Resistin potentiates chemoresistance and stemness of breast cancer cells: implications for racially disparate therapeutic outcomes

Sachin K. Deshmukh[1], **Sanjeev K. Srivastava**[1], **Haseeb Zubair**[1], **Arun Bhardwaj**[1], **Nikhil Tyagi**[1], **Ahmed Al-Ghadhban**[1], **Ajay P. Singh**[1,2], **Donna L. Dyess**[1], **James E. Carterand**[3], and **Seema Singh**[1,2]

[1]Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, Alabama, USA

[2]Department of Biochemistry and Molecular Biology, College of Medicine, University of South Alabama, Mobile, Alabama, USA

[3]Department of Pathology, College of Medicine, University of South Alabama, Mobile, Alabama, USA

Abstract

Breast cancer (BC) continues to be the most frequently diagnosed cancer in American women, which disproportionately affects women of African-American (AA) descent. Previously, we reported greater serum levels of resistin in AA BC patients relative to Caucasian-American (CA) patients, and established its role in growth and aggressiveness of breast tumor cells. Here we have investigated the role of resistin in BC-chemoresistance. MDA-MB-231 and MDA-MB-468 BC cells of CA and AA origin, respectively, were incubated with resistin prior to doxorubicin treatment. Our data suggest that resistin conferred chemoresistance to both BC cell lines; however, the effect on AA cells was more profound. Furthermore, the resistin-induced doxorubicin-resistance was shown to occur due to suppression of apoptosis. Resistin treatment also affected the stemness of BC cells, as suggested by reduced cell surface expression of CD24, induced expression of CD44 and ALDH1, and increased capability of cells to form mammospheres. Mechanistic studies revealed that resistin-induced chemoresistance, apoptosis and stemness of BC cells were mediated through STAT3 activation. Taken together, our findings provide novel insight into the role of resistin in BC biology, and strengthen its role in racially disparate clinical outcomes.

To whom correspondence should be addressed: Seema Singh, Ph.D., Mitchell Cancer Institute, University of South Alabama, 1660 Springhill Avenue, Mobile, AL 36604-1405, U.S.A., Phone: 251-445-9844, Fax: 251-460-6994, seemasingh@health.southalabama.edu.

Keywords

Racial disparity; breast cancer; resistin; chemoresistance; stemness; STAT3

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in women in the United States [1]. According to the American Cancer Society, approximately 252,710 women will be diagnosed with BC, and nearly 40,610 will die with this malignancy in 2017 [1]. More importantly, it disproportionately affects the women of African American (AA) descent with early onset and occurrence of more aggressive disease, poor therapeutic outcome, and a higher risk of recurrence, as compared to Caucasian-American (CA) women [2]. According to Surveillance, Epidemiology and End Results (SEER) 2013 data, despite low incidence rate of BC in AA women, compared to CA women (124.3 vs. 128.1, per 100,000, respectively), AA women face high death rates (28.19 vs. 20.26, per 100,000, respectively), contributing to BC disparity ratio of 39.14% [3]. These data suggest that BC is far more aggressive in AA women. Indeed, highly aggressive triple-negative breast cancer (TNBC), characterized by the absence of ER, progesterone receptor (PR) or HER2, is much more prevalent in AA women [4]. TNBC suffers from lack of targeted therapy and chemotherapy remains the only option for disease management [5–7]. Doxorubicin (adriamycin), a conventional anthracycline, has been an important mainstay in the treatment of BC [8]. However, its efficacy for BC treatment is hindered by inherent or acquired chemoresistance leading to poor clinical outcome [9]. Several mechanisms have been proposed for chemoresistance of BC, which may involve both intrinsic and extrinsic factors [10, 11]. Tumor microenvironment (TME) is comprised of a heterogeneous mixture of many different cells that produce and secrete a number of growth factors and cytokines [12, 13]. Interestingly, a recent study suggested the existence of disparate TME in AA vs. CA breast tumors [13–15]. Emerging data have provided convincing evidence for an important role of TME in cancer chemoresistance, which involves both direct and indirect mechanisms [16, 17]. TME may not only impact drug delivery to the tumor cells, but can also bestow tumor cells with chemoresistant phenotypes by altering their cell signaling [18, 19].

Resistin, initially described as an adipocyte-derived cytokine, has been studied extensively for its role in inflammation and obesity-related cancers [20, 21]. Several studies have shown that resistin is elevated in BC patients, and its elevated levels are associated with increased risk of BC [22–24]. In addition, the levels of resistin correlate with tumor stage, size and metastasis [25]. Importantly, expression of resistin was also found to affect the overall clinical outcome [25]. Further, we and others have reported increased levels of resistin in AA BC patients relative to those in Caucasian patients, and resistin is also shown to promote breast tumor growth and aggressiveness [13, 14, 26, 27]. Interestingly, elevated serum levels of resistin have also been reported in chemotherapy-treated BC patients [28]. In light of these observations, we attempted to investigate the role of resistin in chemoresistance of BC cells. TNBC cell lines of AA and CA origin (MDA-MB-468 and MDA-MB-231, respectively) were used to evaluate the effect of resistin on doxorubicin-sensitivity. Our data

suggest that resistin promotes doxorubicin-resistance in BC cells by suppressing doxorubicin-induced apoptosis. Furthermore, resistin confers stem cell-like characteristics to BC cells. From the mechanistic standpoint, our data support an essential role of STAT3 activation in resistin-induced survival and stemness of BC cells. Altogether, our study suggests a novel role of resistin in BC chemoresistance, which could contribute, at least in part, toward a racially disparate therapeutic outcome in BC patients.

2. Material and methods

2.1 Cell culture

Human breast cancer (BC) cell lines, MDA-MB-231 and MDA-MB-468, were purchased from ATCC (Manassas, VA). Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences, Logan, Utah) supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5 % CO2 at 37 °C. Cells were intermittently tested for mycoplasma contamination at our institutional flow cytometry core facility. Cells were routinely monitored for their typical morphology, and authenticated by in-house short-tandem repeats genotyping.

2.2 Antibodies and siRNAs

Antibodies used were: anti-cleaved caspase 7, -cleaved caspase 3 and -cleaved PARP1 (rabbit polyclonal), anti-BCL2, -BCL-xL, -KLF4, -Nanog (rabbit monoclonal) (Cell Signaling Technology, Danvers, MA); Anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); Anti-human fluorochrome-conjugated antibodies against CD24 (Alexa Fluor-647 conjugated), CD44 (Brilliant violent-421 conjugated) (BioLegend, San Diego, CA) and β-actin (mouse monoclonal) antibody (Sigma-Aldrich, St. Louis, MO). Non-targeting scrambled siRNAs (NT-Scr), or STAT3 targeting siRNAs were purchased from GE Dharmacon (Lafayette, CO).

2.3 Treatments and transfection

MDA-MB-231 and MDA-MB-468 cells were seeded in 96 or 6 -well plates and treated with PBS or resistin (20 ng/ml) (Phoenix Pharma, Burlingame, CA) alone or in the presence of doxorubicin (0–2.0 μM), as mentioned in the pertinent figure legends. To examine the involvement of STAT3 in resistin-induced effects, BC cells were transfected with NT-Scr or STAT3-targeting siRNA (30 nM) and further treated with resistin and/or doxorubicin, and effects on cell viability, sphere formation, and associated proteins were examined.

2.4 Cell viability assay

Cells (1×10^4) were grown in 96-well plates and treated with PBS or resistin (20 ng/ml) for 12 h. After treatment, cells were further treated with doxorubicin (0–2.0 μM) in presence or absence of resistin for 72 h, and cell viability was calculated using WST-1 assay kit (Roche, Indianapolis, IN), as described earlier [29, 30].

2.5 Plating efficiency assay

Plating efficiency assay was performed to examine the effect of resistin on chemoresistance in the long term. For this, cells (5×10^2) were seeded in 6-well plates and cultured for 48 h. Subsequently, cells were treated with PBS or resistin (20 ng/ml) for 12 h before doxorubicin (0–1.0 μM) treatment and grown under normal culture conditions. Resistin was supplemented every 48 h during the culture. After two weeks, colonies were stained with crystal violet, photographed and counted using Image analysis software (Gene Tools, Syngene, Frederick, MD).

2.6 RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen), and reverse-transcribed with high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), as described earlier [31]. Subsequently, qRT-PCR was performed in 96-well plates using cDNA as a template and SYBR Green Master Mix on an iCycler system (Bio-Rad, Hercules, CA, USA) with specific primer pair sets. GAPDH served as an internal control. (Table S1). Following thermal conditions were used: cycle 1: 95 °C for 10 min, cycle 2 (40): 95 °C for 10 sec and 58 °C for 45 sec.

2.7 Immunoblot analysis

Immunoblotting was performed as previously described [13, 31]. Briefly, protein samples were resolved on 10 % polyacrylamide gels, transferred to PVDF membrane, and subjected to immunodetection using specific antibodies. Bands were visualized using ECL plus Western Blotting substrate kit (Thermo Scientific, Logan, UT) with an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

2.8 Measurement of apoptosis

Resistin or vehicle pre- treated (for 12 h) BC cells (1×10^6 cells/well) were exposed to 0.1 μM doxorubicin in presence or absence of resistin (20 ng/ml) for 72 h. After that, cells were stained with Annexin V APC and SYTOX Green (Invitrogen) and analyzed by flow cytometry, according to manufacturer's protocol.

2.9 Detection of CD24⁺ and CD44⁺ cells

MDA-MB-231 and MDA-MB-468 cells were treated with PBS or resistin (20 ng/ml) for 48 h. Post-incubation, cells were washed and suspended in PBS supplemented with 2 % FBS. Subsequently, cells (1×10^6/ml) were incubated with fluorochrome-conjugated anti-CD24 (Alexa Fluor-647), and CD44 antibodies (Brilliant violent-421) for 15 min on ice. After incubation, cells were washed with PBS and subjected to flow cytometry analysis using FACS AriaII™ (BD Bioscience). 7-Aminoactinomycin D (7-AAD; BD Bioscience) was added to exclude non-viable cells before FACS analysis.

2.10 ALDEFLUOR assay

ALDEFLUOR assay was performed using ALDEFLOUR™ kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, cells ($2.5 \times$

10^5) were incubated in ALDH1 assay buffer containing ALDH1 substrate (Bodipy Aminoacetaldehyde; BAAA) at 37 °C for 45 min with intermittent mixing. After incubation, cells were centrifuged at 250 g for 10 min, suspended in 0.5 ml of ALDH1 assay buffer and analyzed using FACS AriaII™. ALDH1 enzyme inhibitor [diethylaminobenzaldehyde (DEAB)] treated cells were used as a control. 7-AAD was added to the cell suspension before FACS analysis for the exclusion of dead cells.

2.11 Sphere formation assay

For sphere formation, MDA-MB-231 and MDA-MB-468 cells (1×10^3/well) were cultured in 6-well Ultra-Low attachment plates (Corning Incorporated, Corning, NY) in stem cell culture medium (DMEM:F-12K, 1:1; Life Technologies, Carlsbad, CA) containing penicillin (100 units/mL), streptomycin (100 μg/mL) and supplemented with basic fibroblast growth factor (bFGF; 20 ng/ml), epidermal growth factor (EGF; 20 ng/ml) (Life Technologies) and resistin (20 ng/ml) or vehicle every alternate day. After 15 days, the mammospheres were counted and photographed using phase contrast microscope, as described previously [32].

2.12 Statistical analysis

All the experiments were performed at least three times, and data are expressed as mean ± SD. Wherever suitable, the data were also subjected to unpaired two-tailed Student's t-test and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Resistin confers doxorubicin-resistance in breast cancer cells

Earlier, we have shown that elevated serum levels of resistin, particularly in AA BC patients, correlate with enhanced growth and aggressiveness of BC cell lines [13]. Also, recently, it was demonstrated that BC patients, who received chemotherapy, had significantly enhanced levels of serum resistin [28]. We, therefore, hypothesized that increased levels of resistin might confer resistance of BC cells against therapy-induced cell death. To test this, we pre-treated BC cell lines of AA (MDA-MB-468) and CA (MDA-MB-231) origin, with (20 ng/ml) resistin or vehicle (water) for 12 h, and then exposed them to increasing doses of doxorubicin (0–2 μM) in presence or absence of resistin for additional 72 h. Effect on cell viability was examined using WST-1 assay. Our data reveal that doxorubicin treatment resulted in a significant cell death in both MDA-MB-468 and MDA-MB-231 BC cells. Notably, resistin treatment significantly protected BC cells from doxorubicin-induced cell death (Fig. 1A). As indicated in Table 1, upon treatment with resistin, the IC_{50} values for doxorubicin significantly increased from 0.12 and 0.17 μM in MDA-MB-231 and MDA-MB-468 BC cells to 0.65 and 1.05 μM, respectively, indicating resistin-mediated resistance to doxorubicin.

Further, we analyzed the effect of resistin on doxorubicin resistance in BC cells by plating efficiency (an ideal test to monitor growth in long-term). Our data show a significant chemoprotective effect of resistin on both the cell lines (Fig. 1B). Importantly, no visible colony was detected in vehicle-treated MDA-MB-231 and MDA-MB-468 cells at 0.5 and

1.0 μM doxorubicin doses. However, a remarkably higher number of colonies were observed in both the BC cell lines with resistin and doxorubicin (0.5 and 1.0 μM dose) (Fig. 1B). Interestingly, a considerably greater resistin-induced doxorubicin resistance was observed in the cell line of AA origin (MDA-MB-468), as compared to MDA-MB-231 cells (CA origin). Altogether, our data thus suggest that enhanced resistin level may be one of the key mechanisms underlying high chemoresistance of this tumor type, especially in patients of AA origin.

3.2 Resistin-protects breast cancer cells from doxorubicin-induced apoptosis

Since induction of apoptotic cell death is a major mechanism of chemotherapeutic drugs [33, 34], we were interested to see if resistin has any effect on doxorubicin-induced apoptosis. For this, resistin pre-treated MDA-MB-231 and MDA-MB-468 cells were treated with doxorubicin alone or in combination with resistin and, the extent of apoptosis was monitored by Annexin V staining. We observed a lower apoptotic index (Annexin V positive/SYTOX negative cells) in BC cells treated with a combination of resistin and doxorubicin, as compared with doxorubicin treatment alone. Data shows 79.9 % and 43.6 % apoptotic cells in MDA-MB-231 and MDA-MB-468 cells, respectively, that were treated with doxorubicin only (Fig. 2A). Importantly, the doxorubicin-induced apoptosis was significantly abolished following co-treatment with resistin, as only 33.0 % (MDA-MB-231 cells) and 29.2 % (MDA-MB-468 cells) apoptosis was observed in the BC cells treated with doxorubicin along with resistin (Fig. 2A).

Activation of caspases (caspase 3 and 7) and subsequent activation of PARP-1, a known cellular substrate of caspases, is considered a hallmark of apoptotic cell-death [35, 36]. We, therefore, examined the effect of resistin and/or doxorubicin on these apoptosis effector molecules. In concordance to our flow-cytometry data, doxorubicin treatment resulted in the enhanced cleavage of caspases-3 and -7, and PARP-1, in both BC cells (Fig. 2B), an effect that was abrogated upon resistin-treatment (Fig. 2B). To study the underlying molecular mechanism, we further analyzed the expression of survival related proteins (BCL2 and BCL-xL) in the above mentioned treatment groups, by qRT-PCR and immunoblot assay. We observed a drastic reduction in the expression of anti-apoptotic proteins BCL2 and BCL-xL, following doxorubicin treatment, at both mRNA (data not shown) and protein levels (Fig. 2C). Interestingly, doxorubicin-mediated downregulation of BCL2 and BCL-xL was abolished in BC cells upon resistin treatment (Fig. 2C). Our findings thus clearly suggest a significant protection of BC cells by of resistin against doxorubicin-induced apoptosis.

3.3 Resistin induces stemness in breast cancer cells

The role of cancer stem cells in chemoresistance is well established [37]. Therefore, we investigated the significance of resistin in stemness potential of BC cells. CD24 (low) and CD44 and ALDH1 (high) expression/activities are well-characterized markers of BC stem cells (BCSCs). We, therefore, first examined the effect of resistin on the expression of stemness-associated markers in BC cells by quantitative RT-PCR and flow-cytometry. Our data reveal that resistin treatment enhanced the expression of CD44 and ALDH1 in MDA-MB-231 and MDA-MB-468 cells at mRNA level, with a concomitant decrease in the CD24 expression, as compared to respective vehicle-treated cells (Fig. 3A). Further, we stained the

BC cells for CD24 and CD44, using Alexa Fluor-647 and Brilliant violent-421 antibodies, respectively, and performed flow-cytometric analysis. Our data indicate that the individual geometric mean fluorescence intensity (MFI) of CD44 was increased in resistin-treated MDA-MB-231 (1.1 fold) and MDA-MB-468 (3.2 fold) cells, as compared to vehicle-treated cells (Fig. 3B). In contrast, reduction in MFI for CD24 (1.1 and 2.0 folds, respectively) was observed upon treatment with resistin in both MDA-MB-231 and MDA-MB-468 cells. BCSCs are enriched with ALDH1 [38], and our data show that the number of ALDH1 positive cells increased from 29.8 % to 37.4 % in the MDA-MB-468 cells upon resistin treatment (Fig. 3C), while opposite effect on ALDH1 activity was seen in MDA-MB-231 cells upon resistin treatment.

Further, to identify if these changes in surface markers also translate into functional alterations, we tested the ability of these BC cell lines to generate mammospheres upon resistin treatment. For this, cells were seeded at low density in ultra-low attachment plate and supplemented with resistin and growth factors every alternate day. The cells were allowed to grow in stem cell culture medium for 15 days, and the number of spheres formed was counted. Data demonstrate greater sphere forming ability (~2.0 fold) in MDA-MB-231 cells treated with resistin, as compared to the control cells (Fig. 4A). Notably, the effect of resistin on the sphere formation was more potent (~2.8 fold) in BC cells of AA origin i.e. MDA-MB-468 (Fig. 4A). Furthermore, we also examined the effect of resistin on the expression of stemness-associated transcription factors (Nanog and KLF4) at mRNA and protein levels. Our data exhibit significant induction in the expression of both Nanog and KLF4 in MDA-MB-231 as well as MDA-MB-468 cells, at both mRNA (Fig. 4B) and protein levels (Fig. 4C). Taken together, our data show that resistin potentiates stemness in BC cells.

3.4 STAT3 signaling mediates resistin-induced drug resistance and stemness in breast cancer cells

In our earlier study [13], we demonstrated that resistin enhances the expression and phosphorylation of STAT3 that mediates resistin-induced growth and aggressiveness of BC cells. Furthermore, studies document the critical role of STAT3 in proliferation and self-renewal of CSCs and the promotion of therapy-resistant phenotype in several cancer types, including BC [39]. Therefore, we sought to examine whether STAT3 is mechanistically involved in resistin-induced drug resistance and stemness. For this, BC cells were transfected with non-target scrambled (NT-Scr) or STAT3-specific siRNA, and silencing was confirmed by immunoblot assay. STAT3 siRNAs efficiently silenced its expression (basal as well as resistin-induced) by 24 h, an effect that sustained at least up to 72 h post-transfection (data not shown). STAT3-silenced BC cells were pre-treated with resistin or vehicle for 12 h and further treated with increasing doses of doxorubicin, and the effect on cell viability was examined. Data show that resistin protects BC cells, that were transfected with NT-Scr, from doxorubicin-induced cytotoxicity (Fig. 5A and Table 2), while no chemoprotective effect of resistin is seen in both MDA-MB-231 and MDA-MB-468 cells transfected with STAT3-specific siRNAs (Fig. 5A and Table 2).

Moreover, we also analyzed the role of STAT3 in resistin-induced stemness potential of BC cells. Data demonstrate that silencing of STAT3 significantly diminished the sphere forming ability of BC cells (Fig. 5B). Notably, when STAT3-silenced BC cells were treated with resistin, no induction in the sphere formation was observed (Fig. 5B). Also, the effect of STAT3 silencing on the resistin-induced expression of survival (BCL2 and BCL-xL) and stemness (Nanog and KLF4)-associated proteins was examined (Fig. 5C). Our immunoblot data showed that silencing of STAT3 efficiently blocks the resistin-induced expression of anti-apoptotic (BCL2 and BCL-xL) (Fig. 5C, upper panel), and stemness-associated transcription factors (Nanog and KLF4) (Fig. 5C, lower panel) in both the BC cell lines. Taken together, our data suggest that STAT3 mediates resistin-induced chemoresistance and stemness in the BC cells by regulating the expression of associated proteins.

4. Discussion

Elevated levels of resistin have been reported in several types of malignancies, including BC [13, 40, 41]. Further, the levels of resistin correlate with breast tumor size, stage and lymph node metastasis [25]. Expression of resistin also affects disease-free as well as overall survival rates in BC patients [25]. Studies also report a role of resistin in the neovascularization process [42]. Our earlier work demonstrated a significantly disparate overexpression of resistin in AA BC serum, as compared to CA patients, in addition to its role in growth and aggressiveness of BC cell lines [13]. Interestingly, treatment of BC patients with chemotherapeutic drugs has been shown to result in increased expression of resistin [28]. One of the known side-effects of anthracycline-based therapy, such as doxorubicin, is the associated cardiotoxicity. Interestingly, a role of resistin in doxorubicin-induced cardiotoxicity has been reported in a transgenic mouse model [43]. Doxorubicin treatment was shown to result in four-fold induction of resistin in mice. The study also evaluated resistin levels in BC patients undergoing anthracycline-containing chemotherapy, and found significantly elevated resistin levels in such patients [43]. These studies and our own findings, presented herein, establish resistin, as a key determinant of BC therapeutic response. While our study provides first evidence for a role of resistin in cancer chemoresistance, its role in insulin resistance is well characterized [44]. Incidentally, insulin resistance is also associated with high risk of human cancers [45], including BC [46].

Development of drug-resistance often compromises the clinical efficacy of chemotherapeutic drugs/regimens. A number of possible mechanisms have been suggested for both primary and acquired chemoresistance, and these include enhanced DNA repair, overexpression of drug efflux pump related genes and induction of survival pathways [47]. Cancer cells escape apoptosis by activating several signaling pathways and stemness-associated factors, resulting in reduced sensitivity to doxorubicin. Here, we report the effects of resistin on doxorubicin resistance, protection against doxorubicin-induced apoptosis and enhanced stemness potential of BC cells. Apoptosis is a tightly regulated and highly complex process of cell death that has a critical role in cell growth and tissue homeostasis [48]. Tumor cells have inherent ability to resist apoptosis, which has also been associated with chemoresistance [49–51]. In this study, we observed that resistin conferred doxorubicin resistance through suppression of apoptosis. Another interesting finding from our study was that we see a significantly greater effect of resistin in imparting chemoresistance in the MDA-MB-468 BC

cells, which are of AA racial background as compared to MDA-MB-231 cells of CA origin. These findings are significant, and are supported by our earlier observation that BC cells of AA origin exhibit greater expression of CAP1 (receptor for resistin) [13]. Therefore, it appears that differential expression of CAP1 by tumor cells, along with disparate levels of resistin, may serve as determinant of racially disparate therapeutic outcome.

Chemoresistance in BC is also suggested to be due to stem cells phenotype acquired by the tumor cells [52–54]. Calcagno et al. reported that long-term continuous culture of MCF7 cells resulted in doxorubicin resistant MCF7/ADR cells that exhibited cancer stem cell-like population and contained a larger CD44+/CD24− population. Moreover, these cells were more invasive than MCF7 cells *in vivo* [55]. Furthermore, it has been suggested that BC cells with $CD44^{high}/CD24^{low}/ALDH^{high}$ phenotype exhibit greater self-renewal capacity and tumorigenicity, compared to cells that are $CD44^{low}/CD24^{high}/ALDH^{low}$ [56–58]. Wang et al. characterized the $CD44^{+}CD24^{−}$ stem cells derived from BC cells, which rapidly formed mammospheres and had potent tumorigenicity *in vivo* [59]. Therefore, therapeutic targeting of factors that are associated with BCSCs and therapy resistance would have profound clinical implications for better management of this disease. A direct induction of stemness associated markers ($CD44^{high}$ and $CD24^{low}$) by resistin, as reported here, provides a rationale for targeting of resistin in an attempt to control BCSCs. It is known that the therapeutic resistance of cancer cells is largely attributed to self-renewing subpopulations of CSCs present in the bulk tumor, and enhanced sphere-forming potential is one of the important characteristics of CSCs. Data from our study demonstrate that resistin enhanced sphere forming capacity of BC cells.

In our earlier study, we have established that STAT3 is a critical mediator of resistin-induced growth and aggressiveness of BC cells. STAT3 is constitutively activated in numerous cancer types, including BC [60, 61]. Further, BC cells expressing activated STAT3 exhibit poorer therapeutic response to neo-adjuvant chemotherapy [61], and a role of STAT3 in the maintenance of BC stemness has also been well explored [39, 62]. Guha et al. demonstrated a critical role of STAT3 in apoptosis resistance and development and maintenance of CSC characteristics in breast cancer cells [63]. In other cancers, such as pancreatic cancer, STAT3 has been shown to be important in maintaining stem cell phenotypes [32]. In addition to maintaining the stemness phenotypes, activation of STAT3 is also linked to the conversion of non-CSCs to CSCs [64]. Our data from the present study clearly demonstrates STAT3 to be a crucial mediator of resistin induced chemoresistance and stemness potential (Fig. 5D).

In conclusion, our study establishes a role of resistin in doxorubicin resistance and BC stemness, an effect that is mediated through STAT3 activation. Thus, selective targeting of resistin could be of great significance in BC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Resistin confers doxorubicin-resistance in breast cancer cells

- Effect of resistin on doxorubicin-resistance is due to suppression of apoptosis

- Resistin promotes stemness in breast cancer cells

- STAT3 mediates resistin-induced drug resistance and stemness in breast cancer cells
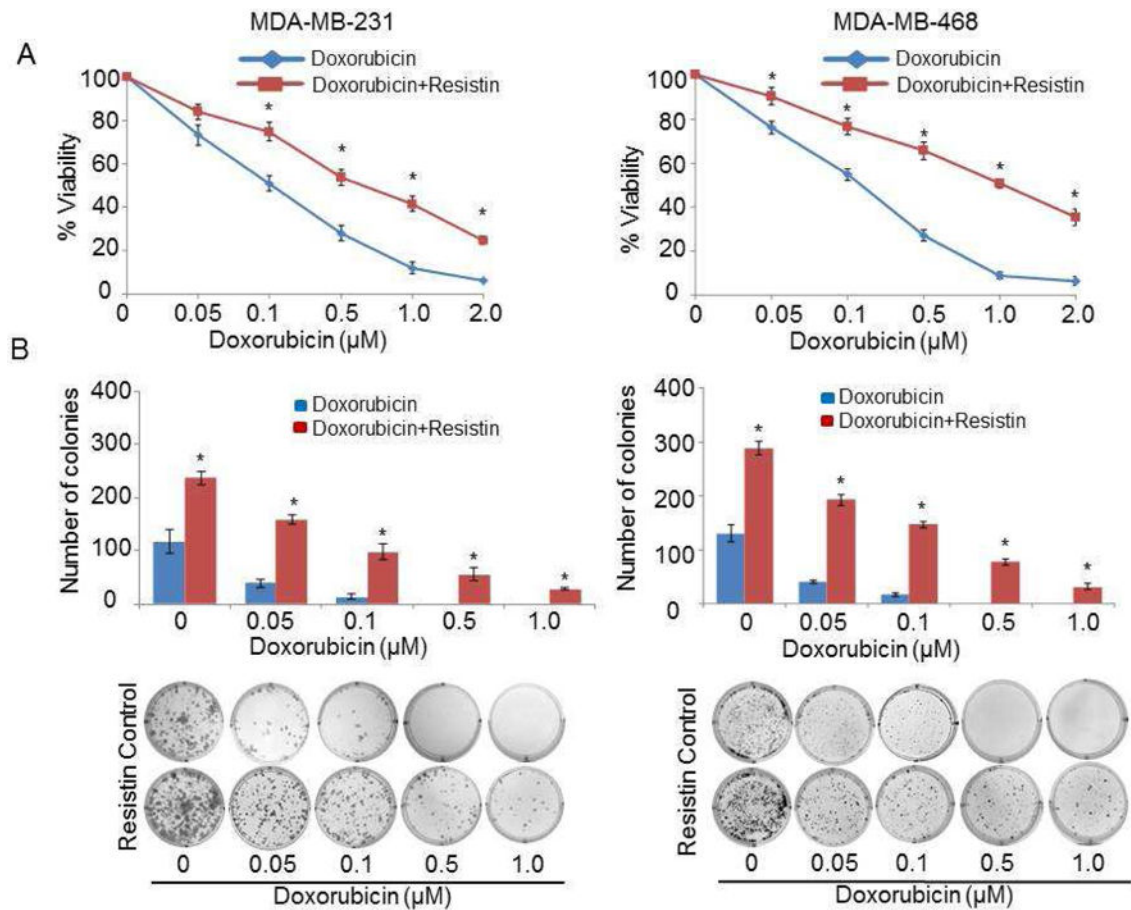
Figure 1. Resistin promotes doxorubicin resistance in breast cancer cells

(A) MDA-MB-231 and MDA-MB-468 cells pretreated with resistin (20 ng/ml) for 12 h were further treated with indicated doses of doxorubicin (0–2.0 μM) for 72 h in the presence of resistin, and cell viability was determined by WST-1 assay. Cells untreated with resistin served as control. OD value of untreated cells was taken as 100 % viable. Data represent the mean ± SD; n = 3; *p < 0.01. **(B)** Cells were seeded (500 cells/well) in 6 well plates and allowed to adhere and establish for 48 h. Subsequently, cells were treated with resistin (20 ng/ml) for 12 h and then with varying doses of doxorubicin. Resistin was then supplemented to the media after every 48 h. After two weeks, colonies were stained with crystal violet and visualized and photographed using imaging system. Bars represent mean ± S.D. n=3, *p < 0.01. Representative images of colonies, formed in 2 weeks after treatment with increasing concentrations of doxorubicin in presence or absence of resistin, are shown at the bottom.
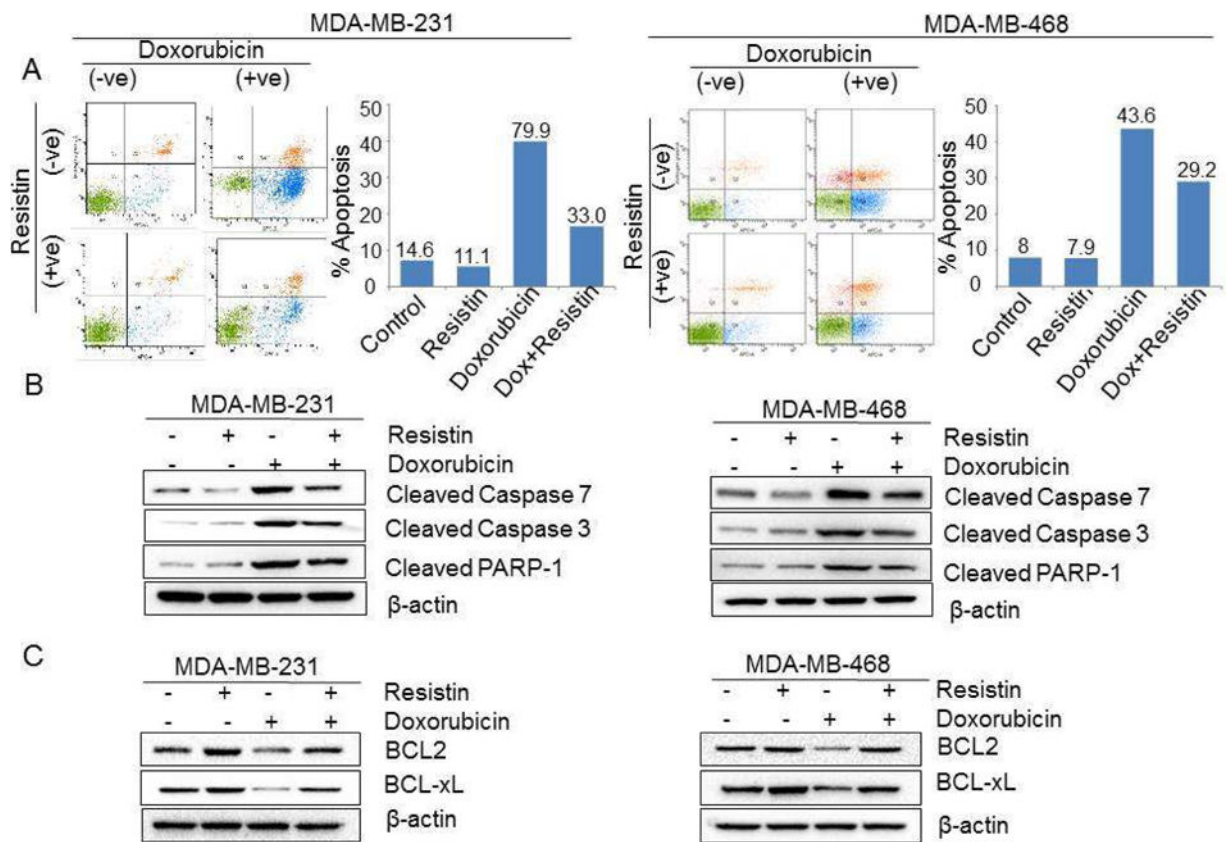
Figure 2. Resistin abrogates doxorubicin-induced apoptosis in BC cells

(**A**) Resistin-pretreated breast cancer cells were treated with doxorubicin (0.1 μM) in the presence of resistin for 72 h. Following treatment, the number of apoptotic cells was determined by Annexin V-binding assay, using flow cytometry. (**B and C**) Resistin-pretreated breast cancer cells were exposed to doxorubicin (0.1 μM) in presence and absence of resistin for 48 h. After that, total protein was isolated, and immunoblot analysis was performed to analyze the expression of (**B**) cleaved-caspase 7, caspase 3 and PARP1 and (**C**) BCL2 and BCL-xL. β-actin was used as loading control.
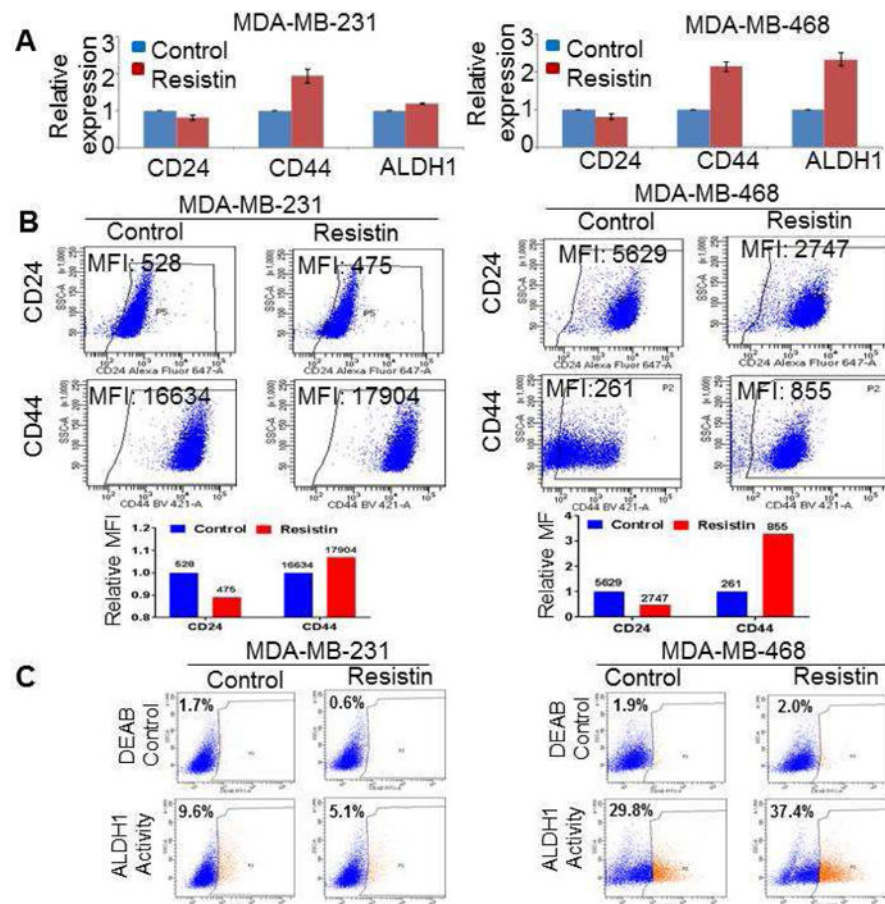
Figure 3. Effects of resistin on the breast cancer-specific stemness-markers
(A) Breast cancer cells were treated with resistin for 48 h, total RNA was isolated, c-DNA prepared and expression of stemness-associated markers (CD24, CD44, and ALDH1) examined by real-time quantitative RT-PCR assay. GAPDH was used as an internal control. **(B and C)** Resistin-treated BC cells were incubated with fluorochrome-conjugated **(B)** anti–CD44, –CD24 and **(C)** –ALDH1 antibodies for 15 min on ice and analyzed by flow cytometry. The data obtained from FACS analysis is presented as a geometric mean of fluorescence intensity (MFI) or percentage of cells.
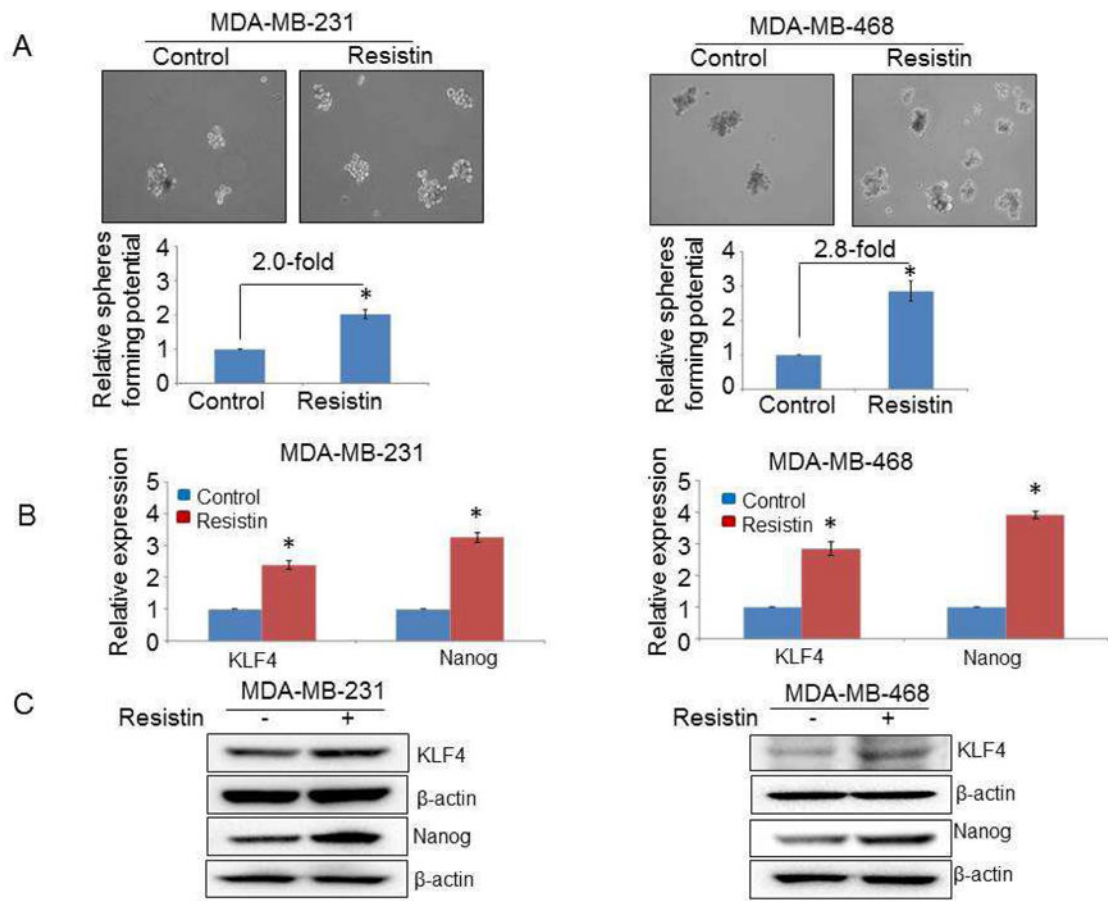
Figure 4. Resistin enhances stemness potential in breast cancer cells
(**A**) MDA-MB-231 and MDA-MB-468 cells (1×10^3) were seeded in ultra-low attachment plate and treated with resistin or vehicle, and allowed to grow for 15 days in stem cell culture medium. Resistin (20 ng/ml) was added every alternate day during incubation. Images were taken under a light microscope at 200x magnification, and the number of spheres (size > 50 μm) was counted. Error bars represent the mean ± SD; n = 3; *p < 0.05. (**B and C**) Breast cancer cells were treated with resistin and the expression of stemness-associated markers was examined (**B**) at mRNA level by quantitative RT-PCR (**C**) and protein level by immunoblot assay. GAPDH (for mRNA) and β-actin (for protein) were used as an internal control.
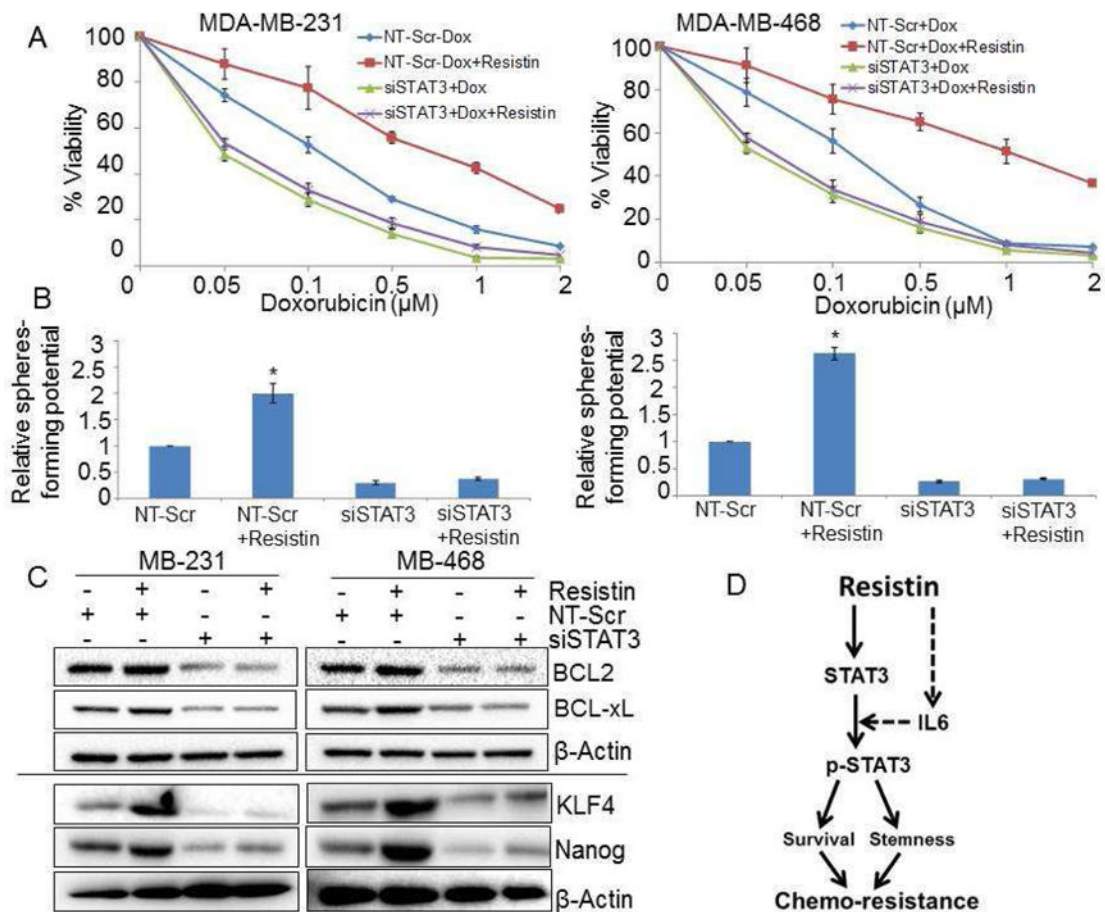
Figure 5. Silencing of STAT3 abrogates resistin-induced effects in breast cancer cells

(A) Cells were seeded in 96 well plates and transfected with non-targeting (NT)-Scr or siSTAT3. 24 h after transfection, cells were treated with resistin (20 ng/ml) for 12 h, followed by incubation with various concentration of doxorubicin (0.05-2 μM) in presence or absence of resistin for 72 h and cell viability was measured by WST-1 assay. Bars represent mean ± S.D (n=3). **(B)** To examine the role of STAT3 in resistin-induced stemness, BC cells were transfected with NT-Scr or siSTAT3 and sphere formation assay was performed in presence or absence of resistin as described earlier. Sphere >50 μm in diameter was counted at 200× magnification. Error bars represent the mean ± SD; n = 3; *p < 0.05. **(C)** MDA-MB-231 and MDA-MB-468 cells were transfected with NT-Scr or STAT3-targeting siRNAs for 24 h, post transfection, cells were treated with resistin (20 ng/ml) for next 48 h and expression of survival (BCL2 and BCL-xL) and stemness (KLF4 and Nanog) associated genes were observed by immunoblot assay. **(D)** A schematic diagram showing the molecular basis of resistin induced-chemoresistance in breast cancer cells. Resistin not only regulates the expression of STAT3 but also enhances its activation. Phosphorylation of resistin-induced STAT3 is mediated through IL-6. Further, activation of STAT3 results in enhanced stemness potential, survival and chemoresistance. Arrow dash indicates earlier published findings [13]

Table 1

Doxorubicin IC_{50} values for MDA-MB-231 and MDA-MB-468 breast cancer cells.

Cell lines	IC_{50} (in μM) of Doxorubicin at 72 h	
	Vehicle	Resistin
MDA-MB-231	0.12	0.65
MDA-MB-468	0.17	1.05

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Table 2

Doxorubicin IC$_{50}$ values for control Vs. STAT3 silenced MDA-MB-231 and MDA-MB-468 breast cancer cells

Cell lines	IC$_{50}$ (in µM) of Doxorubicin at 72 h			
	NT-Scr		siSTAT3	
	Vehicle	Resistin	Vehicle	Resistin
MDA-MB-231	0.15	0.72	0.05	0.06
MDA-MB-468	0.19	1.1	0.06	0.07