Cancer-associated Fibroblasts Induce Trastuzumab Resistance by Expanding Cancer Stem Cells and Activating Multiple Pathways in HER2 Positive Breast Cancer

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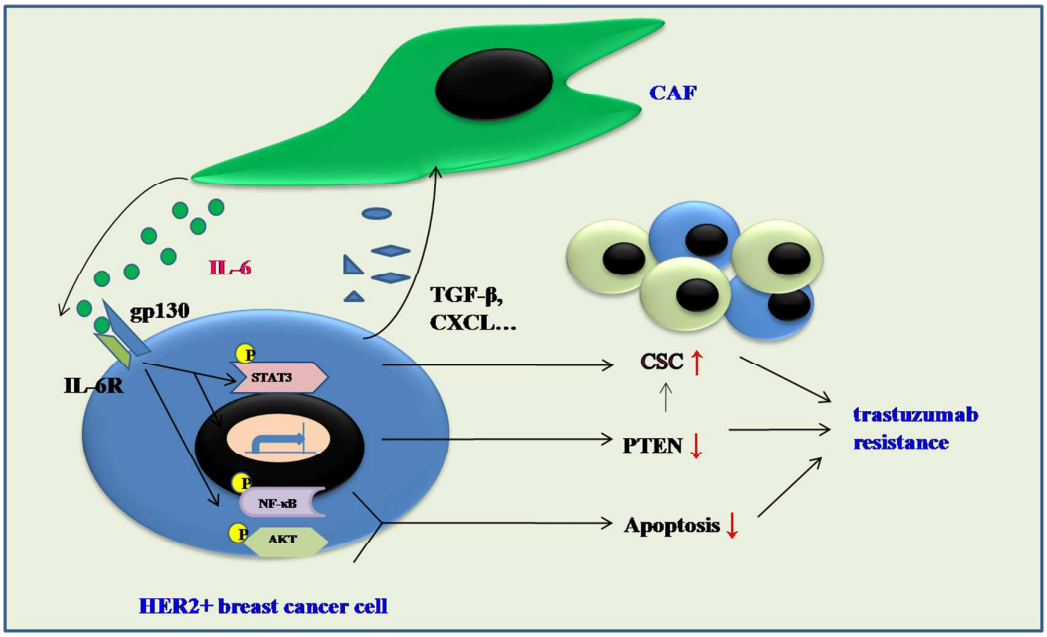
Graphical abstract



In our manuscript, we found that CAFs isolated from HER2+ patients secreted higher level of IL-6 which expanded cancer stem cells and activated multiple pathways, such as NF-κB, JAK/STAT3 and PI3K/AKT, then induced trastiuzumab resistance; combination of an anti-IL6 antibody, or multiple pathway inhibitors and trastuzumab maybe a novel strategy to reverse trastuzumab resistance in HER2 positive breast cancer.

Cancer-associated Fibroblasts Induce Trastuzumab Resistance by Expanding Cancer Stem Cells and Activating Multiple Pathways in HER2 Positive Breast Cancer

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CAFs induce trastuzumab resistance

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Abstract

Whether and how cancer-associated fibroblasts induce trastuzumab resistance in HER2+ breast cancer is still elusive. We analyzed the percentage of CSC and multiple pathway status before and after trastuzumab treatment in HER2 positive breast cancer cells co-cultured with conditional medium (CM) from CAFs. The results suggest that CAFs induce trastuzumab resistance by expanding cancer stem cells and activating multiple pathways, such as NF-κB, JAK/STAT3 and PI3K/AKT; combination of an anti-IL6 antibody, or multiple pathway inhibitors and trastuzumab in HER2 positive breast cancer maybe a novel strategy to reverse trastuzumab resistance.

Key words: trastuzumab resistance; cancer-associated fibroblasts; breast cancer

1. Introduction

HER2 gene amplification breast cancers like triple negative breast cancers (TNBCs) are the most virulent subtype of this malignancy[1]. HER2 positive patients account for about 20-25% of breast cancer, and HER2 target therapy such as trastuzumab[2, 3] and lapatinib [4] are initially effective in most HER2 positive patients with primary and metastatic diseases; however, approximately 15% of women diagnosed with early HER2-positive disease and >70% of HER2-overexpressing metastatic breast carcinomas show resistance to trastuzumab [3, 5, 6]**.** It is urgent to find out new ways to improve the prognosis of these patients. Metastatic tumor relapses are characterized by rapidly proliferating, drug-resistant cancers, associated with a high mortality rate. An increasing body of evidence suggests that CAFs, the majority of tumor stromal cells in breast cancer, interact with tumor cells and promote cancer progression by secreting cytokines and activating multiple pathways[7]. In this study, we sought to identify clinically targetable molecules or pathways driving the survival of trastuzumab-resistant cells induced by CAFs in HER2 positive patients.

Recent studies suggest that CAFs play important roles in breast cancer drug-resistance. Our group and other investigators showed that breast cancer cells co-cultured with CAFs were resistant to tamoxifen treatment [8, 9]. Moreover, CAFs are potent inducers of an epithelial-to-mesenchymal transition (EMT) and increasing cancer stem cells in mammary cells which have been associated with acquisition of tumor drug resistance and metastasis[10]. Furthermore, CAFs can activate multiple pathways, such as PI3K/AKT/ mTOR pathway and JAK/STAT3 pathway. PI3K/AKT/ mTOR pathway and other pathways were often detected activated in trastuzumab resistant cancer cells[11]. PTEN (Phosphatase and Tensin Homolog) loss also took part in trastuzumab resistance by activating IL6 inflammatory loop[12]. These data suggest the possibility that CAFs in tumors may be involved in trastuzumab resistance in breast carcinomas by expanding cancer stem cells and activating multiple pathways.

PI3K/AKT/mTOR pathway inhibitors have been proposed and are being developed as anti-metastatic therapies in patients with breast cancer, such as Everolimus[13], BEZ235 and BKM120[14]. However, the impact of these inhibitors on drug-resistance induced

by CAFs in HER2+ breast cancer has not yet been explored. We determined the roles of CAFs in trastuzumab resistance in HER2+ breast cancer cells; and tested the effect of combined trastuzumab with multiple pathway inhibitors in these cells. The results showed that PI3K/AKT/mTOR pathway inhibitors BEZ235, BKM120, JAK/STAT3 pathway inhibitor S3I-201, NF-kB pathway inhibitor bortezomib (BTZ), IL-6 neutralizing antibody combined with trastuzumab can reverse trastuzumab resistance induced by CAFs. These studies are the first to our knowledge to demonstrate the role of CAFs in trastuzumab resistance and the efficacy of pathway inhibitors combined with trastuzumab in HER2 positive breast cancer. They provide a basis for future clinical studies to treat resistant HER2+ breast cancer patients with trastuzumab and multiple pathway inhibitors.

2. Materials and Methods

Some reagents and antibodies are listed in Supplementary methods.

2.1 Cell cultures and agents

Human breast cancer cell lines BT474 and SK-BR3 were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 (Hyclone) supplement with 10% FBS (GIBCO) and 1% penicillin/streptomycin. They were cultured at 37℃ water-saturated 5% CO2 atmosphere.

2.2 RNA extraction and real-time quantitative PCR (qPCR).

Please see Supplementary methods for procedures.

2.3 Western blot assays

Please see Supplementary methods for procedures.

2.4 Isolation of primary fibroblasts

CAFs were isolated from human invasive mammary ductal carcinomas tissue center, non-cancer-associated fibroblasts (NAFs) from samples obtained from 3 reduction mammoplasty, in which only normal mammary tissue was detectable. All tissues were obtained from the Ruijin Hospital with approval of the hospital ethical committee and by the patient's written informed consent (Shanghai, China). The details of these patients were showed in Table S1. All fibroblasts cultured in

DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS, GIBCO) and 5μg/mL insulin (Tocris Bioscience, UK) (Supplementary methods).

2.5 ELISA

Quantification of IL-6 level in the supernatants of fibroblasts or breast cancer cells were calculated by ELISA assay according to the protocol in the quantikine ELISA kit (Cat#:DY206, R&D Systems). All experiments were done with 4 wells per experiment and repeated three times.

2.6 Proliferation Assays

After a 24h serum withdrawal, BT474 and SK-BR3 were digested and plated at 5000 cells/well into 96-well flat-bottomed cell culture plates with 5 replications in 100ul complete culture medium for a night. Media was exchanged with 1% of FBS containing CM from CAFs, NAFs and normal DMEM/F12. After at least 48 h different concentrations of trastuzumab or vehicle (IgG) or various combination of trastuzumab (10ug/ml), IL-6 neutralizing antibody (100ng/ml), STAT3 inhibitor (S3I-201,10μmol/L), PI3K inhibitors (BKM120, 1μmol/L, and BEZ-235, 1μmol/L), NF-κB inhibitor (Bortezomib, 10nmol/L), MEK inhibitor (U0126, 1μmol/L) were added into the CM. Five days later, 10 μl of cell counting kit-8 (CCK-8) (Cat#: CK04, Dojindo Molecular Technologies, Japan) reagent was added into each well and the plates were incubated in a 37℃ incubator with 5% CO2 for 3 h. Cell proliferation was determined by CCK-8 assay and read on multi-well scanning spectrophotometer (Thermo) at A450 for wavelength correction. All experiments were repeated at least three times.

2.7 Luciferase reporter assays

Constructs of the PTEN promoter region at -1986/+14 were generated from genomic DNA of SK-BR3 cells. The PTEN promoter was cloned into the pGL3 basic reporter gene vector and verified by sequencing.SK-BR3 and BT474 cells were maintained in RIPM 1640 containing 10% FBS and transfected by Lipofectamine™ 2000 Reagent

(Invitrogen Corp, Carlsbad, CA) in 12-well plates. PTEN promoter constructs: Renilla plasmid DNA (200ng: 20ng) were co-transfected for 6−9 h and then incubated with IL6 (4ng/ml) for 36−48 h in fresh complete medium. Cells were then rinsed in cold PBS and lysed with the luciferase assay buffer. Luciferase activities were measured by using a dual luciferase assay kit (Cat#: E1960, Promega, Wisconsin, USA) with a Berthold chemiluminometer (Berthold Detection Systems GmbH). The results were expressed as ratio of firefly luciferase activity to Renillaluciferase activity. Data were expressed as the mean values and standard deviations from at least three independent transfections performed in triplicate.

2.8 Tumor sphere assay

Tumor sphere culture was performed as previously described[15, 16]. CM collected from differentially fibroblasts, or recombinant IL6 were added to ultralow attachment 6-well plates filled with 2 ml mammary epithelial growth medium (MEGM), and supplemented with B27 supplement (10 ng/ml EGF, 10 ng/ml bFGF, 10 μg/ml Insulin). (Corning; Corning, NY) containing freshly plated single BC cells at the beginning of the tumor sphere formation assay. On day 10, numbers of tumor spheres (diameter $\geqslant$100 μm) were counted, and tumor sphere efficacy was calculated based on the numbers of initially seeded cells.

For secondary sphere formation, first-passage tumor spheres from day 10 cultures were collected by gentle centrifugation (320g) and dissociated into single cells by incubation in trypsin-EDTA solution (Invitrogen; Carlsbad, CA), before plating into new wells. Similar analysis was done as mentioned in primary tumor sphere assay.

2.9 Flow cytometry

Single-cell suspensions prepared from cell culture were stained with FITC-conjugated human CD44 antibody (Catalog #; 555742, BD Biosciences, Franklin Lakes, NJ), PE-conjugated CD24 antibody (Catalog #; 555574, BD Biosciences, Franklin Lakes, NJ) or isotype controls following the manufacturer's protocol. Flow cytometry assays were performed using a CyAn ADP cytometer (Dako; Carpinteria, CA) and analyzed

with FlowJo software (TreeStar; Ashland, OR).

2.10 Cell Cycle Analysis

BT474 and SK-BR3 single-cell suspension were cultured in 6-well plate at a density of 10,000 cells per well for a night. Then media were exchanged for medium with 1% of FBS containing CM from CAFs, NAFs and Normal DMEM/F12 medium. At least 48h later, different concentrations of trastuzumab or vehicle (IgG) or various combination of trastuzumab (10ug/ml), IL-6 neutralizing antibody (100ng/ml), STAT3 inhibitor (S3I-201, 10μmol/L), PI3K inhibitors (BKM120, 1μmol/L, and BEZ-235, 1μmol/L), NF-κB inhibitor (Bortezomib, 10nmol/L), MEK inhibitor (U0126, 1μmol/L) were added. After 24 h treatment, all cells were harvested and washed twice with cooled PBS and fixed in 75% ethanol for 2 h at 4 ℃. The fixed cells were washed with cooled PBS and were then stained with the staining solution containing 0.05 μg/mL PI (Sigma-Aldrich), 1 μg/mL DNase-free RNase (Sigma-Aldrich) for 30 min at RT. Ten thousand events were acquired using a FACSCalibur analyzer (Becton-Dickinson, San Jose, CA, USA), and cell cycle data were determined using Modfit software (Verity Software House, Topsham, ME, USA).

2.11 Apoptosis Analysis

All cells had the same treatment mentioned above in cell cycle analysis. After 24h treatment, cells were detached with EDTA-free trypsin and washed twice with cooled PBS. Cells were resuspended in 400 μL 1× loading buffer with 5 μL Annexin V and 5 μL PI (BD Pharmingen, San Diego, CA, USA) for 15 min on ice in dark. Analyses were performed at FACSCalibur analyzer (Becton-Dickinson)

2.12 Survival Analysis

2014 version of the database including 88 HER2+ patients with enough information were analyzed for overall survival (OS) with 66% of IL-6, STAT3, NF-κB and AKT gene expression as a cutoff. The results were presented as Kaplan–Meier plots and tested significance using log-rank tests. The analysis was performed according to the

manufacture's instructions (http://kmplot.com/analysis/index.php?p)[17].

3. Results

3.1 Higher expression of α-SMA and lower expression of caveolin 1 (CAV1) in CAFs

It is well known that CAFs play important roles in cancer progression. But there was none identical markers to separate CAFs from non-cancer-associated fibroblasts (NAFs). However, increasing evidence suggest that compared to NAFs, CAFs were characterized by higher expression of α-SMA, or loss expression of CAV1[7]. To study the interaction of CAFs and breast cancer cells, we first isolated CAFs from HER2 positive breast cancer tissue and non-cancer associated fibroblast from patients with vice breast. Then we confirmed them by markers mentioned above. Surprisingly, α-SMA was strong and CAV1 was weak in CAF11, CAF23 and CAF64 compared with NAF1 (Fig.1). These findings indicate that CAFs isolated from HER2+ breast cancer were really activated.

3.2 Cancer-associated Fibroblasts Reduce Sensitivity to Trastuzumab in HER2+ Breast Cancer Cells

HER2 positive breast cancer is exquisitely sensitive to trastuzumab. However, at least 50% of these patients develop trastuzumab resistance during treatment[6]. Since more and more studies suggested that CAFs involved in drug resistance, we asked whether CAFs in tumor can mediate trastuzumab resistance in HER2 positive breast cancer. To test this hypothesis, we first collected conditional medium (CM) from two cancer-associated fibroblasts (CAF11, CAF64) from HER2+ breast cancer and NAF1 from vice breast. Then we co-cultured BT474 and SK-BR3 with these medium. Concomitantly, we observed that medium from CAFs can decrease sensitivity to trastuzumab in BT474 and SK-BR3 cells compared with medium from NAF1 and control by CCK-8 assay (Fig.2), indicating that fibroblasts in tumor were different from them in normal breast tissue and may participate in trastuzumab resistance.

3.3 IL-6 is the Major Different Cytokines Secreted from CAFs and NAFs

As we know that CAFs take part in cancer development and progression mainly by secreting cytokines and growth factors[7]. Since IL6 have been reported play important roles in cancer progression, we confirmed the results by ELISA in CAFs (Fig.3A, B). The results showed that IL6 was really higher secreted from CAFs. Since some study showed there existed autocrine of IL6 in BT474 and SK-BR3 cells, we then compared IL6 level among these two cell lines and CAFs. Interestingly, compared with CAFs, BT474 and SK-BR3 cells secret little of IL6 (Fig.3C), which suggested that paracrine way of IL6 may play more important roles in trastuzumab resistance. Moreover, recombined IL6 can partly induce trastuzumab resistance in BT474 and SK-BR3 cells (Fig.3D). The data mentioned above indicated that IL6 may be the major factor contributing to trastuzumab resistance.

3.4 CAF-CM and IL6 Expand Cancer Stem Cells (CSC) Population

Cancer stem cells are one of the most important populations contributing to cancer recurrence and drug-resistance, and IL6 have been reported to increase CSC population by activating JAK/STAT3 pathway[18], so we examined their changes in BT474 and SK-BR3 cells treated with IL6. The tumor sphere numbers were increased in both BT474 and SK-BR3 cells after 10 days of IL6 treatment (Fig. 4A). Moreover, CAF11-CM and CAF64-CM also increased tumor sphere number (Fig.4B) and subpopulation of $CD44^+/CD24^-$ cells in these two cell lines after 10 days of culture (Fig. 4E,F). More importantly, after 72 hours treatment of trastuzumab, BT474 cells treated with IL6 compared with PBS still have almost 2-folds of secondary tumor sphere number (Fig. 4C). Similar results were also found in SK-BR3 cells. Interestingly, these two cell lines cultured with CAF11-CM, CAF64-CM also have at least 3-folds of secondary tumor sphere number compared with control (Fig. 4D) and subpopulation of $CD44^+CD24^-$ cells were also highly increased even after72 hours treatment of trastuzumab. Furthermore, we tested other CSC-related gene expressions in cultured breast cancer cells with CAF-CM and IL6.

RNA extracted from them showed that *CD44 and ALDH1A1* gene expressions were upregulated after interaction with CAF-CM and IL6 in both cell lines (Supplementary Figure 1). All these results suggested that CAF-CM and IL6 both can increase CSC population and then may decrease the sensitivity of trastuzumab in HER2 positive breast cancer cells.

3.5 IL6 Expands Cancer Stem Cells Population by Activating STAT3 Pathway and Downregulating PTEN through Decreasing Its Transcriptional Activity

To explore the mechanism of CSC expanded by IL6, we first tested CSC-related pathways. It has been reported that IL6 increased CSC population by activating JAK/STAT3 pathway[18]. So we test this pathway and found that IL6 really increased p-STAT3 level (Fig. 5A). More importantly, CAF23-CM also activated JAK/STAT3 pathway in both BT474 and SK-BR3 cell lines (Fig.5B). Moreover, whether IL6 play important roles in increasing CSC by activating these pathways, we tested tumor sphere number and p-STAT3 protein expression after adding IL-6 neutralizing antibody to CAF11-CM and CAF64-CM. The results showed that adding IL6 neutralizing antibody, p-STAT3 expression and tumor sphere number were downregulated in BT474 and SK-BR3 cells compared with CAF-CM alone (Fig. 6E), which suggested that IL6 expands CSC population by activating STAT3 pathway.

PTEN loss plays important roles in cancer progression and drug resistance[19]. Moreover, one study suggested PTEN loss also promotes CSC expanding[12]. Therefore, we made further study to find out whether IL6 affected PTEN expression. The results showed that PTEN protein expression was downregulated by CAF-CM and IL6 treatment in BT474 and SK-BR3 cells (Fig.5C, D). We tested the mRNA level of *PTEN* in BT474 treated with IL6 or CAF-CM. Fig.5E, F showed that IL-6 and CAF-CM both can downregulate *PTEN* mRNA level, not only in BT474 but also in SK-BR3 cells. So, we did the luciferase reporter assay to find out whether there were transcriptional activity changes in PTEN promoter. As the results showed that, IL6 did decrease transcriptional activity in the promoter of *PTEN* in both cell lines (Fig5 G).The mechanisms in this process need further study.

3.6 Anti-IL6 treatment can rescue trastuzumab sensitivity by downregualting CSC

Since we found that CAFs induced trastuzumab resistance by increasing IL6, we want to answer whether block IL6 can reverse its sensitivity in HER2+ breast cancer. We use IL-6 neutralizing antibody to block its function and test its role in trastuzumab resistance. In Fig.6A, SK-BR3 cultured with CAF64-CM and IL-6 neutralizing antibody was more sensitive to trastuzumab compared with SK-BR3 cultured with CAF64-CM. Similar results were also showed in BT474 (Fig.6B). Furthermore, the tumor sphere number in these two HER2+ cell lines were also decreased after adding IL-6 neutralizing antibody to CAF64-CM (Fig. 6C, D). Meanwhile, PTEN expression were reversed and JAK/STAT3 pathway were inhibited, too. These data support our previous hypothesis and that anti-IL6 treatment maybe a novel strategy to reverse trastuzumab resistance in HER2+ breast cancer cells.

3.7 Multiple Activated Pathways Participate in Trastuzumab Resistance Induced by CAFs

Since PI3K/AKT/mTOR pathway activation has been found promote cancer progression, and mTOR inhibitor Everolimus improved DFS in endocrine resistant patients [13], we want to found whether this pathway also play important roles in CAF induced trastuzumab resistance or there still exist other pathways. The results indicate that CAF11-CM and CAF64-CM from HER2 positive patients can activate PI3K/AKT/mTOR pathway whatever with or without trastuzumab treatment. More importantly, we also found JAK/STAT3, NF-κB and ERK pathways activated under HER2-target treatment (Fig. 7A). More importantly, recombined IL6 also activated these multiple pathways even under trastuzumab treatment (Fig. 7B). These results suggest that IL6 mediates trastuzumab resistance not only by expanding CSC, but also by activating multiple pathways. So we want to test whether combined pathways inhibitors and trastuzumab can improve treatment response in trastuzumab resistance cells induced by CAFs. We first test cell viability under treatment with combined

trastuzumab and these pathway inhibitors: JAK/STAT3 inhibitor S3I-201, IL6 neutralizing antibody anti-IL-6, PI3K/AKT pathway inhibitor BEZ235 and BKM120, NF-κB pathway inhibitor BTZ and MEK pathway inhibitor U0126. Fig. 7C showed that dual inhibitor of HER2 and one of these pathways can really improved trastuzumab activity, such as S3I-206, BEK235, BKM120 and BTZ. However, MEK inhibitor U0126 did not have this effect. To find out the mechanisms, we tested the pathways status, apoptosis and cell cycle in these cells. As showed in Fig. 7D, every multiple pathway inhibitor can partly block its own pathway activation. However, adding Anti-IL6, S3I-206, BEK235, BKM120 and BTZ compared with trastuzumab alone increased early or late apoptosis in BT474 and SK-BR3 cells cultured with CAF64-CM, but U0126 had little effect in this field (Fig.S2). We also tested cell cycle changes in BT474 and SK-BR3 cells under the same treatment. The results showed that there were little changes in G1 or G2/M phase, especially in U0126 treated group. Other inhibitors, like BTZ, BKM120 almost play part of roles in an accumulation of cells in the G2/M phase fraction with concomitant reduction of cell numbers in G1 phase, but not very obviously (Fig. S3). These findings suggested that CAFs mediate trastuzumab resistance also by activating PI3K/AKT/mTOR, JAK/STAT3 and NF-κB pathways; adding these pathways inhibitors compared with trastuzumab alone had better treatment effect through increasing apoptosis.

3.8 Increasing IL-6 gene expression indicates poor overall survival in HER2+ patients

To test our hypothesis in patients, we analyzed the gene expression of IL-6, STAT3, NF-κB and AKT in HER2 + patients. The results showed that patients with IL-6 and NF-κB gene overexpression had poor OS (n=88, HR=2.72, 95%CI, 1.18, 6.3, p=0.015; HR=2.37, 95%CI, 1.03, 5.48, p=0.037) for 5 years follow-up (Fig.8A,C), while patients with STAT3 and AKT gene high expression (Fig.8B,D) with a trend to have poor OS, but not reach the significant difference. These results may indicate that IL-6 play important roles in HER2+ patients.

4. Discussion

It has been proposed that tumor recurrences following treatment with anti-HER2 drugs are driven by a subpopulation of CSCs. CSCs are thought to possess intrinsic resistance to trastuzumab and other drugs compared with the bulk of tumor cell population[20, 21]. Tumor microenvironment has been reported to increase CSC population by secreting CCL2 in breast cancer and correlate with poor survival [10]. In 2008, a stroma-derived prognostic predictor (SDPP) that stratifies disease outcome independently of standard clinical prognostic factors which highlights the importance of stromal biology in tumor progression was found[22]. Therefore, to know more about the functional roles of stromal cells, such as cancer-associated fibroblasts, in breast cancer is an urgent problem to solve. To date, many studies showed that CAFs play roles in drug-resistance, such as chemoresistance[23], tamoxifen resistance[8, 9] and EGFR kinase inhibitor resistance[24]. However, there was no data about their roles in trastuzumab resistance. In order to find out whether CAFs in HER2+ breast cancer patients participate in trastuzumab resistance, we examined cell viability of BT474 and SK-BR3 cells cultured with CAF-CM with or without trastuzumab treatment. The results showed that CM from CAFs in tumor had the ability to decrease trastuzumab response. To test whether CSC play roles in this process, we also tested CSC-related gene expression in HER2+ breast cancer cells cultured with CAF-CM. RNA extracted from cultured BT474 and SK-BR3 cells showed enrichment of CSC-related genes, such as *CD44* and *ALDH1A1*. Meanwhile, $CD44^+/CD24^-$ cell population and tumor sphere numbers were also increased. In an effort to discover why CSC increased in this process, we examined several CSC-related pathways. The results suggested that JAK/STAT3 pathway were activated in cultured BT474 and SK-BR3 cells even under tratuzumab treatment. These results concur with recent findings that IL-6 inflammatory loop participates in trastuzumab by expanding CSC population through JAK/STAT3 and NF-kB pathways[12]. In this study, we found out the major cytokines secreted from CAFs was also IL-6, which have been reported correlated with CSC population expanding[12, 18]. Of note, IL-6 is a pleiotropic cytokine and involves in a wide range of biological activities including inflammation, immune regulation,

hematopoiesis and oncogenesis. Clinical studies have shown that high serum IL-6 levels and high IL6 expression in tumor cells are indicative of poor prognosis and survival in breast and other kinds of cancer[25, 26]. Blockade of IL-6 has also been reported to abrogate PTEN loss induced enrichment of CSCs and reverse trastuzumab resistance in mouse model[12]. Also, there are phase I/II clinical trials testing the effect of anti-IL6 therapy in prostate cancer[27], myeloma (NCT00911859) and ovarian cancer[25]. These data suggest that IL6 may be a promising new target in cancer. Furthermore, our work also found that IL6 downregulated PTEN expression by affecting its transcriptional activity in the luciferase reporter assay which helps to increase CSC population. Recombined IL6 also expanded CSC population in BT474 and SK-BR3 cells. Also, adding IL-6 neutralizing antibody compared with trastuzumab alone significantly reduced stem cell markers, and the tumor-initiating potential of BT474 and SK-BR3 cancer cells. However, our data do not exclude the autocrine cytokines changes in BT474 and SK-BR3 cells after cultured with CAFs. Taken together, these data suggest that upregulation of autocrine/paracrine IL-6 signaling at tumor sites contribute to a tumor microenvironment that sustains CSCs in the primary cancer and also facilitates progression at metastatic sites.

In recent years, the progression in cancer treatment was targeting the activated signaling pathways. In BOLORE 2, everolimus combined with exemestane showed good response in endocrine resistant patients[13]. Moreover, BOLERO-3 study showed that mTOR inhibition in combination with trastuzumab plus vinorelbine treatment significantly improved PFS compared to trastuzumab or vinorelbine alone in trastuzumab-resistant MBC patients[28]. More interestingly, in our study, PI3K/AKT/mTOR pathway inhibitor BEZ235, BKM120; JAK/STAT3 inhibitors S3I-201; NF-κB pathway inhibitor BTZ combined with trastuzumab also had good effects to reverse trastuzumab resistance induced by CAFs. To find the mechanisms, we did further study about the changes in signaling pathways status, apoptosis and cell cycle. The data suggested that these pathway inhibitors improved trastuzumab efficacy by increasing apoptosis in cancer cells. Besides, many preclinical and phase

I/II studies showed good effect of BKM120, BEZ235 and BTZ in trastuzumab resistant cells[29-32].

5. Conclusions

In summary, results presented herein link anti-HER2 therapy, CAFs, paracrine IL-6 signaling, the expansion of CSC, multiple signaling pathways activation with trastuzumab resistance (Fig. 9). We surmise that, these data imply that anti-HER2 therapy in combination with systemic antagonists of IL-6 signaling, either small molecular kinase inhibitor or therapeutic antibodies; PI3K/AKT pathway inhibitors BEZ235, BKM120; NF-κB pathway inhibitor BTZ may inhibit HER2+ breast tumor recurrences following trastuzumab treatment, and, as a result, improve the outcome of patients with this subtype of breast cancer.

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Figure Legends

Fig. 1 Expression of CAF markers (α-SMA and CAV1) in isolated fibroblasts

Western blot analysis of lysates from CAF11, CAF23, CAF64 and NAF1 cells using anti-α-SMA and anti-CAV1 antibodies. GAPDH was used as a loading control.

Fig.2 Response to different concentrations of trastuzumab after culture of SK-BR3 and BT474 cells with conditioned media from CAFs and NAFs

(A, B) Cell viability assay in SK-BR3 cells treated with different concentrations of trastuzumab after culture with conditioned media (CM) from control, CAF11, CAF64, and NAF1 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. * indicates $p<0.05$.

(C, D) Cell viability assay in BT474 cells treated with different concentrations of trastuzumab after culture with CM from control, CAF11, CAF64, and NAF1 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. * indicates $p<0.05$.

Fig. 3 IL-6 is a key cytokine secreted from CAFs

(A) The ELISA results of IL6 secreted from CAFs and NAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d.

(B) The ELISA results of average IL6 secreted from CAFs and NAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d. * indicates $p<0.05$.

(C) The ELISA results of IL6 secreted from BT474, SK-BR3 and CAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d. * indicates $p<0.05$.

(D) Cell viability assay in SK-BR3 (left) and BT474 (right) cells treated with different concentrations of trastuzumab after incubation in PBS or IL-6 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. * indicates $p<0.05$.

Fig. 4 IL-6 secreted by CAFs expands cancer stem cells population.

(A) Tumor sphere assay in SK-BR3 (left) and BT474 (right) cells treated with PBS or IL6 (4ng/ml) for 10 days. Three independent experiments were performed in triplicate.

(B) Tumor sphere assay in SK-BR3 (left) and BT474 (right) cells treated with control, CAF11-CM or NAF1-CM for 10 days. Three independent experiments were performed in triplicate.

(C) Secondary tumor sphere assay in SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.

(D) Secondary tumor sphere assay in SK-BR3 (left) and BT474 (right) cells cultured with control, CAF11-CM or NAF1-CM under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.

(E) Percentage of $CD44^{+}/CD24^{-}$ population in BT474 cells cultured with control, CAF64-CM or NAF-CM under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.

(F) Percentage of CD44+/CD24- population in SK-BR3 cells cultured with control, CAF11-CM or NAF-CM for 10 day by flow cytometry.. Three independent experiments were performed in triplicate.

Fig. 5 IL-6 activates JAK/STAT3 pathway in HER2+ breast cancer cells

(A) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control, CAF23-CM (CM23) or NAF1-CM (CM1) under trastuzumab (10ug/ml) treatment for 24 h using indicated antibodies. GAPDH was used as a loading control.

(B) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) under trastuzumab (10ug/ml) treatment for 24h using indicated antibodies. GAPDH was used as a loading control.

(C) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control or CAF11-CM for 24 h using anti-PTEN antibodies. GAPDH

was used as a loading control.

(D) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with different concentrations of IL6 (0, 0.2, 2, 4ng/ml) for 24 h using anti-PTEN antibodies. GAPDH was used as a loading control.

(E) Quantitative real-time PCR (qRT-PCR) measuring PTEN mRNA levels in SK-BR3 (left) and BT474 (right) cells treated with control, CAF11-CM or NAF1-CM for 24 h. Data were normalized to GAPDH mRNA levels and expressed as fold change compared with controls (log2 scale). Data are shown as mean ± SE of 3 independent replicates for each condition.

(F) Quantitative real-time PCR (qRT-PCR) measuring PTEN mRNA levels in SK-BR3 (left) and BT474 (right) cells treated with PBS or IL6 (4ng/ml) for 24 h. Data were normalized to GAPDH mRNA levels and expressed as fold change compared with controls (log2 scale). Data are shown as mean ± SE of 3 independent replicates for each condition.

(G) PTEN promoter activity after transfection of the full length construct (-1086/+14) alone or together with IL6 (4ng/ml). pGL3-Basic, control for promoter constructs;

The results are expressed as a relative ratio of firefly luciferase to Renilla luciferase. Three independent experiments were performed in triplicate.

Fig. 6 IL-6 neutralizing antibody decreased CSC population and restored trastuzumab sensitivity

(A) Cell viability assay in SK-BR3 (left) and BT474 (right) cells treated with different concentrations of trastuzumab after culture with conditioned media (CM) from control, CAF64 or CAF64 combined with IL6 neutralizing antibody (100ng/ml) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. * indicates $p<0.05$.

(B) Relative changes of secondary tumor sphere in SK-BR3 (left) and BT474 (right) cells treated with trastuzumab (10ug/ml) alone or combined with IL6 neutralizing antibody (100ng/ml) after culture with conditioned media (CM) from control, CAF11 or CAF64 for 10 days. Three independent experiments were performed in triplicate.

(C) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells

treated with trastuzumab (10ug/ml) after culture with conditioned media (CM) from control, CAF11 (CM11) , CAF64 (CM64) or combined with IL6 neutralizing antibody (100ng/ml) for 24h using indicated antibodies. GAPDH was used as a loading control.

Fig. 7 CAF-CM activated multiple pathways and combined trastuzumab with inhibitors of these pathways restored trastuzumab resistance.

(A) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control or CM from CAF11 (CM11),CAF64 (CM64) and treated with trastuzumab (10ug/ml) or IgG for 24h using indicated antibodies. GAPDH was used as a loading control.

(B) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) and treated with trastuzumab (10ug/ml) or IgG for 24h using indicated antibodies. GAPDH was used as a loading control.

(C) Cell viability assay in SK-BR3 (left) and BT474 (right) cells cultured with control or CM from CAF11 and treated with a series of concentrations of trastuzumab with DMSO, IL-6 neutralizing antibody (100 ng/ml), STAT3 inhibitor S3I-201 (10 μM), BEZ235 (1μM) or BKM120 (1μM), NF-κB inhibitor Bortezomib (10 nM), MEK inhibitor U0126 (1μM) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. * indicates $p<0.05$.

(D) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells treated with CM from CAF64 (CM64) and treated with trastuzumab (10ug/ml), or trastuzumab (10ug/ml) combined with IL-6 neutralizing antibody (100 ng/ml), STAT3 inhibitor S3I-201 (10 μM), BEZ235 (1μM) or BKM120 (1μM), NF-κB inhibitor Bortezomib (10 nM), MEK inhibitor U0126 (1μM) for 1 h using the indicated antibodies. GAPDH was used as a loading control.

Fig. 8 Survival outcome in HER2+ patients

5 year overall survival outcome affected by IL-6 (A), STAT3 (B), NF-κB (C) and AKT (D) gene expression in 88 HER2+ patients.

Fig. 9 Schematic representation showing the role of CAFs in mediating trastuzumab resistance.

IL-6 secreted from CAFs can increase CSC population by activating the JAK/STAT3 pathway and downregualting PTEN expression in HER2+ breast cancer cells, which induced trastuzumab resistance. Meanwhile, CAFs-CM also activate PI3K/AKT/mTOR, NF-κB pathways which may decrease apoptosis in HER2+ breast cancer cells and also contribute to trastuzumab resistance.

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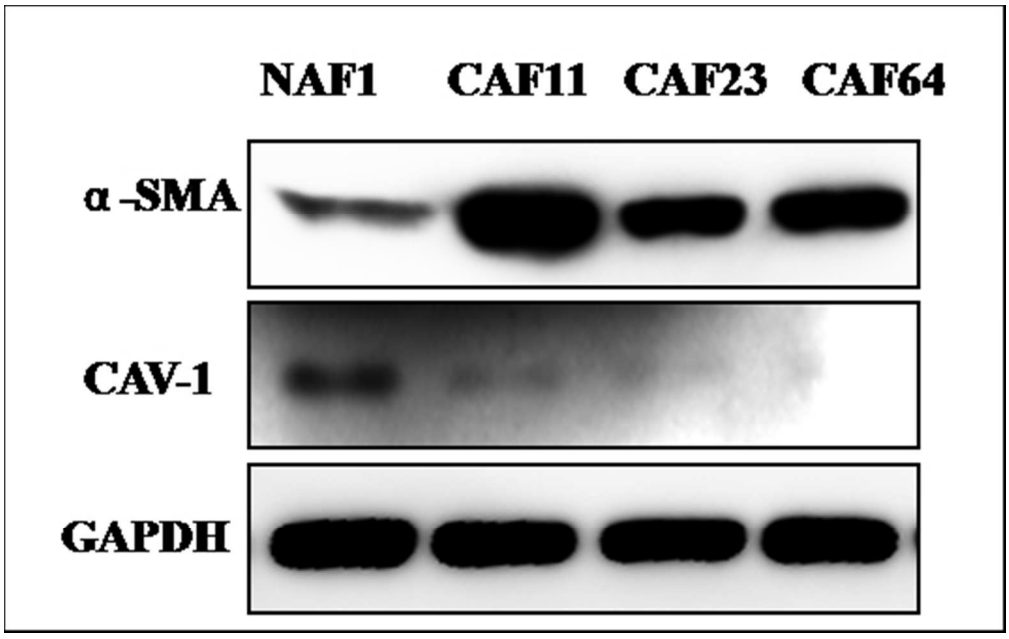

Expression of CAF markers (α-SMA and CAV1) in isolated fibroblasts
Western blot analysis of lysates from CAF11, CAF23, CAF64 and NAF1 cells using anti-α-SMA and anti-CAV1 antibodies. GAPDH was used as a loading control.

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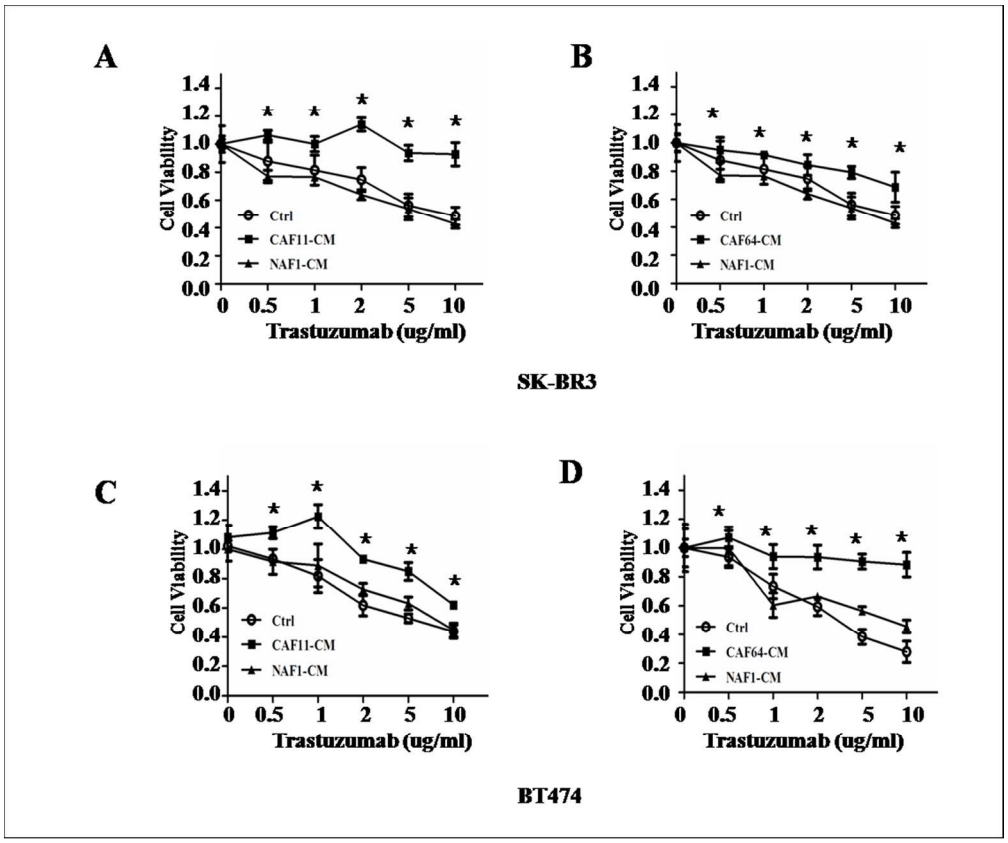

Response to different concentrations of trastuzumab after culture of SK-BR3 and BT474 cells with conditioned media from CAFs and NAFs

(A, B) Cell viability assay in SK-BR3 cells treated with different concentrations of trastuzumab after culture with conditioned media (CM) from control, CAF11, CAF64, and NAF1 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. ∗ indicates $p<0.05$.

(C, D) Cell viability assay in BT474 cells treated with different concentrations of trastuzumab after culture with CM from control, CAF11, CAF64, and NAF1 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. ∗ indicates $p<0.05$.

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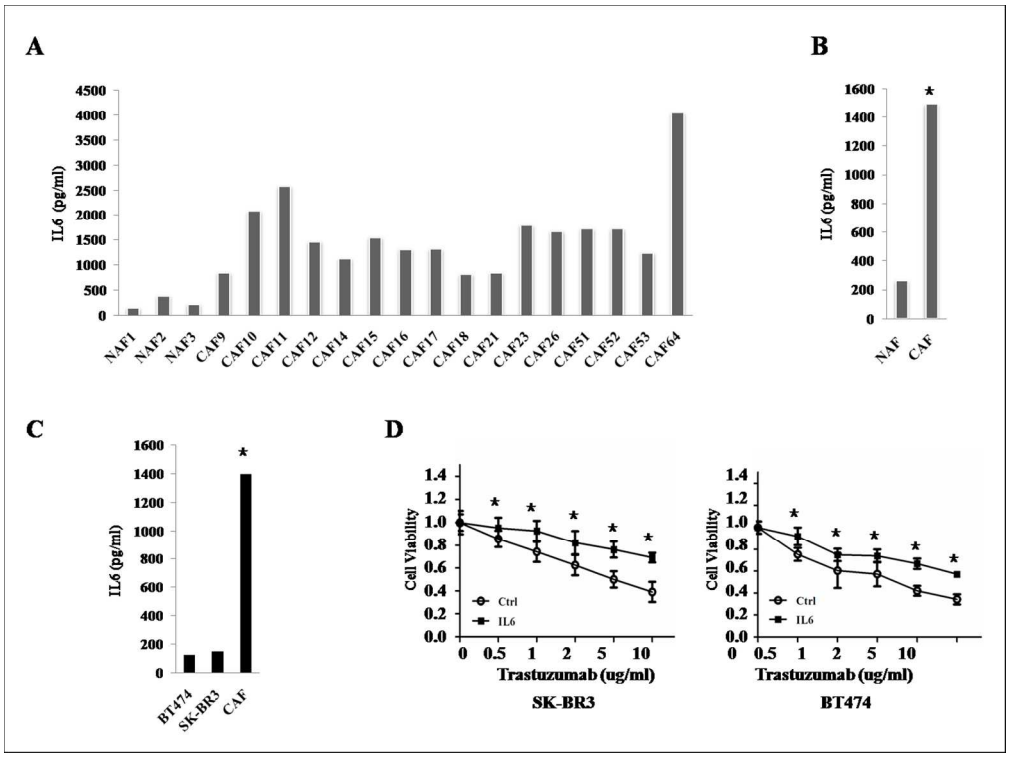

IL-6 is a key cytokine secreted from CAFs

(A) The ELISA results of IL6 secreted from CAFs and NAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d.

(B) The ELISA results of average IL6 secreted from CAFs and NAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d. * indicates $p<0.05$.

(C) The ELISA results of IL6 secreted from BT474, SK-BR3 and CAFs. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d. * indicates $p<0.05$.

(D) Cell viability assay in SK-BR3 (left) and BT474 (right) cells treated with different concentrations of trastuzumab after incubation in PBS or IL-6 for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. ∗ indicates $p<0.05$.

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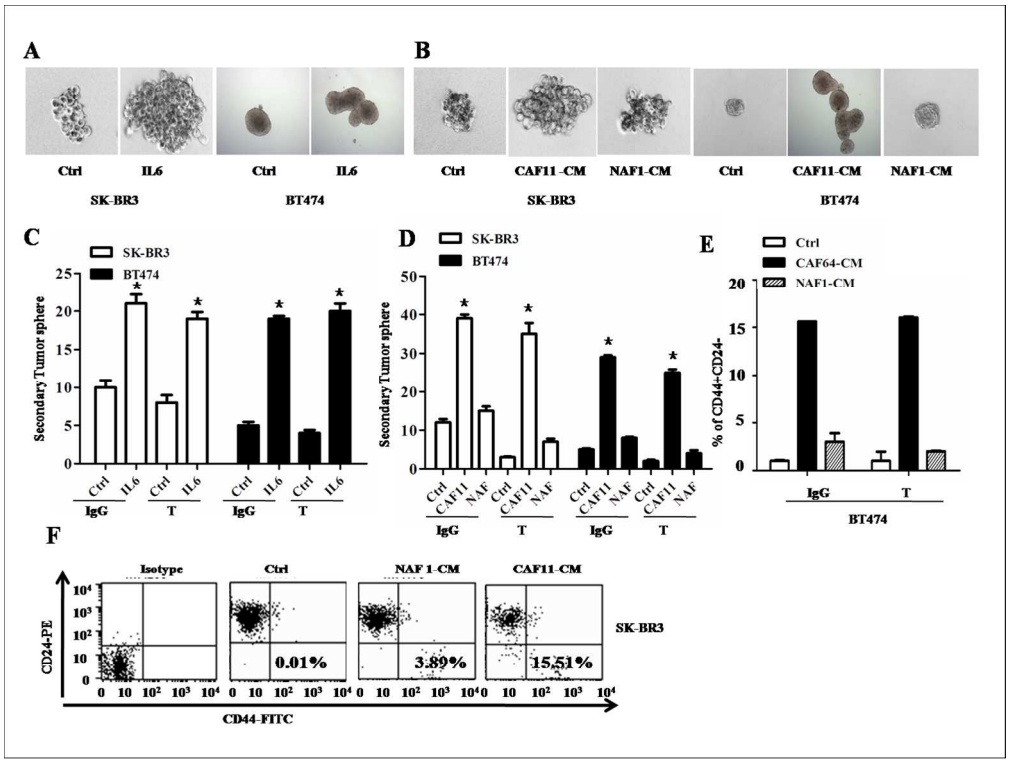

IL-6 secreted by CAFs expands cancer stem cells population.
(A) Tumor sphere assay in SK-BR3 (left) and BT474 (right) cells treated with PBS or IL6 (4ng/ml) for 10 days. Three independent experiments were performed in triplicate.
(B) Tumor sphere assay in SK-BR3 (left) and BT474 (right) cells treated with control, CAF11-CM or NAF1-CM for 10 days. Three independent experiments were performed in triplicate.
(C) Secondary tumor sphere assay in SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.
(D) Secondary tumor sphere assay in SK-BR3 (left) and BT474 (right) cells cultured with control, CAF11-CM or NAF1-CM under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.
(E) Percentage of CD44+/CD24- population in BT474 cells cultured with control, CAF64-CM or NAF-CM under IgG or trastuzumab (10ug/ml) treatment for 10 days. Three independent experiments were performed in triplicate.
(F) Percentage of CD44+/CD24- population in SK-BR3 cells cultured with control, CAF11-CM or NAF-CM for 10 day by flow cytometry.. Three independent experiments were performed in triplicate.

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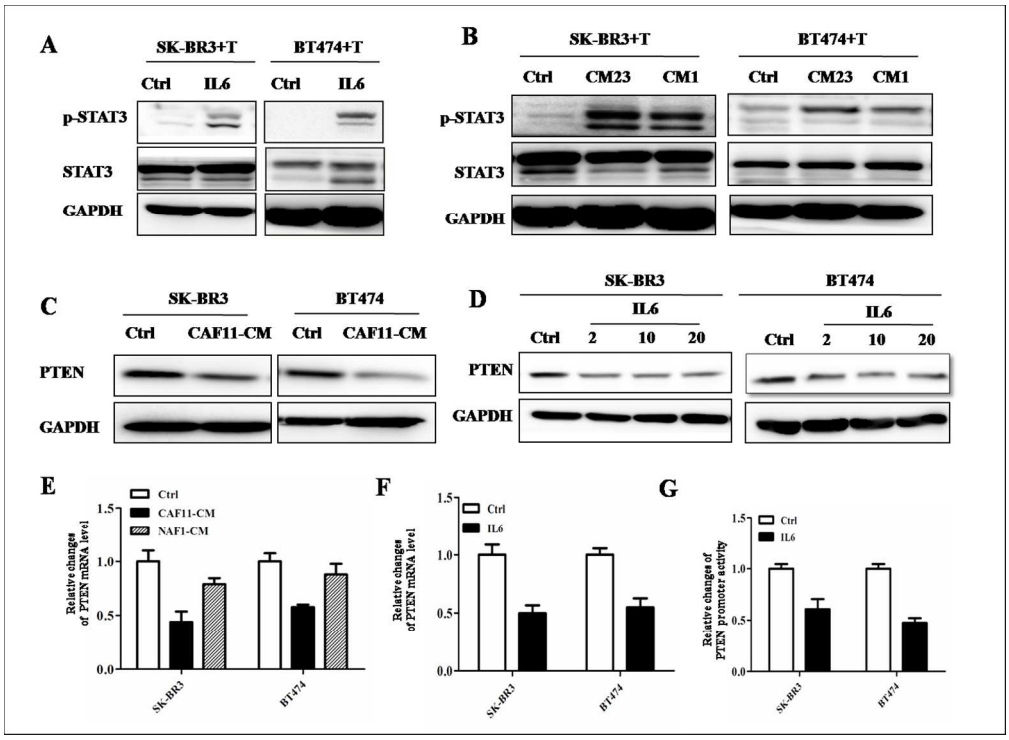

IL-6 activates JAK/STAT3 pathway in HER2+ breast cancer cells

(A) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control, CAF23-CM (CM23) or NAF1-CM (CM1) under trastuzumab (10ug/ml) treatment for 24 h using indicated antibodies. GAPDH was used as a loading control.

(B) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) under trastuzumab (10ug/ml) treatment for 24h using indicated antibodies. GAPDH was used as a loading control.

(C) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control or CAF11-CM for 24 h using anti-PTEN antibodies. GAPDH was used as a loading control.

(D) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with different concentrations of IL6 (0, 0.2, 2, 4ng/ml) for 24 h using anti-PTEN antibodies. GAPDH was used as a loading control.

(E) Quantitative real-time PCR (qRT-PCR) measuring PTEN mRNA levels in SK-BR3 (left) and BT474 (right) cells treated with control, CAF11-CM or NAF1-CM for 24 h. Data were normalized to GAPDH mRNA levels and expressed as fold change compared with controls (log2 scale). Data are shown as mean ± SE of 3 independent replicates for each condition.

(F) Quantitative real-time PCR (qRT-PCR) measuring PTEN mRNA levels in SK-BR3 (left) and BT474 (right) cells treated with PBS or IL6 (4ng/ml) for 24 h. Data were normalized to GAPDH mRNA levels and expressed as fold change compared with controls (log2 scale). Data are shown as mean ± SE of 3 independent replicates for each condition.

(G) PTEN promoter activity after transfection of the full length construct (-1086/+14) alone or together with IL6 (4ng/ml). pGL3-Basic, control for promoter constructs;

The results are expressed as a relative ratio of firefly luciferase to Renilla luciferase. Three independent experiments were performed in triplicate.

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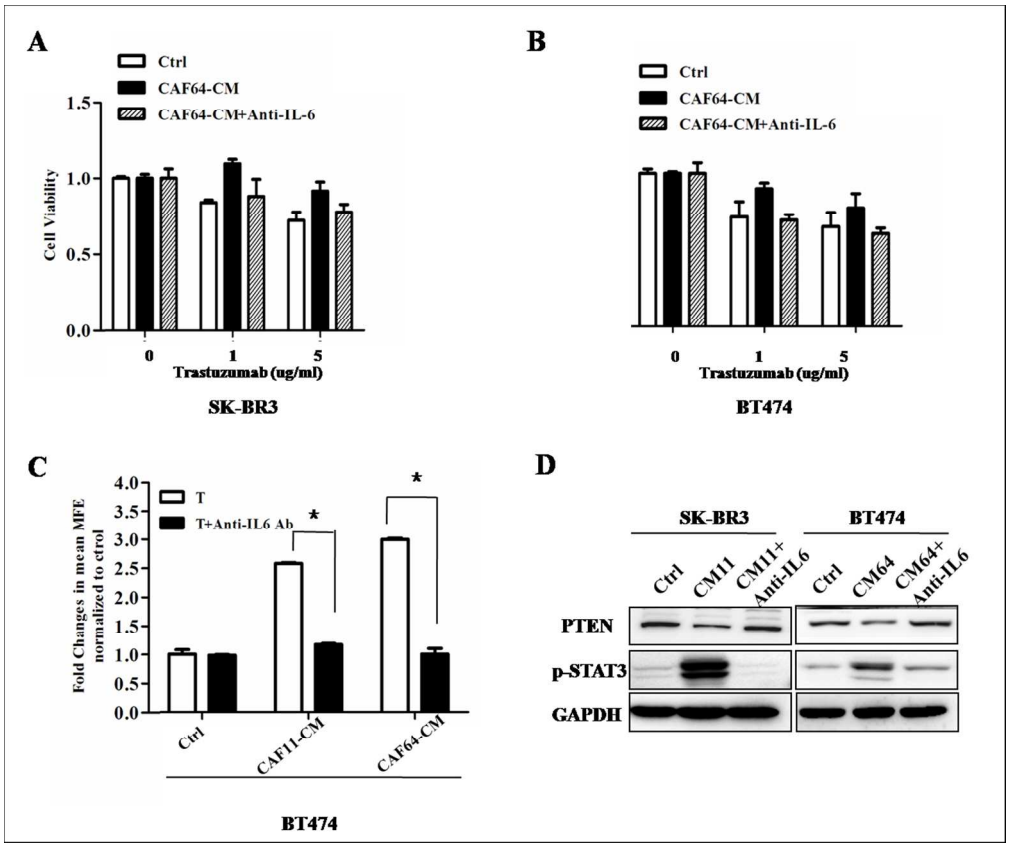

IL-6 neutralizing antibody decreased CSC population and restored trastuzumab sensitivity
(A) Cell viability assay in SK-BR3 (left) and BT474 (right) cells treated with different concentrations of trastuzumab after culture with conditioned media (CM) from control, CAF64 or CAF64 combined with IL6 neutralizing antibody (100ng/ml) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. ∗ indicates $p<0.05$.
(B) Relative changes of secondary tumor sphere in SK-BR3 (left) and BT474 (right) cells treated with trastuzumab (10ug/ml) alone or combined with IL6 neutralizing antibody (100ng/ml) after culture with conditioned media (CM) from control, CAF11 or CAF64 for 10 days. Three independent experiments were performed in triplicate.
(C) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells treated with trastuzumab (10ug/ml) after culture with conditioned media (CM) from control, CAF11 (CM11) , CAF64 (CM64) or combined with IL6 neutralizing antibody (100ng/ml) for 24h using indicated antibodies. GAPDH was used as a loading control.

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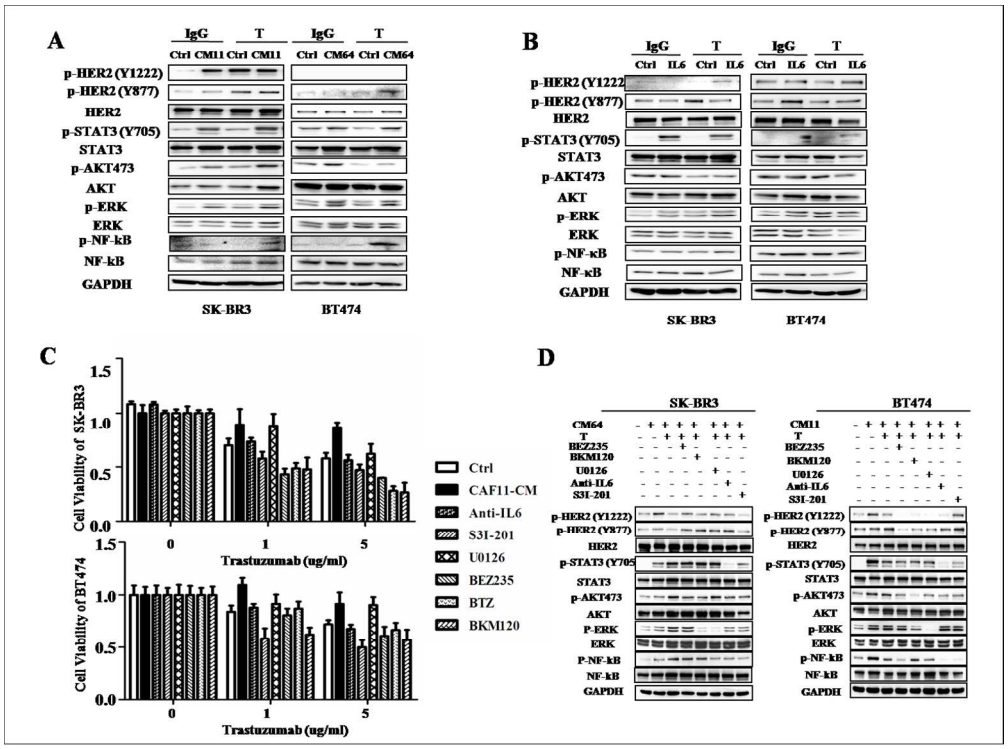

CAF-CM activated multiple pathways and combined trastuzumab with inhibitors of these pathways restored trastuzumab resistance.

(A) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with control or CM from CAF11 (CM11),CAF64 (CM64) and treated with trastuzumab (10ug/ml) or IgG for 24h using indicated antibodies. GAPDH was used as a loading control.

(B) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells cultured with PBS or IL6 (4ng/ml) and treated with trastuzumab (10ug/ml) or IgG for 24h using indicated antibodies. GAPDH was used as a loading control.

(C) Cell viability assay in SK-BR3 (left) and BT474 (right) cells cultured with control or CM from CAF11 and treated with a series of concentrations of trastuzumab with DMSO, IL-6 neutralizing antibody (100 ng/ml), STAT3 inhibitor S3I-201 (10 μM), BEZ235 (1μM) or BKM120 (1μM), NF-κB inhibitor Bortezomib (10 nM), MEK inhibitor U0126 (1μM) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means ± s.d. ∗ indicates $p<0.05$.

(D) Western blot analysis of lysates from SK-BR3 (left) and BT474 (right) cells treated with CM from CAF64 (CM64) and treated with trastuzumab (10ug/ml), or trastuzumab (10ug/ml) combined with IL-6 neutralizing antibody (100 ng/ml), STAT3 inhibitor S3I-201 (10 μM), BEZ235 (1μM) or BKM120 (1μM), NF-κB inhibitor Bortezomib (10 nM), MEK inhibitor U0126 (1μM) for 1 h using the indicated antibodies. GAPDH was used as a loading control.

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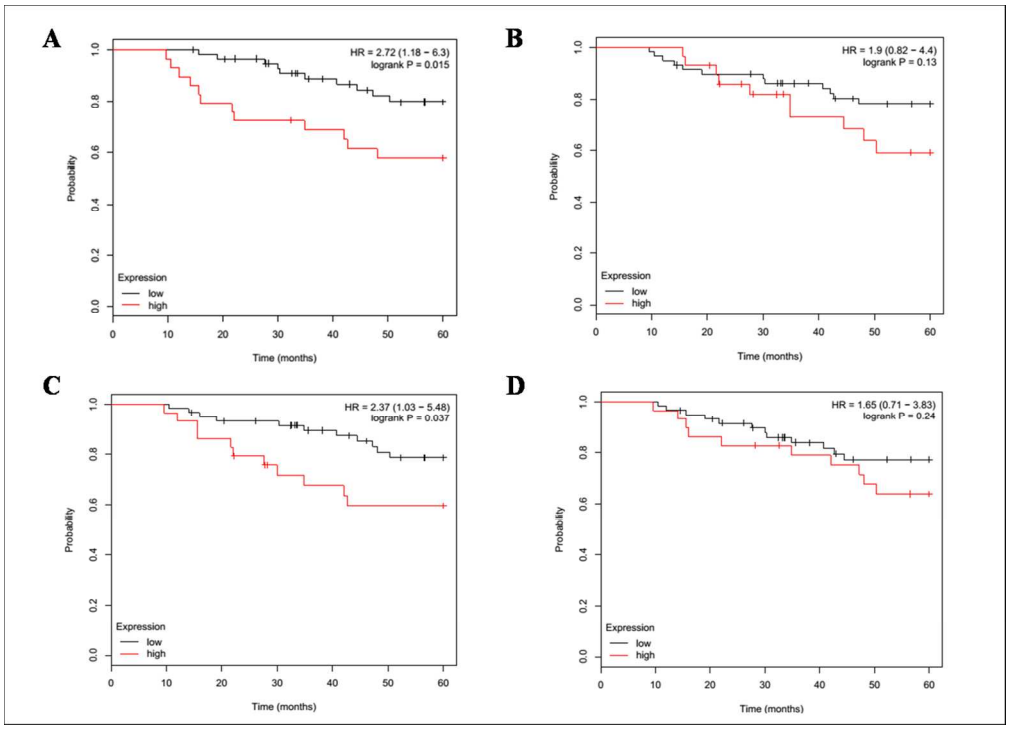

Survival outcome in HER2+ patients
5 year overall survival outcome affected by IL-6 (A), STAT3 (B), NF-κB (C) and AKT (D) gene expression in 88 HER2+ patients.

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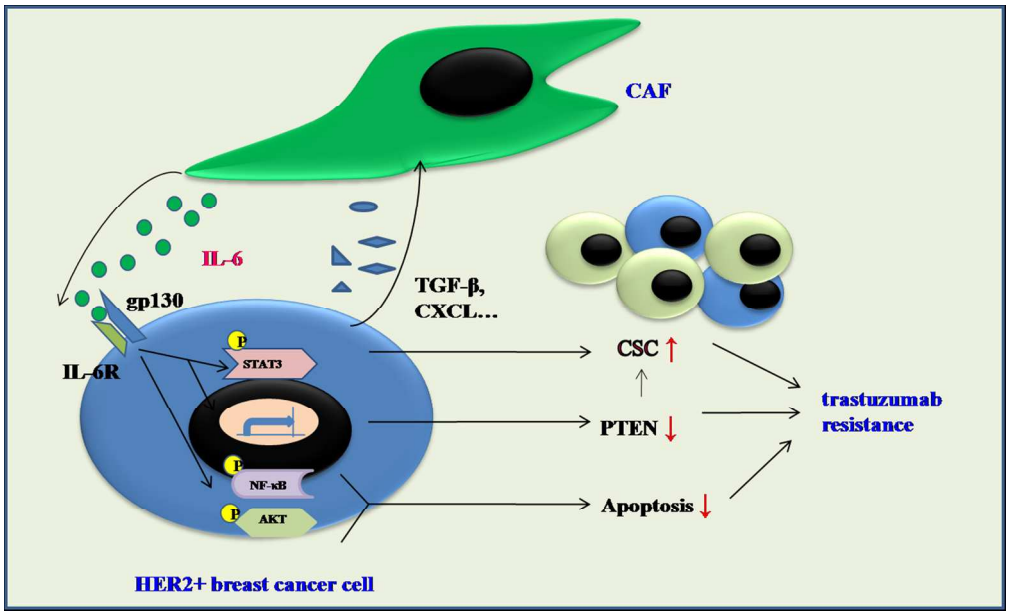

Schematic representation showing the role of CAFs in mediating trastuzumab resistance. IL-6 secreted from CAFs can increase CSC population by activating the JAK/STAT3 pathway and downregualting PTEN expression in HER2+ breast cancer cells, which induced trastuzumab resistance. Meanwhile, CAFs-CM also activate PI3K/AKT/mTOR, NF-κB pathways which may decrease apoptosis in HER2+ breast cancer cells and also contribute to trastuzumab resistance.

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