supplementary Fig. S6A depicted that 2.5 mmol/L dose of aspirin efficiently downregulated nuclear NfκB as well as Bcl-2, and Cox2, in CSCs by decreasing IkBα phosphorylation level and disrupting NfκB–IL6 inflammatory signaling (Fig. 5D). Incubation of NSCCs with CM of CSCs treated with aspirin prior to FAC treatment decreased the induction of CSC phenotype (Fig. 5E, left) and inhibited the upregulation of chemoresistance markers in newly generated CSCs (Fig. 5F). In contrast, CM of aspirin-treated (i) NKfB-overexpressed or (ii) rIL6-exposed CSCs failed to resist in inducing CSC phenotype in NSCCs (Fig. 5E, left). Aspirin pretreatment was also effective when NSCCs were cocultured with CSCs, as evident by decrease in GFP-CSCs in the mixed population postchemotherapy in comparison with FAC-treated sets (Fig. 5E, right). In accumulation, pretreatment of aspirin disrupted NfκB–IL6 inflammatory feedback loop in preexisting CSCs, thereby
hindering the expansion of chemotherapy-mediated de novo generation of aggressive CSC pool.

Aspirin pretreatment sensitizes preexisting CSCs

Recent report (37) demonstrating that aspirin at its 2.5 mmol/L (physiologically achievable) dose, fails to induce apoptosis in CSCs, prompted us to explore the possibility of its sensitizing CSCs towards chemotherapy. For this ex vivo study, 30 primary human cell lines of luminal histotype, comprising of luminal A and luminal B subtype cells (Supplementary Fig. S1A), derived from untreated patients were included (Supplementary Table S1B). After assessing the in vitro expression levels of chemoresistant markers (Supplementary Fig. S6B), and response to in vitro combinational therapy, 11 were categorized as CR and 19 as CS. ER+/PR+/HER2+ status was often predictive of poor response to in vitro chemotherapy, whereas ER+/PR−/HER2− status showed

Figure 3.
Pre-existing CSCs drive therapy-mediated induction of CSC phenotype in NSCCs. A, flow cytometric analysis of percent CSCs (left), relative mean fluorescence intensities (MFI) of Aldh1 (middle), and Oct-4 (right) in MCF-7 cells under FAC/mitomycin c/mitomycin c-exposed FAC-treated conditions. B, changes in the number of CSCs purified from MCF-7 cells under the specified experimental conditions were determined by employing Trypan blue dye exclusion and flow cytometric assays. C, schematic representation of the experimental protocol (left). Flow cytometric analysis of percent CSCs in primary NSCCs upon FAC treatment (right). D, graphical representation of relative mean fluorescence intensities of ABCG2 and ABCC1 (left) and E-cadherin and vimentin (middle) in untreated and FAC-treated primary NSCCs. Migration of NSCCs purified from untreated and FAC-treated primary NSCCs (right). E, schematic representation of the experimental protocol (top). Flow cytometric analysis of percent CSCs in primary NSCCs upon FAC treatment (bottom). F, illustration showing transwell contact-independent coculture system (top). Percent CSCs (bottom left), relative mean fluorescence intensities of Aldh1 (bottom middle), and Oct-4 (bottom right) in NSCCs of MCF-7 cells under the specified experimental conditions. Data are presented as mean ± SEM or representative of three independent experiments. **P < 0.01 and ***P < 0.001. AU, arbitrary units.
Figure 4.
Chemotherapy triggers NFκB-dependent IL6 inflammatory feedback loop in preexisting CSCs. A, immunohistology (brown color for antibody staining; counterstaining with hematoxylin) for NFκB in pre and posttreatment CS (top) and CR-primary tumor samples (bottom). Scale bar, 100 μm. B, nuclear and cytosolic NFκB in untreated and FAC-treated CSCs and NSCCs of MCF-7 cells (Western blot analysis data, left; quantitative data, right). C, Western blot data of Bcl-2, Cox2, and XIAP in CSCs and NSCCs of pre and posttreatment primary tumors of CR-patients (left) and untreated and FAC-treated MCF-7 cells (right). Right panels of both represent quantitative data of respective Western blots. D, representative RT-PCR data of IL6 mRNA expression in pre and posttreatment primary tumors of CS- and CR-patient (left) and box-and-whisker plot of mean IL6/GAPDH ratio of all pre and posttreatment CS- and CR-tumors (right). E, relative mean fluorescence intensities (MFI) of IL6 levels in FAC-treated MCF-7 cells (left) and fold change of same in different cells upon 3 hours of FAC treatment. F, ELISA of secretory levels of IL6 in spent media of untreated and FAC-treated CSCs and NSCC populations of MCF-7 cells. G, relative levels of IL6 secreted in spent media of untreated and FAC-treated CSCs of MCF-7 cells under the indicated experimental conditions (left). Western blot analysis data (middle) and quantitative data (right) of the same nuclear NFκB in CSCs of MCF-7 cells under the indicated experimental conditions; α-actin/histone H1/GAPDH were internal loading controls. Data are presented as mean ± SEM or representative of three independent experiments. *P < 0.05; **, P < 0.01; and ***, P < 0.001. AU, arbitrary units.
Figure 5.
Aspirin perturbs preexisting CSC-induced NSCC-to-CSC conversion by inhibiting NFκB-dependent IL6. A, diagrammatic representation of the experimental protocol (left). Percent CSCs in NSCCs of MCF-7 cells (middle) and relative mean fluorescence intensities (MFI) of ABCG2 in newly generated CSCs from NSCCs (right) when incubated with conditioned media of the indicated experimental sets. B, Western blot analysis of aspirin-treated CSCs of MCF-7 cells (top) and quantitative data of the same (bottom). C, time-dependent profiles of NFκB in nuclear and cytosolic fraction of CSCs isolated from MCF-7 (top left) and Western blot analysis data of nuclear NFκB, Bcl-2, and Cox2 in primary CSCs (top right) upon treatment with 2.5 mmol/L aspirin. Bottom panels represent quantitative data. D, relative levels of IL6 in spent media of untreated and DMSO/FAC/aspirin/combinatorial therapy–treated CSCs of MCF-7 cells by ELISA. E, percent CSCs in NSCCs of MCF-7 cells incubated with conditioned media of the indicated experimental sets (left) and percent GFP−/CSCs in NSCCs of MCF-7 cells under the specified experimental conditions (right). F, relative mean fluorescence intensities of ABCG2 in newly generated CSCs from NSCCs when incubated with conditioned media of the indicated experimental sets; α-actin/histone H1 were internal loading controls. Data are presented as mean ± SEM or representative of three independent experiments. *p < 0.01 and **p < 0.001. AU, arbitrary units.
comparatively better response to treatment (Supplementary Table S1B). Thereafter, CSCs purified from CS- and CR-primary human cell lines and from different breast cancer cell lines, with almost similar characteristics as that of primary cell lines, were exposed to aspirin treatment prior to FAC. Supporting our hypothesis, results of aspirin-preexposed CSCs showed significant increase in Annexin V positivity upon FAC treatment while aspirin alone failed to do so (Fig. 6A and 6B, top and Supplementary Fig. S6C), indicating that aspirin sensitized CSCs towards chemotherapy regimen. Such combinatorial therapy also decreased CSC content even below the level present in their untreated counterparts (Fig. 6A and 6B, bottom and Supplementary Fig. S6D). Further analysis with MCF-7 cells revealed that such sensitization of preexisting CSCs resulted from significant decrease in the expression levels of chemoresistance markers (Fig. 6C). Interestingly, aspirin-induced decrease in chemoresistance of preexisting CSCs was inhibited by rIL6 or upon NFκB overexpression (Fig. 6D). ChIP assay further showed that although FAC treatment increased NFκB recruitment on the promoter regions of ABCG2 and ABCC1 in CSCs (Fig. 6E), such bindings were decreased in aspirin-pretreated sets. These results signified that aspirin pretreatment decreased the expression of drug efflux pumps, thereby sensitizing preexisting CSCs to genotoxic therapy.

All these results unveiled the hitherto unexplored role of aspirin in hindering the acquisition of aggressive phenotype by luminal histotype breast cancer cells during the course of therapy by (i) perturbing preexisting CSC-assisted de novo conversion of NSCCs to aggressive CSCs and (ii) sensitizing inherently chemoresistant preexisting CSCs. These findings not only identified preexisting CSCs as the probable candidates escalating therapy-induced resistance but also provided a new strategy to inhibit therapy-acquired resistance by aspirin in breast cancer stem cells.

Figure 6. Aspirin chemosensitizes preexisting CSCs towards genotoxic therapy. Graphical representation of percent apoptosis (top) and percent CSCs (bottom) of CS- and CR-patients (A) and different cells (B) under the indicated experimental conditions. C, relative mean fluorescence intensities (MFI) of ABCG2 and ABCC1 in preexisting untreated and DMSO/FAC/aspirin/combinatorial therapy–exposed CSCs of MCF-7 cells (left). The mRNA expression profiles of ABCG2 and ABCC1 in CSCs of MCF-7 cells under the indicated experimental conditions (middle) and quantitative data of the same (right). D, relative mean fluorescence intensities of ABCG2 in preexisting CSCs under the indicated experimental sets. E, pictorial representation of specific NFκB binding sites on ABCG2 and ABCC1 (left). ChIP assay of untreated and FAC/aspirin/combinatorial therapy–treated CSCs of MCF-7 cells (middle) and quantitative data of the same (right). Histone H1 was used as an internal loading control. Data are presented as mean ± SEM or representative of three independent experiments. **, P < 0.01; and ***, P < 0.001. AU, arbitrary units. IP, immunoprecipitation.
recurrence in breast cancer patients but also raised the possibility of combining aspirin with genotoxic therapies to efficiently improve relapse-free survival in NACT-treated breast cancer patients.

**Discussion**

Resistance of breast tumors toward therapy is one of the major causes of the incurability of the disease. In addition to being inherently resistant to therapies during the course of chemotherapeutic treatments, tumors also acquire resistance towards such therapies. The consequences are 2-fold; first, the tumors acquire resistance with progressive treatment and second, provide impetus to the growth of recurrent tumors (1–4). CSC subpopulation of the tumor has been held responsible for both therapy resistance and development of tumor recurrences (5–7, 26, 38). Therefore, the resistance of CSCs to chemotherapy/radiotherapy might be a plausible mechanism to explain breast cancer recurrence.

In keeping with these reports, we observed that acquired chemoresistance-resistant patients are at a greater risk of developing tumor recurrence than chemotherapy-sensitive patients. An explanation for this observation is partly provided by existing reports that NACT leads to enrichment of CSCs. This observation is partly provided by existing reports of the tumor has been held responsible for both therapy resistance and development of tumor recurrences. In this preclinical study, we identified that in patient samples and cell lines of luminal histotype, NACT treatment results in agmation of aggressive CSC (15, 40, 41), which besides impeding therapeutic outcome, also helps them to hide in distant sites to escape surgery, thus providing an unintended advantage of recurrence.

Next, we sought to explore the mechanisms responsible for the enrichment of CSCs following NACT. Several studies have demonstrated that therapeutic intervention may contribute to CSC genesis. In addition to earlier reports that suggest the CSC enrichment following NACT results from selection as the NSCCs naturally succumb to these anticancer therapies, we found conversion of nonstem breast cancer population to CSCs, which also concurrently acquire resistance and aggressive properties. These findings are further supported by earlier reports describing the spontaneous conversion of nonstem tumor cells to CSCs (15, 40, 41).

An in-depth analysis to divulge the mechanisms responsible for NACT-induced conversion of NSCCs-to-CSCs revealed that this effect was brought about through paracrine secretion of IL6, NFκB downstream effector (13–15). Lopoulous and colleagues have shown that the spent media from CSCs promote non-CSC-to-CSC conversion in IL6-dependent manner. Interestingly, in many types of cancer, elevated level of serum IL6 was found (42), and the same was strongly correlated with poor overall survival and accelerated disease progression (43).

Interestingly, NFκB–IL6 signaling has been implicated in the self-renewal, chemoresistance, and tumorigenesis (14, 44–47). The involvement of NFκB–IL6 signaling in our *ex vivo* and *in vitro* models prompted us to evaluate the role of aspirin in abrogating this pathway to chemosensitize resistant breast tumors. In our study, physiologic doses of aspirin (36) efficiently perturbed nuclear translocation of NFκB, resulting in the abrogation of NFκB–IL6 feedback loop culminating in the inhibition of paracrine induction of NSCC-to-CSC conversion and also perturbing the gain of chemoresistance and migration potential. Moreover, aspirin potentially chemosensitized preexisting CSC pool by perturbing NFκB-mediated expression of chemoresistance promoting factors. Our study, therefore, signifies the possibility of a combination strategy that induces apoptosis in preexisting CSCs and NSCCs after FAC administration. This approach was further justified by a recent study by Maity and colleagues (37), where aspirin treatment prevented tumor growth in nude mice xenograft model by reducing the self-renewal capacity and growth of breast CSCs but failed to induce apoptosis by itself even in inherently resistant CSCs at physiologically achievable dose (37). Taken together, our study thus provides a scientific rationale for combining aspirin with standard chemotherapy for successful targeting of both CSC and NSCC pool to restrain the acquisition of aggressive phenotype by breast cancer cells during the course of chemotherapy. These data corroborated previous clinical studies with breast cancer patients, in which a link between aspirin use and lower incidences of cancer initiation, recurrence, and metastasis, the three attributes associated with CSCs, has been found (16, 17, 48, 49).

In conclusion, tumor relapse is a common and devastating phenomenon in clinical oncology following initial success with cytotoxic therapies. We provide evidence that aspirin-mediated inhibition of NFκB–IL6 inflammatory signaling in preexisting CSC meets the criteria for the development of potential treatment strategies by sensitizing preexisting CSCs in one hand and inhibiting NSCC-to-CSC conversion on the other. Moreover, controlling chemoresistance of CSCs and the generation of new CSCs during chemotherapy treatment may ultimately improve curability and allow deescalation of currently given chemotherapeutic dose, thereby reducing acute and long-term adverse effects. Therefore, cotreatment of aspirin and chemotherapeutic regimen can be a potentially safe and attractive treatment strategy to tackle cancer in a multipronged approach, targeting both CSC and NSCC populations.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S. Saha, T. Das

Development of methodology: S. Saha, S. Mukherjee, T. Das

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Saha, P. Khan, K. Kajal, M. Mazumdar, A. Manna

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Saha, S. Mukherjee, S. Mukherjee, D. K. Sarkar, T. Das

Writing, review, and/or revision of the manuscript: S. Saha, S. Mukherjee, T. Das

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Mukherjee, S. De, D. Jana, D. K. Sarkar

Study supervision: T. Das

**Acknowledgments**

The authors thank R. Dutta for technical help related to flow cytometry.

**Grant Support**

Grants were received from Council of Scientific and Industrial Research (S. Saha, S. Mukherjee, and P. Khan), Department of Science and Technology (K. Kajal, A. Manna, and T. Das), and Department of Biotechnology (M. Mazumdar), India.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 27, 2015; revised October 24, 2015; accepted December 28, 2015; published OnlineFirst February 3, 2016.
Aspirin Sensitizes Breast Cancer Stem Cells

References

18. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE. \( \text{References} \)
Aspirin Suppresses the Acquisition of Chemoresistance in Breast Cancer by Disrupting an NFκB–IL6 Signaling Axis Responsible for the Generation of Cancer Stem Cells

Shilpi Saha, Shravanti Mukherjee, Poulami Khan, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-15-1360

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2017/06/06/0008-5472.CAN-15-1360.DC1

Cited articles
This article cites 45 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/76/7/2000.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/76/7/2000.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.