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ANXA2 could act as a moderator of EGFR-directed therapy resistance in triple negative breast cancer

Yue Zhang\textsuperscript{a}, Jiajia Bi\textsuperscript{b}, Hongtao Zhu\textsuperscript{a}, Mei Shi\textsuperscript{a} and Xianlu Zeng\textsuperscript{a}

\textsuperscript{a}School of Life Science, Institute of Genetics and Cytology, Northeast Normal University, Changchun, China; \textsuperscript{b}Synthetic Biology Engineering Lab of Henan Province, School of Life Sciences and Technology, Xinxiang Medical University, Xinxiang, China

**ABSTRACT**

Triple negative breast cancer (TNBC) patients cannot benefit from EGFR-targeted therapy even though the EGFR is highly expressed, because patients exhibit resistance to these drugs. Unfortunately, the molecular mechanisms remain relatively unknown. ANXA2, highly expressed in invasive breast cancer cells, is closely related with poor prognosis, and acts as a molecular switch to EGFR activation. In this study, MDA-MB-231 cells and MCF7 cells were used. Our results showed that ANXA2 expression is inversely correlated with cell sensitivity to gefitinib. Knockdown of ANXA2 expression in MDA-MB-231 cells increased the gefitinib induced cell death. When ANXA2 was overexpressed in MCF7 cells, the gefitinib induced cell death was decreased. Furthermore, we demonstrated that phosphorylation of ANXA2 at Tyr23 is negatively correlated with the sensitivity of TNBC to gefitinib. Altogether, our results suggest a new role of ANXA2 in regulating sensitivity of TNBC MDA-MB-231 cells to the EGFR inhibitor gefitinib.

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ANXA2; EGFR; TNBC; gefitinib; tyrosine phosphorylation

Triple negative breast cancer (TNBC) is characterized by the lack of estrogen, progesterone and HER2 receptor [1-3]. Patients with TNBC cannot be treated with endocrine therapy or targeted therapies due to lacking related receptors [4,5]. Such, only a small proportion of patients benefit from current cytotoxic chemotherapeutics [4]. Moreover, TNBC possesses aggressive phenotypes of high histological grade, metastatic potential and an earlier recurrence [6–9], and result in the poor prognosis [4]. Therefore, it is necessary to identify the molecular markers which will contribute to effective targeted therapies in TNBC.

Over the past decade, preclinical findings have suggested various protein targets and pathways for possible TNBC treatments, such as the epidermal growth factor receptor (EGFR). EGFR is highly expressed in approximately 50% of TNBC patients, and inversely correlated with the prognosis, making this receptor a potential therapeutic target for the treatment of this aggressive breast cancer [10,11]. There are two kinds of EGFR-targeted therapies, one targets the extracellular domain of the receptor by monoclonal antibodies, and the other one targets the kinase activity of the receptor by tyrosine kinase inhibitors, such as gefitinib [12,13]. Phase II clinical studies have reported that gefitinib exhibits antitumor activity in the patients with oestrogen receptor positive and EGFR positive primary breast cancer [14], but little therapeutic benefit has been noted in TNBC patients [15]. One proposed mechanism for this resistance to gefitinib in TNBC is crosstalk between EGFR and other signalling proteins [16–19]. However, the underlying mechanisms of gefitinib resistance in TNBC have not yet to be fully characterized.

Recently, ANXA2, a member of the Annexin family of calcium-dependent phospholipid binding proteins, has been demonstrated to colocalize with EGFR and play a role in EGF-induced EGFR homodimerization and phosphorylation [20,21]. Notably, gefitinib inhibits the activity of EGFR by inhibiting autophosphorylation of EGFR and blocks downstream signalling [22]. Furthermore, ANXA2 is highly expressed in TNBC and depletion of ANXA2 markedly impairs the migration, invasion and proliferation of highly invasive breast cancer cells [20,21]. However, whether ANXA2 functions in the sensitivity of TNBC to gefitinib is unclear.

In this study, we showed that knockdown of ANXA2 remarkably increased the sensitivity of TNBC to gefitinib, and ANXA2 phosphorylation at Tyrosine 23 in MCF7 cells decreased their sensitivity to gefitinib significantly. Our results suggest that ANXA2 functions in the sensitivity of TNBC to EGFR tyrosine kinase inhibitors, and the effectiveness of EGFR inhibitors might be enhanced by targeting the ANXA2 phosphorylation at Tyrosine 23 simultaneously.
Materials and methods

Material

Rabbit polyclonal antibody to ANXA2 (H-50), purchased from Santa Cruz Biotechnology, was used in western blotting. Rabbit monoclonal antibody to ANXA2 (D11G2), purchased from Cell Signalling Technology, was used in immunofluorescence. Mouse monoclonal antibody to ANXA2 (3D5/4), purchased from novus biologicals, was used in immunofluorescence and immunoprecipitation. Mouse monoclonal antibody to ANXA2 (11. Tyr 24) was obtained from Santa Cruz Biotechnology. Rabbit monoclonal antibody to EGFR (D381B1) was obtained from Cell Signalling Technology. Normal mouse IgG2a (sc-3878) was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody to actin (AC-40) was purchased from Sigma Aldrich. DAPI (D1306) and FITC-conjugated anti-rabbit IgG antibody were obtained from Invitrogen. Gefitinib (4765) was purchased from Cell Signalling Technology. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS, G358) was purchased from Promega. PP2 (sc-202,769) was purchased from Santa Cruz Biotechnology. The protein A/G-Sepharose beads and ECL Plus Western blotting reagents (RPN2232) were purchased from GE Healthcare Life Sciences.

Cell culture

MCF7 and MDA-MB-231 cells were purchased from the Cell Bank of type culture collection of Chinese Academy of Science (Shanghai, China). MCF7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), and MDA-MB-231 cells were maintained in L15 medium containing 10% FBS.

Small interference RNA (siRNA)

siRNA approaches were used to knockdown endogenous ANXA2. The sense and antisense sequences of ANXA2 siRNA oligonucleotides were 5'-AGACCAAAGGUGGGAGAUU-3' and 5'-UCAUUCACCACCUUGGCUUU-3'. Control cells were transfected with unspecific oligonucleotides, the sense and antisense sequences of unspecific oligonucleotides were 5'-UUCUCCGAACGUGACACGUTT-3' and 5'-ACGUGACACGUUCGAGAATT-3'. SiRNA duplex oligonucleotides were synthesized by GenePharma (China). Transfection of siRNA duplexes was performed using the Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions. Experiments were performed 48 h after transfection.

Lentiviral infection and establishment of cell lines

Producing Lentiviral Particles: Day 1, HEK-293T cells were plated in DMEM + 10% FBS without antibiotics in a 6 cm tissue culture plate, cells were incubated at 37°C, 5% CO₂ overnight. Day 2, the transfection was performed by making a cocktail for each transfection (1 μg pCDH-CMV-plasmid, 750 ng psPAX2 packaging plasmid and 250 ng pMD2.G envelope plasmid to 20 μL serum-free OPTI-MEM). Transfection was performed using the Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions. Cells were incubated at 37°C, 5% CO₂ for 12–15 hours. Day 3, changing the media of the cells to remove the transfection reagent. Cells were incubated at 37°C, 5% CO₂ for 24 hours. Day 4, the media was harvested from cells and transferred to a polystyrene storage tube. The media containing the lentiviral particles was filtered through a 0.45 μm filter to remove the cells and then stored at −80°C.

Infecing Target Cells: Day 1, target cells were plated in 6 cm plate and grow at 37°C, 5% CO₂ overnight. Day 2, Target cells should be approximately 70% confluent. Changing to fresh culture media for the cells. Lentiviral particle solution was added in volume ratio of 1:1 with the culture media. Cells were incubated at 37°C, 5% CO₂ overnight. Changing to fresh media for the cells after 24 hours.

Selections by puromycin: First step is determining the Optimal Puromycin concentration for cells. Puromycin was diluted in the preferred culture media for target cells. The final concentration of puromycin should be from 1–10 μg/mL in 1 μg/mL increments. The target cells should be approximately 80–90% confluent. Examine cells each day and change to fresh puromycin-containing media every other day. The minimum concentration of puromycin that results in complete cell death after 3–5 days is the concentration that should be used for selection cells. Second step is adding puromycin to the media at the concentration determined, changing to fresh puromycin-containing media for the cells as needed every few days. Experiments were performed after cells screened by puromycin (1μg/mL).

Immunofluorescence

Cells growing on glass coverslips in 24-well plates were fixed with 10% formaldehyde for 10 min, blocked with blocking buffer (1×PBS/5% normal serum/0.3% Triton X-100) for 60 min and then stained with primary antibodies at 4°C overnight, followed by secondary antibodies at 22°C for 60 min. The cells were then washed for three times and mounted on the stage of a confocal microscope (Olympus, Japan). Images were collected and analysed using the Olympus confocal software.
**Immunoprecipitation**

Cells were cultured overnight and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na3VO4, 1 mM β-glycerophosphate, 1 mM PMSF, and 20 μg/mL aprotinin/leupeptin) on ice for 30 min, followed by centrifugation at 12,000 g for 30 min. The supernatant was collected and incubated with the indicated antibodies at 4°C for 3 h, after that the supernatant was incubated with 30 μl of protein A/G-Sepharose beads for another 3 h. Wash the beads with lysis buffer for three times by centrifugation at 2,000 rpm for 3 min every time. Isopyknic 2xloading buffer was added into the beads and boiled for 5 min.

**Western blotting**

Briefly, the cells were washed in ice-cold PBS and lysed in lysis buffer. After incubation on ice for 30 min, the lysates were centrifuged at 12,000 × g for 30 min. Lysates from MDA-MB-231 cells and MCF7 cells were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% fat-free milk for 45 min, and then incubated with the primary antibodies for 60 min prior to detection with secondary antibodies. Chemiluminescent detection was performed by using ECL Plus Western blotting reagents.

**The MTS assay**

The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. Day1, cells were seeded into 96-well culture plate to 80% confluence and grown in DMEM/L15 medium supplemented with 10% FBS. Day 2, the medium for the cells was changed and the new medium is DMEM/L15 supplemented with 2% FBS and different concentration of gefitinib (6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM). Day 3 to 5, the cells were incubated at 37°C for 72 h, and control groups were incubated with DMSO in equivalent concentration to corresponding treatment groups in the meantime. Day 6, cells were incubated with MTS, which was dissolved in DMEM/L15 in ratio of 1:50, at 37°C for 4 h, then the absorbance at 490 nm was measured using a ELx800 absorbance microplate reader (BioTek™ Instruments, VT, USA). Optical density values of the control cells were calculated as 100% viability. The absorbance is in proportion to the number of living cells in a sample. Each test was repeated for six times.

**Statistical analysis**

Statistical analysis was performed using a 2-way ANOVA followed by a Bonferroni’s multiple comparison test using GraphPad Prism version 5 (San Diego, CA). The calculation and t-test analysis of IC50 value were also performed using GraphPad Prism version 5.

**Result**

**ANXA2 expression in the TNBC is inversely correlated with cell sensitivity to gefitinib**

The EGFR is highly expressed in the majority of TNBC patients, but these patients exhibit resistance to EGFR inhibitor [10,12]. ANXA2, an activator of EGFR, is also highly expressed in TNBC [20,21,23]. We wonder whether ANXA2 plays a role in the sensitivity of TNBC to EGFR inhibitor. The two cell lines with different expression level of ANXA2 were used in our experiments. MDA-MB-231 cells express high level of ANXA2, and MCF7 cells, which belong to other subtype of breast cancer, only express limited amount of ANXA2 (Figure 1(a)). To investigate whether this difference in ANXA2 expression is related to the sensitivity of TNBC to EGFR inhibitors, the MTS assay was performed in MDA-MB-231 cells and MCF7 cells to detect the cell viability at different dose of gefitinib (6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM). As shown in Figure 1(b), with the treatment of 25 μM gefitinib, the viability of MDA-MB-231 cells was much higher than MCF7 cells, and the apoptosis of the two cell lines was also significantly different under 12.5 μM and 25 μM of gefitinib (Figure 1(c)). Figure 1(d–e) showed that the mean IC50 value for gefitinib treatment were 24.99 μM for MDA-MB-231 and 17.60 μM for MCF7, respectively. These results suggest that ANXA2 expression is inversely correlated with TNBC cells sensitivity to gefitinib.

**ANXA2 knockdown increases the sensitivity of TNBC cells to gefitinib treatment**

To further investigate the role of ANXA2, siRNA was used to knockdown ANXA2 in MDA-MB-231 cells. The knockdown efficiency was shown in Figure 2(a). Then the viability of cells treated under different dose of gefitinib were analysed by MTS assay. After ANXA2 knockdown, the TNBC cells exhibited better sensitivity to gefitinib treatment. At the same dose of gefitinib (6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM), the viability of ANXA2 knockdown cells were obviously lower than the one of ANXA2 normal cells (Figure 2(b)). The apoptosis experiments also showed significant difference under these doses of gefitinib (Figure 2(c)). The mean IC50 value for gefitinib treatment were 24.93 μM in control cells and...
12.77 μM in ANXA2 siRNA transfected cells, respectively (Figure 2(d, e)). Notably, these results indicate that ANXA2 knockdown can effectively increase the sensitivity of TNBC cells to gefitinib treatment.

Overexpression of ANXA2 decreases the sensitivity of MCF7 cells to gefitinib

MCF7 cells have been reported to express low level of ANXA2 and to be sensitive to gefitinib [24]. To further test the role of ANXA2 in cell resistance to gefitinib, a lentivirus was used to constitutively express ANXA2WT in MCF7 cells. The MCF7 cells transfected with ANXA2WT were termed as ANXA2WT. The transfect efficiency was detect by immunofluorescence. The result showed that ANXA2 was overexpressed in ANXA2WT cells (Figure 3(a)). Then the viability and apoptosis of the transfected cells and untransfected cells were analysed. As shown in Figure 3(b,c), the cells survived better under gefitinib after overexpressing ANXA2, especially under the dose of 12.5 μM. The mean IC50 of control cells and ANXA2WT cells changed from 17.54 μM to 20.03 μM (Figure 3(d,e)). These results indicate that the sensitivity of MCF7 cells decreased after overexpression of ANXA2.

ANXA2 exists in the same protein complex with EGFR

Previous studies have reported that ANXA2 regulated EGFR homodimerization and phosphorylation [21]. Whether ANXA2 plays roles in the sensitivity of TNBC to gefitinib through EGFR? To investigate the relationship between ANXA2 and EGFR, the colocalization status of the two proteins was observed by using the experiment of immunofluorescence. As shown in Figure 4(a), ANXA2 colocalized with EGFR at the cell surface. To determine the interaction status of ANXA2 and EGFR, the experiment of immunoprecipitation was performed, the result showed that ANXA2 and EGFR were in the same protein complex (Figure 4(b)). These results suggest that the interaction of ANXA2 with EGFR seems to be a partial mechanism of ANXA2 regulating resistance of TNBCs to EGFR-mediated therapy.
Phosphorylation of ANXA2 at Tyr23 makes MCF7 cells resistant to gefitinib treatment

The phosphorylation of ANXA2 at Tyr23 is critical for most of its functions [23,25–27]. To define the Tyr23 phosphorylation status of ANXA2 in TNBC cells, western blotting experiment was performed. As shown in Figure 5(a), Tyr23 was phosphorylated in MDA-MB-231 cells, the level of phosphorylation decreased after treatment with PP2, an inhibitor of Src family kinases. The result indicates that Tyr23 of ANXA2 is phosphorylated in MDA-MB-231 cells, and Src kinase plays a role in the phosphorylation of ANXA2 at Tyr23. To confirm whether the phosphorylation functions in the sensitivity to gefitinib, two mutants, ANXA2Y23A and ANXA2Y23E were constructed. ANXA2Y23A was a mutant in which Tyr23 was altered to an alanine residue to make a non-phosphorylation site, and ANXA2Y23E was another mutant in which Tyr23 was altered to a glutamic acid residue to mimic constitutive phosphorylation [28]. Then the two plasmids were transfected into MCF7 cells, respectively. The two transfected cells were termed as ANXA2Y23A and ANXA2Y23E respectively. It is reported that ANXA2 translocated to cell surface after phosphorylation at Tyr23 [28]. So the location of ANXA2 in the cells was checked by the experiment of immunofluorescence. Figure 5(b) showed that the ANXA2Y23A is mainly localized in the cytoplasm, whereas ANXA2Y23E is predominantly localized on the cell surface of MCF7 cells. After overexpression of ANXA2Y23E, the cells got resistance to gefitinib, and when compared ANXA2Y23E cells with control cells, the viability increases from 20% to 80% at the dose of 25 μM and from 15% to 45% at the dose of 50 μM. While when compared ANXA2Y23A cells with control cells, the viability changed at the dose of 12.5 μM (Figure 5(d)). The ANXA2Y23E cells significantly reduced apoptosis at doses of gefitinib ranging from 12.5 μM to 100 μM, when compared with control cells. While a decrease in apoptosis was observed in ANXA2Y23A cells treated with 12.5 μM of gefitinib, and cell apoptosis was at the same level at doses of gefitinib ranging from 25 μM to 200 μM (Figure 5(d)). The mean IC50 value of MCF7, ANXA2Y23A and ANXA2Y23E is...
17.51 μM, 19.05 μM and 48.42 μM, respectively (Figure 5(e, f)). These results suggest that Tyr23 phosphorylation is crucial for the function of ANXA2 in cell resistance to gefitinib. In addition, we noted that the expression level of ANXA2 also participated in the regulation of cells resistance to gefitinib. Overexpression of ANXA2 increases the resistance of MCF7 cells to gefitinib. (a) The overexpression of ANXA2 was detected by immunofluorescence. Cells were cultured on coverslips and stained with anti-ANXA2 antibody followed by FITC labelled second antibody. Scale bar = 10 μm. Images were collected and analysed using the Olympus confocal software (60×). (b) The viability of MCF7 cells transfected with controls or ANXA2WT plasmid were analysed by MTS assay. The cells were treated with indicated concentrations of gefitinib at 37°C for 72 h. Then percent of cell viability was analysed by GraphPad Prism version 5. (c) Two-way ANOVA analysis of cell apoptosis rate of MCF7 cells transfected with controls or ANXA2WT plasmid obtained from three independent experiments was performed. (d–e). The IC50 value was determined by cytotoxicity assay in MCF7 cells transfected with controls or ANXA2WT plasmid, and the calculation and t-test analysis of IC50 value were performed using GraphPad Prism version 5. ** means P < 0.01, *** means P < 0.001.

ANXA2 was in the same protein complex with EGFR. (a) The colocation status was detected by the experiment of immunofluorescence. MDA-MB-231 cells were cultured on coverslips and stained with anti-ANXA2 antibody followed by TRITC labelled second antibody. The coverslips were washed three times with PBS, then stained with anti-EGFR antibody followed by FITC labelled second antibody. Scale bar = 10 μm. Images were collected and analysed using the Olympus confocal software (60×). “Zoom” was an enlargement of the area in the white square in the merge image. (b) The interaction status between ANXA2 and EGFR in MDA-MB-231 cells were detected by the experiment of immunoprecipitation. Mouse anti-ANXA2 Antibody was used to immunoprecipitation ANXA2 from cell lysates of MDA-MB-231 cells. Immunoblots were probed with rabbit anti-EGFR antibody.
gefitinib. Thus, targeting ANXA2, especially the phosphorylation of ANXA2, might significantly improve the therapeutic efficacy of gefitinib.

**Discussion**

Although EGFR inhibitors are still a therapeutic choice for TNBC, the resistance of TNBC to these inhibitors has limited their broad use in patients. Thus, to understand the mechanisms mediating the resistance is very necessary. In TNBC, ANXA2 is highly expressed and correlated with the poor prognosis [21]. Previous studies reported that ANXA2 antibody inhibits the EGF-induced EGFR homodimerization and phosphorylation [20,21]. ANXA2 was also implicated in multi-drug-resistance in gastric cancer through p38MAPK and AKT pathway.

![Figure 5](image_url)

**Figure 5.** Phosphorylation of ANXA2 at Tyr23 makes MCF7 cells resistant to gefitinib treatment. (a). The phosphorylation status of ANXA2 at Tyr23 was detected by western blotting. Cells were treated by 5 μM PP2 at 37°C for 1 h, and β-Actin was used as the loading control. (b). The expression and location of ANXA2 was detected by the experiment of immunofluorescence. Cells were cultured on coverslips and stained with anti-ANXA2 antibody followed by FITC labelled second antibody. Scale bar = 10 μm. Images were collected and analysed using the Olympus confocal software (60×). (c). The viability of MCF7 cells transfected with control, ANXA2Y23A or ANXA2Y23E plasmid were analysed by MTS assay. The cells were treated with indicated concentrations of gefitinib at 37°C for 72 h. Then percent of cell viability was analysed by GraphPad Prism version 5. D. Two-way ANOVA analysis of cell apoptosis rate of MCF7 cells transfected with plasmid of control, ANXA2Y23A or ANXA2Y23E obtained from three independent experiments was performed. E-F. The IC50 value was determined by cytotoxicity assay in MCF7 cells transfected with plasmids of controls, ANXA2Y23A, ANXA2Y23E respectively, and the calculation and t-test analysis of IC50 value were performed using GraphPad Prism version 5. * means P < 0.05, *** means P < 0.001.
[29], and in reducing sensitivities to irradiation and chemotherapeutic drugs in nasopharyngeal carcinoma [30]. However, whether ANXA2 is involved in the drug resistance of targeted therapy has not been reported to date. Here, we showed the high expression of ANXA2 was inversely correlated with the sensitivity of TNBC to gefitinib. Knockdown of ANXA2 significantly enhanced the sensitivity of MDA-MB-231 cells to gefitinib. Therefore, ANXA2 affects the prognosis of TNBC patients not only through improving tumor metastasis and invasion [23,25,28], but also through decreasing the sensitivity of targeted therapy.

Tyr23 phosphorylation of ANXA2 functions throughout tumor progression, such as malignant transformation and epithelial-mesenchymal transition (EMT), tumor metastasis and invasion [28]. Previous researches showed that after phosphorylation, ANXA2 is required for Rho-regulated EMT process in mouse model of pancreatic cancer [28,31,32]. Tyr23 phosphorylated ANXA2 has also been reported to enhance STAT3 phosphorylation and promote proliferation, as well as migration and invasion, of breast cancer cells [32]. In this study, our results showed a novel function of Tyr23 phosphorylation of ANXA2 in regulating sensitivity of TNBC to gefitinib. That is, Tyr23 phosphorylation of ANXA2 plays a crucial role in increasing the cells resistance to gefitinib. In addition, we also found the expression level of ANXA2 also participates in the regulation of cells resistance to gefitinib. Thus, ANXA2 plays a complex role in mediating of gefitinib resistance in TNBC. The mechanism by which phosphorylation of ANXA2 at Tyr23 plays a crucial role in gefitinib resistance is still unclear. Our results indicated that ANXA2 colocalized with EGFR at the cell surface. An early study showed that ANXA2Y23E could make protein complex with EGFR, ANXA2Y23A was not in the same protein complex with EGFR [20]. Thus, we infer that phosphorylation of ANXA2 at Tyr23 is necessary for the interaction of ANXA2 with EGFR.

An early study showed that ANXA2 was a Src kinase substrate [33]. Our result of western blotting experiment shows that ANXA2 is phosphorylated in MDA-MB-231 cells, but phosphorylation of ANXA2 at Tyr23 was not completely inhibited by PP2, suggesting that Tyr23 phosphorylation of ANXA2 could be regulated by different kinases in MDA-MB-231 cells. Our speculation was supported by the report of Karasik, A, et al, in which they point out that Tyr23 of ANXA2 could also be phosphorylated by Insulin receptor tyrosine kinase [34].

Gefitinib was thought to inhibit cellular proliferation and tumour growth in human non-small cell cancer (NSCLC) dose-dependently [22], and potentiated the cytotoxic effects of chemotherapy and/or radiation. Previous study set the gefitinib dose as geometric progression (0 µM, 0.1 µM, 1 µM, 10 µM, 100 µM) to investigate the IC50 value of breast cancer cell lines [35]. In the present research, to explore the viability of cells at higher concentration, we set series concentration of gefitinib (6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM). At doses of gefitinib ranging from 6.25 µM to 100 µM, gefitinib inhibits cells with different ANXA2 expression and activity dose-dependently. Unfortunately, the viability of cell lines with different ANXA2 expression and activity under 200 µM of gefitinib was all 0%, it seems that 200 µM of gefitinib is too much for these cells to survive, the effect of gefitinib was regardless of ANXA2 expression and activity status at dose of 200 µM.

Even though the mechanism that ANXA2 regulates EGFR-directed therapy resistance in TNBC is still unclear, based on our and other reports, it is very possible that limiting Tyr23 phosphorylation of ANXA2 could improve the therapeutic efficacy of EGFR inhibitors, and inhibit the proliferation, as well as migration and invasion of TNBC simultaneously.

Author Contribution
Xianlu Zeng and Yue Zhang designed the study. Yue Zhang, Jiajia Bi, Hontao Zhu and Mei Shi carried out the research experiments. Xianlu Zeng and Yue Zhang wrote the manuscript. Jiajia Bi edited and gave scientific advice. All authors reviewed the results and approved the final version of the manuscript.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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