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# Expression of the CD59 Glycoprotein Precursor is Upregulated in an Estrogen Receptor-alpha (ER- $\alpha$ )-Negative and a Tamoxifen-Resistant Breast Cancer Cell Line *In Vitro*

Authors' Contribution:  
Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

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**Background:** Breast cancer is the most prevalent cancer and the leading cause of cancer death among women. Tamoxifen (TAM) therapy is one of the most widely and successfully used endocrine treatments for estrogen receptor  $\alpha$  (ER $\alpha$ )-positive breast cancer. However, resistance to TAM has been a major challenge. In addition, the mechanisms underlying endocrine resistance remain unclear. Here, we report that CD59, a phosphatidylinositol-anchored glycoprotein, is a candidate resistant gene for TAM therapies.

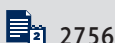
**Material/Methods:** The breast cancer cell line MCF-7, the MCF-10A cell line, and the TAM-resistant breast cancer cell line TAMR-MCF-7 were cultured. The TAMR-MCF-7 cells were transfected with CD59 siRNA and control siRNA. Then, the CD59 glycoprotein precursor expression was detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Cell counting kit-8 and flow cytometry assay were performed to examine cell proliferation, cell apoptosis, and cell cycle. In addition, the expressions of Bax, Bcl2, cleaved-caspase-8, cleaved-caspase-6, cleaved-caspase-3, and cleaved-PARP were analyzed by western blot analysis in the TAMR-MCF-7 cells treated with CD59 siRNA.

**Results:** In the present study, we found that the CD59 glycoprotein precursor was aberrantly upregulated in the ER $\alpha$ -negative breast cancer MCF-10A cells but not the MCF-7 cells. Furthermore, the CD59 glycoprotein precursor expression was elevated in the TAM-resistant breast cancer cells. Importantly, RNAi-mediated attenuation of CD59 was sufficient to rescue the resistance to TAM in the TAMR-MCF-7 cells.

**Conclusions:** In summary, our results proposed a candidate biomarker for predicting TAM resistance in ER $\alpha$ -positive breast cancer via targeting CD59, therefore it could be a novel therapeutic option.

**MeSH Keywords:** **Antigens, CD59 • Extensively Drug-Resistant Tuberculosis • Tamoxifen • Triple Negative Breast Neoplasms**

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## Background

Breast cancer is the most common cause of cancer-related death among women, with an estimated 252 710 new cases diagnosed in 2017 (30% of all malignancies in women); breast cancer ranks second overall in mortality (14%), with an estimated 40 610 deaths [1]. Therefore, it is very important to search for improved treatment option. At least 70% of breast cancers are classified as estrogen receptor (ER)-positive type [2] which renders ER $\alpha$  an ideal target for endocrine treatment. ER $\alpha$  is a nuclear receptor that exerts a profound influence on the progression and initiation of breast cancer [2–4]. Targeting the ER signaling pathway with tamoxifen (TAM), one of the most commonly used drugs in ER $\alpha$ -positive breast cancer, is efficacious in both prevention and treatment of breast cancer [5]. Unfortunately, one-third of women still develop recurrent disease in the next 15 years [6], and the major problem lies in TAM resistance.

At present, the mechanism of TAM resistance has not been fully elucidated. On one hand, with ER $\alpha$  phosphorylation, a substantial proportion of patients are intrinsically resistant to TAM therapy [7–9]. On the other hand, a significant number of patients with advanced disease eventually develop acquired resistance [10]. Although several miRNAs [11] and signaling pathways, including PI3K/mTOR/Akt, Hedgehog, HER2/ERB [12–14], are implicated in TAM resistance, there are few biological predictors for the TAM efficacy. Therefore, there is an urgent need for prognostic biomarkers in breast cancer TAM therapy.

CD59, a phosphatidylinositol-anchored glycoprotein, is a member of the cell membrane-bound complement regulatory proteins and has been demonstrated to overexpress in most solid tumors including breast cancer [15]. CD59 glycoprotein, also known as MAC-inhibitory protein (MAC-IP), membrane inhibitor of reactive lysis (MIRL), or protectin, is a protein that in humans is encoded by the *CD59* gene. CD59 blocks the terminal complement pathway and prevents the formation of the MAC [16]. In addition, *CD59* has been described as a prognostic biomarker in breast cancer [17,18]. In patients with B-cell malignancy, *CD59* expression is associated with resistance to rituximab treatment [19]. The targeting of tumor cells by trastuzumab or pertuzumab alone has little effect on the complement-dependent cytotoxicity (CDC) [20]. CD59 glycoprotein becomes attached to the cell membranes by a glycosphosphatidylinositol (GPI) glycolipid anchor. In addition, several previous studies have investigated the lack of CDC by including both complement decay-accelerating factor (CD55) and CD59 glycoprotein precursor expression on trastuzumab-induced CDC [20]. Some studies have suggested that *CD59* might be a candidate resistant gene in TAM therapies [21]. However, the exact role of *CD59* in breast cancer growth and

drug resistance remains unclear. Here, we investigated CD59 protein in TAM resistance and tried to regulate the protein in order to restrain the tumor resistance.

## Material and Methods

### Cell culture and reagents

The breast cancer cell line MCF-7 and the MCF-10A cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM, Solarbio Life Sciences) containing 10% fetal bovine serum (FBS, Solarbio Life Sciences). 1% (v/v) penicillin-streptomycin-amphotericin B mixture solution (Solarbio Life Sciences) was added to cells and then cultured in a 37°C-incubator supplemented with 95% humidity and 5% CO<sub>2</sub>. Tamoxifen was purchased from Sigma-Aldrich Corporation (USA). TAM-resistant breast cancer cell line TAMR-MCF-7 cells were generated by exposing MCF-7 cells (1×10<sup>7</sup>) to TAM (1  $\mu$ M). TAMR-MCF-7 cells were maintained in RPMI 1640 supplemented with 1  $\mu$ M TAM.

### RNA interference

For *CD59* silencing, TAMR-MCF-7 cells were seeded in 96-well plate, transfected with CD59 siRNA and control siRNA (Thermo Fisher Scientific, Inc.) by Lipofectamine RNAiMAX Transfection Reagent (Invitrogen™), sustained for 72 hours. Experimental grouping: CD59 siRNA transfected TAMR-MCF-7 cells (siRNA) group, untransfected TAMR-MCF-7 cells (NC) group, and control siRNA transfected TAMR-MCF-7 cells (BL) group.

### TAM treatment

MCF-7 cells were seeded in 6-well plates and cultured overnight in serum-free phenol red medium. The following day, the culture medium was replaced with phenol red-free medium containing 10 nM/mL E2 (Sigma-Aldrich) with or without 100 nM/mL TAM.

### CCK-8 assay

Cell number was measured using the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Approximately 5×10<sup>3</sup> cells were seeded into 96-well plates for 24 hours, transfected with the indicated CD59 siRNA and incubated for 48 hours. Then 10  $\mu$ L CCK-8 solution was added into each well and the cells were incubated at 37°C for 2 hours. Absorbance was read at 450 nm using a Bio-Rad iMark plate reader.

## Flow cytometry assay

Cell apoptosis was assessed by FITC apoptosis detection kit (Oncogene Research Products, San Diego, CA, USA) in accordance with manufacturer's instructions. Samples were analyzed by a flow cytometry apparatus (Becton Dickinson FACS Vantage SE, San Jose, CA, USA). Dual analysis was adopted: necrotic cells were propidium iodide (PI)-positive, early apoptotic cells were Annexin-V-FITC-positive, cells at late apoptosis stage were positive for Annexin-V-FITC/PI. Cells ( $2 \times 10^5$ ) were harvested and washed twice with cold PBS, and then stained with either Annexin-V-FITC (10  $\mu$ L) or PI (10  $\mu$ L) were classified as live cells. After 15 minutes of incubation, the majority of live cells fell into FITC/PI negative area which indicated the gating strategy was correct in the current study. Cell number in each category was recorded.

## Western blotting

Radio immunoprecipitation assay lysis buffer (Gibco; Thermo Fisher Scientific, Inc.) was used to extract the total protein of cells and tissues. Then, proteins were separated by 8% SDS-PAGE and immediately transferred onto PVDF membranes, which were then immunoblotted with respective antibodies. Blots were developed with the SuperSignal™ West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific, Inc.) and the images were obtained by ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare Life Sciences). Relative expression was calculated by comparison with the internal reference protein. Each experiment was performed 3 times.

## Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cells were cultured in 6-well plates and total RNA was isolated using an Invitrogen TRIzol reagent (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Total RNA was then reverse-transcribed into cDNA using PrimeScript™ RT Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's instruction. qRT-PCR was performed at 95°C for an initial 10 minutes followed by 40 cycles of denaturation for 30 seconds at 95°C, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds with the SYBR Premix Ex Taq (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. GAPDH as the endogenous controls for calculating the CD59 genes miRNA expression. Following primers were used: CD59 forward primer, 5'-CAGTGCTACAACCTGCTAACC-3' and reverse primer, 5'-TGAGACACGCATCAAATCAGAT-3' (product size, 79 bp); for SYBR PrimeScript miRNA RT-PCR kit universal primer: GAPDH forward primer, 5'-ACAACCTTGGTATCGTGAAGG-3' and reverse

primer, 5'-GCCATCAGCCACAGTTTC-3', (product size, 101 bp) (Thermo Fisher Scientific, USA).

## Statistical analysis

Data are expressed as mean  $\pm$  SEM of triplicate samples. Data significance between 2 groups were analyzed using Student's *t*-test, while differences between multiple groups were assessed by one-way analysis of variance followed by the Dunnett's post hoc test. Statistical analysis was performed by IBM SPSS Statistics v20.0 (SPSS Inc., Chicago, IL, USA).  $P < 0.05$  was considered as a significant difference.

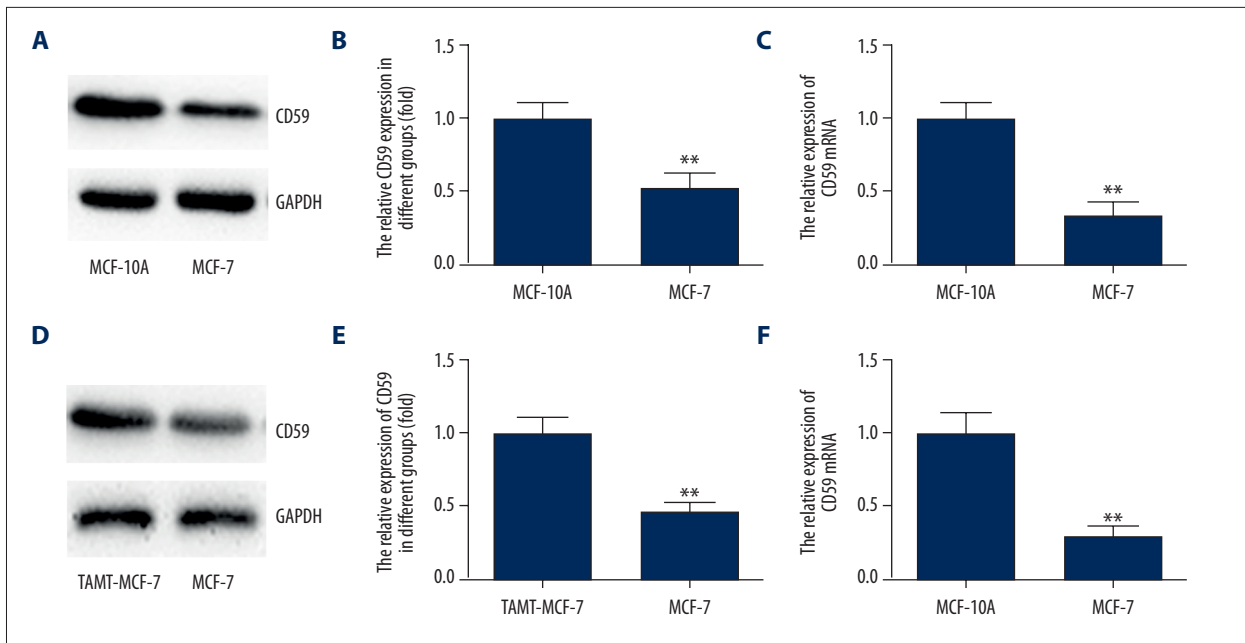
## Results

### CD59 expression was increased in TAM-resistant BC cells

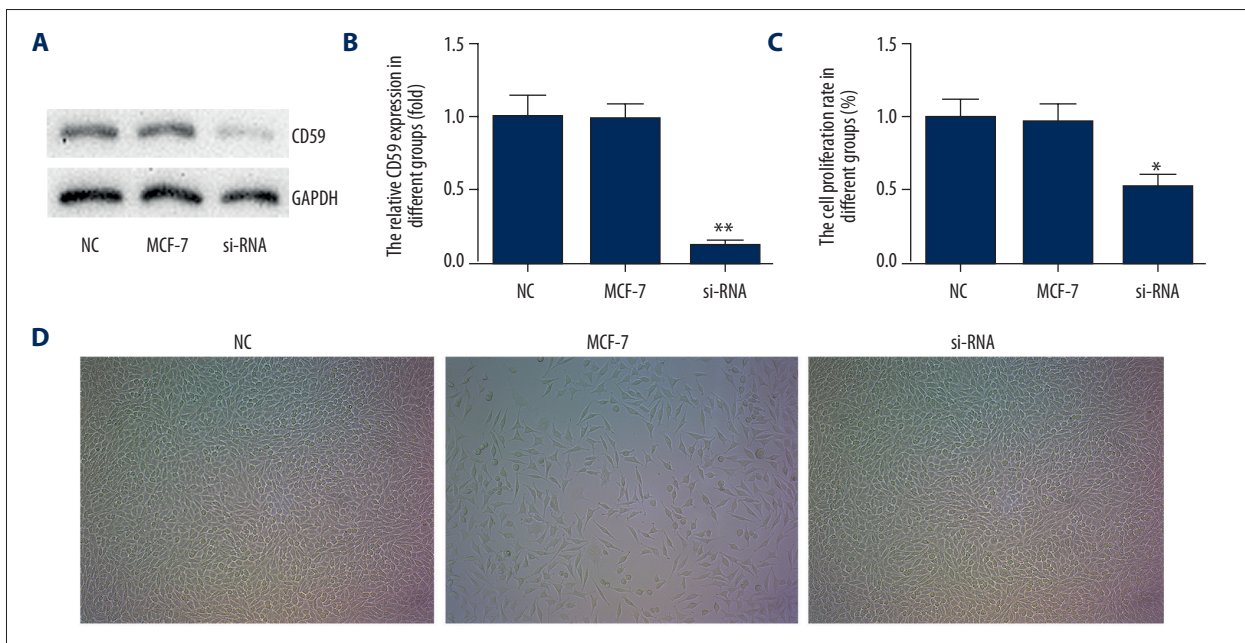
To explore the potential correlations between CD59 and ER $\alpha$  expression in breast cancer, immunoblotting assays and qRT-PCR were performed with total cell lysates in ER $\alpha$ -positive breast cancer cell line MCF-7 and ER $\alpha$ -negative breast cancer cell line MCF-10A. We found that the protein and mRNA level of CD59 were in much lower levels in MCF-7 cells compared with MCF-10A cells (Figure 1A–1C, \*\*  $P < 0.01$ ). To further explore the role of CD59 in TAM resistance, we detected the expression of CD59 in the MCF-7 and the TAMR-MCF-7 cells. As expected, the expression of CD59 in both protein and mRNA levels were much higher in the TAMR-MCF-7 cells (Figure 1D–1F, \*\*  $P < 0.01$ ). These findings showed that CD59 was increased in TAM-resistant breast cancer cells, suggesting that CD59 may play a key role in the drug resistance of TAM in MCF-7 cells.

### CD59 silencing partially promoted TAM sensitivity

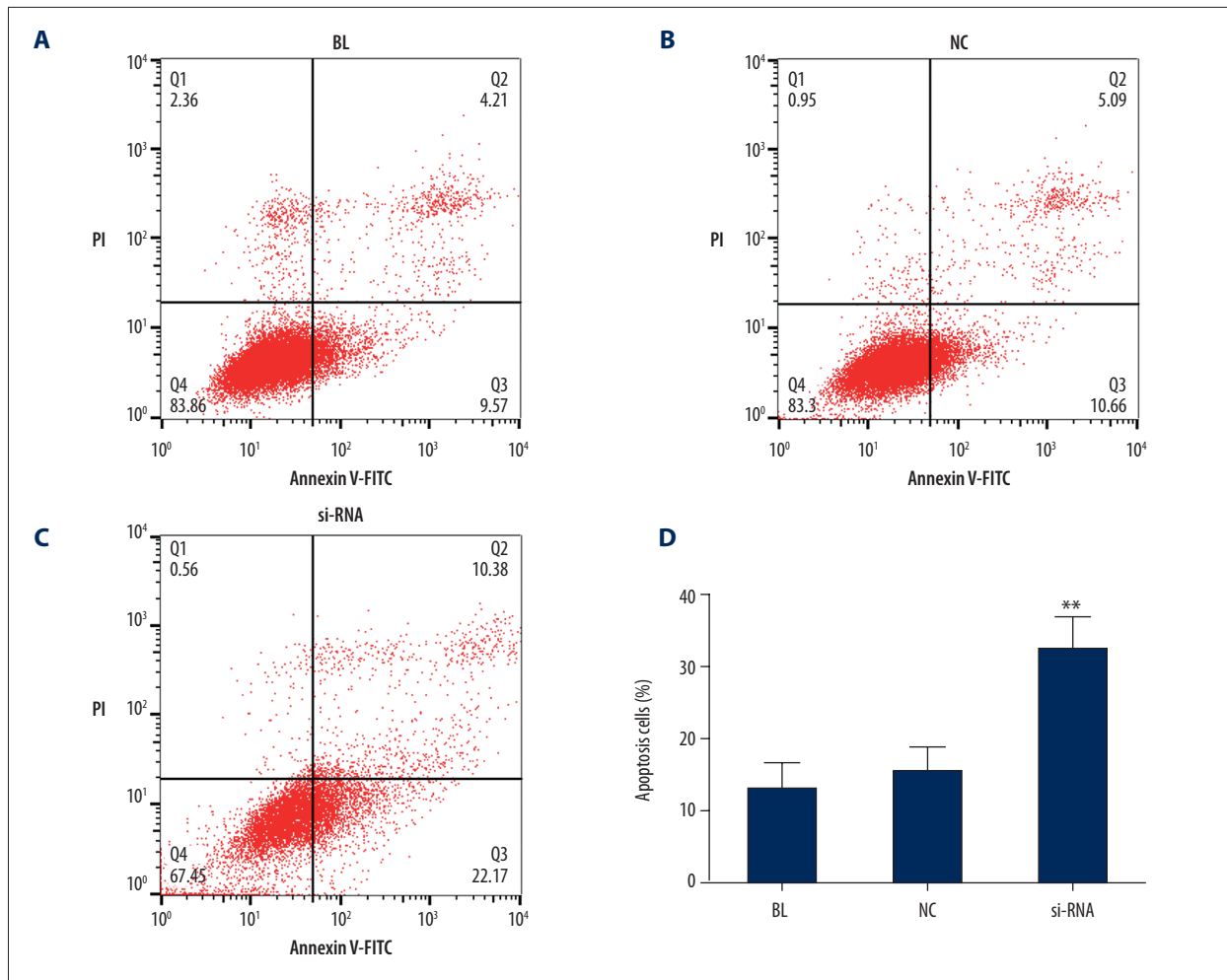
The association between CD59 and TAM resistance suggests that CD59 could be a new target for TAM anti-resistance. To determine whether CD59 specifically induces cell proliferation and inhibits apoptosis in TAMR-MCF-7 cells, CD59 knockdown cell model was established by transfecting with CD59 siRNA. First, CD59 expression was detected by western blot. As shown in Figure 2A and 2B, CD59 expression in the CD59 siRNA transfected group exhibited a dramatic decrease when contrasted to the BL and NC groups (\*\*  $P < 0.01$ ), indicating the CD59 knockdown TAMR-MCF-7 cells models were established successfully. To understand the functional role of CD59, the effect of CD59 on cell proliferation was evaluated using CCK-8 assay in TAMR-MCF-7 cells. Our results showed that CD59 silencing could suppress cell proliferation significantly (Figure 2C, 2D, \*\*  $P < 0.01$ ). This indicated that CD59 silencing could inhibit the growth of TAMR-MCF-7 cells.



**Figure 1.** The mRNA and protein expression levels of CD59 were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. (A, B) The protein expression of CD59 in the MCF-7 cell was decreased. (C) The mRNA levels of CD59 were decreased in the MCF-7 cells. \*\* $P < 0.01$ , MCF-7 vs. MCF-10A. The expression CD59 in both protein (D, E) and mRNA (F) levels were much higher in the TAMR-MCF-7 cells. \*\* $P < 0.01$ , MCF-7 vs. TAMR-MCF-7. All data are presented as the mean  $\pm$  standard deviation of 3 independent experiments. TAMR-MCF-7, MCF-7 cell treated with tamoxifen.



**Figure 2.** The protein expression of CD59 were determined by western blot analysis and cell proliferation was analyzed by cell counting kit-8 assay at 48 hours after transfection with CD59 siRNA. (A, B) CD59 expression in TAMR-MCF-7 cells was significantly decreased in the CD59 siRNA group. (C, D) The proliferation rate of TAMR-MCF-7 cells in the CD59 siRNA group was inhibited when compared with that in the NC and BL groups. Values are expressed as the mean  $\pm$  standard deviation of 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , CD59 siRNA vs. NC and BL. siRNA – CD59 small interfering RNA; NC – negative control; BL – control siRNA.



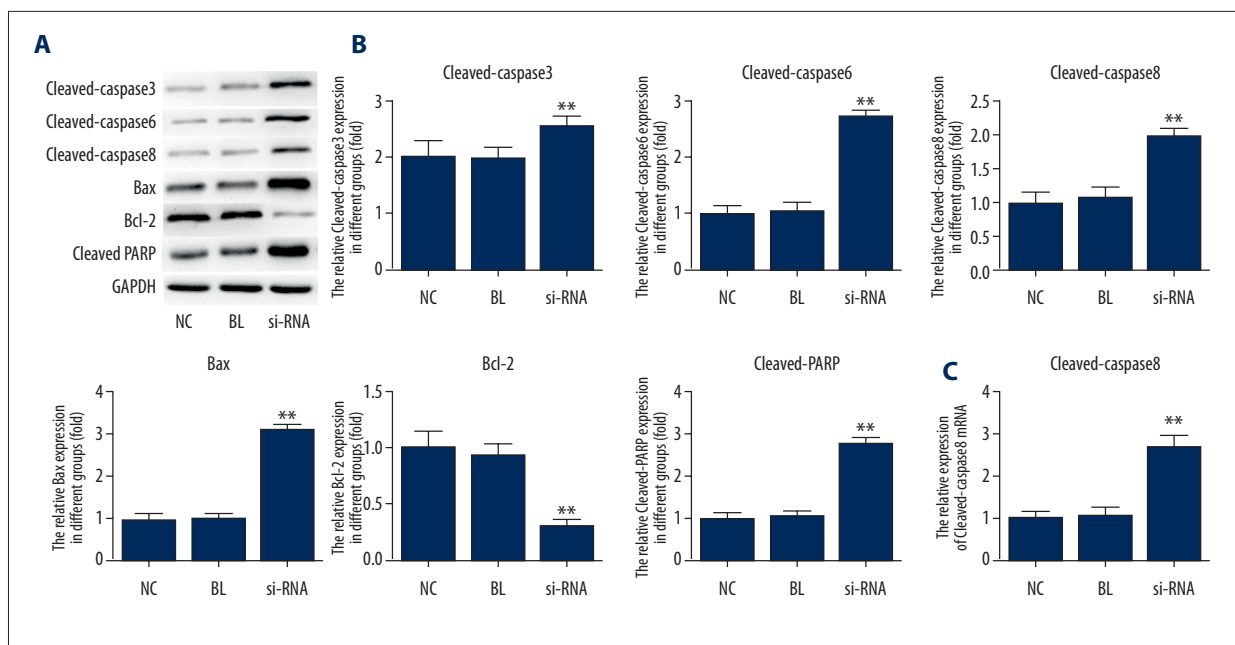
**Figure 3.** Knockdown of CD59 results in the apoptosis of TAMR-MCF-7 cells. After staining with Annexin V-FITC and PI, apoptotic cells were analyzed using a flow cytometer. **(A)** The percentages of apoptotic cells were displayed in the bar graph. **(B–D)** Annexin V assay was performed 3 times and values are expressed as the mean  $\pm$  standard deviation of three independent experiments. \*\*  $P < 0.01$ , CD59 siRNA vs. NC and BL. siRNA – CD59 small interfering RNA; NC – negative control; BL – control siRNA.

### CD59 silencing induced cell apoptosis in TAMR-MCF-7 cells

As TAM is known to induce apoptosis in breast cancer cells [22], the potential roles of CD59 in TAMR-MCF-7 cell in cell apoptosis and cell cycle were analyzed using flow cytometry. In our study, the capability of apoptosis was also tested by flow cytometry. The results showed that the apoptotic percentage was higher in the CD59 silencing cells than in the BL and NC groups (Figure 3A–3D, \*\*  $P < 0.01$ ). These results demonstrated that the knockdown of CD59 resulted in the apoptosis of TAMR-MCF-7 cells. Thus, CD59 plays an important role in changing the response of MCF-7 cells to TAM.

### CD59 downregulation triggered apoptosis through caspases and PARP activation (CD59 downregulation was accompanied by changes in apoptosis-related genes)

The inhibition of cell proliferation should be associated with cells programmed death machinery activation. Changes in the cell apoptosis-related genes after downregulation of CD59 may assess the activation of programmed cell death. To explore the effect of CD59 siRNA on cell apoptosis, we studied some proteins that can act as inducers and effectors of the apoptotic pathway. Specifically, the expression of Bax, Bcl2, cleaved-caspase-8, cleaved-caspase-6, cleaved-caspase-3, and cleaved-PARP were analyzed by western blot analysis in TAMR-MCF-7 cells treated with CD59 siRNA and compared to BL and NC (Figure 4A). The reduction of the anti-apoptotic gene Bcl2 was found to be significant with CD59 siRNA treatment



**Figure 4.** Knockdown of CD59 promotes the protein expression related to cell apoptosis. The expressions of cleaved-caspase3, cleaved-caspase6, cleaved-caspase8, cleaved-PARP, and Bax (A, B), and decreases the protein expression of Bcl-2 (A, B) in the TAMR-MCF-7 cells transfected with CD59 siRNA. \*\*  $P < 0.01$ , CD59 siRNA vs. NC and BL. (C) Similar to the effect on protein expression, reverse transcription-quantitative polymerase chain reaction analysis indicated that knockdown of CD59 increased the mRNA expression of cleaved-caspase8 in TAMR-MCF-7 cells. All data are presented as the mean  $\pm$  standard deviation of 3 independent experiments. \*\*  $P < 0.01$ , CD59 siRNA vs. NC and BL. siRNA – CD59 small interfering RNA; NC – negative control; BL – control siRNA.

(Figure 4B, \*\*  $P < 0.01$ ). This status evolved into an increased ratio of Bax/Bcl2, which indicated the activation of cell apoptosis (Figure 4B, \*\*  $P < 0.01$ ). As the influence factors of cell programmed death, cleaved-caspase-8, cleaved-caspase-6, cleaved-caspase-3, and cleaved-PARP proteins were examined and the results showed that their cleaved-active form increased in the CD59-silenced TAMR-MCF-7 cells (Figure 4B, \*\*  $P < 0.01$ ). The result of cleaved-caspase-8 mRNA expression from RT-PCR measurement was consistent with that of western blot analysis (Figure 4C, \*\*  $P < 0.01$ ). These results demonstrated that the knockdown of CD59 induced cell active apoptosis through changes in apoptosis-related genes.

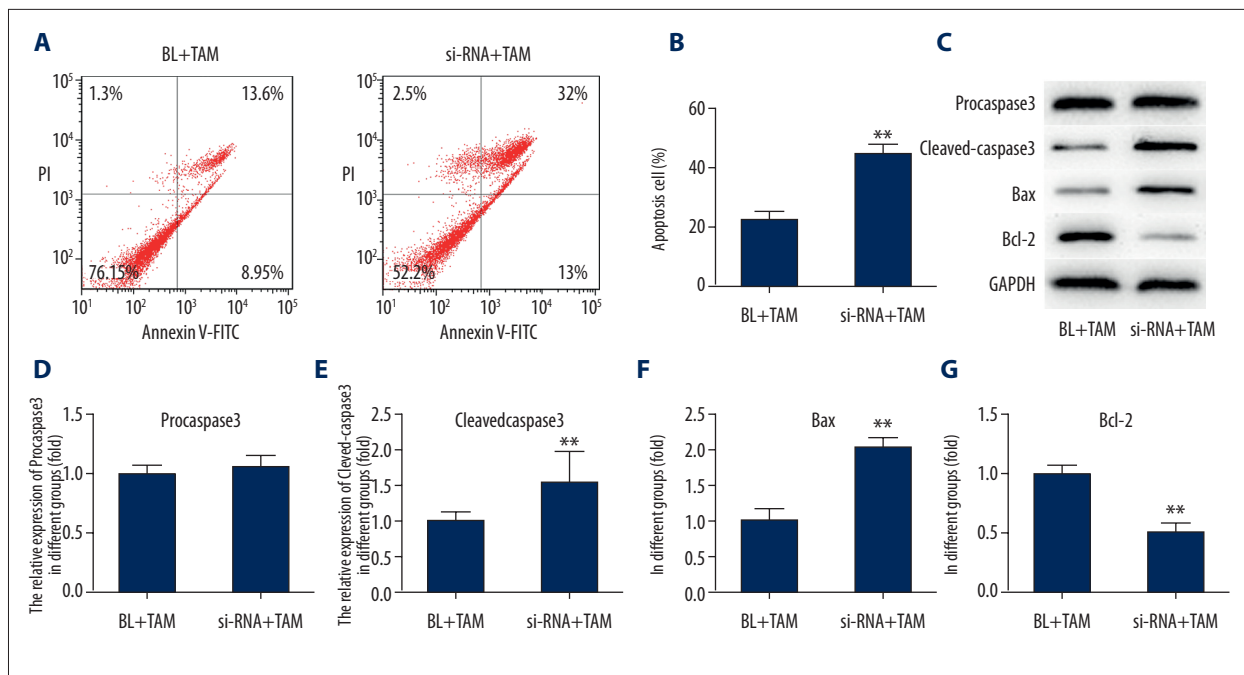
#### Attenuation of CD59 partially rescued the resistance to TAM in TAMR-MCF-7 cells by the activation of caspase-3 and an increase of Bax/Bcl2 ratio

In view of CD59 silencing resulting in decreased cell proliferation and induced cell apoptosis in TAMR-MCF-7 cells, we forecast that CD59 would play a therapeutically resistant role in TAM treatment. To understand the functions of CD59 upon TAM therapy, the impact of CD59 on cell apoptosis was detected using flow cytometry in the TAMR-MCF-7 cells. Transfecting control siRNA group (BL) and transfecting CD59 siRNA group were treated with 100 nM/mL TAM for 48 hours; cell apoptosis was

then analyzed under the same conditions. The results showed that the apoptotic percentage of CD59 siRNA group was higher than that in the BL group (Figure 5A, 5B, \*\*  $P < 0.01$ ). The pro-caspase-3, cleaved-caspase-3, Bax, and Bcl2 proteins were also detected by western blot (Figure 5C). The results showed that even if the procaspase-3 expression did not change (Figure 5D,  $P > 0.05$ ), its cleaved-active form of cleaved-caspase-3 increased in CD59-silenced cells (Figure 5E, \*\*  $P < 0.01$ ). The Bax/Bcl2 ratio also increased significantly in CD59-silenced cells (Figure 5F, 5G, \*\*  $P < 0.01$ ). This series of analyses showed that CD59 indeed plays a key role in altering the response of MCF-7 cells to TAM.

#### Discussion

The efficiency of CDCs for tumors depends primarily on the activation of the complement system, which is regulated by membrane-associated complement regulatory proteins (mCRPs), including complement decay accelerating factor (CD55) and CD59 glycoprotein precursor (CD59) [23]. Complement exerts its biological activity not only by its direct cytotoxic action but also by opsonizing target structures. Patients with breast cancer that overexpressed CD59 exhibited a higher relapse rate following trastuzumab treatment compared with those with low expression of CD59 [24].



**Figure 5.** CD59 downregulation rescues the resistance to tamoxifen in TAMR-MCF-7 cells. (A, B) Flow cytometry was conducted to detect the cell apoptosis, and cell apoptosis rate was calculated, knockdown of CD59 promotes the cell apoptosis of TAMR-MCF-7 cells transfected with CD59 siRNA. \*\*  $P < 0.01$ , CD59 siRNA+TAM vs. BL+TAM. All data are presented as the mean  $\pm$  standard deviation of 3 independent experiments. (C–G) Knockdown of CD59 increases the protein expression of cleaved-caspase3 and Bax/Bcl-2 ratio, \*\*  $P < 0.01$ , CD59 siRNA+TAM vs. BL+TAM, and has no effect on the expression of pro-caspase3,  $P > 0.05$ , CD59 siRNA+TAM vs. BL+TAM. siRNA – CD59 small interfering RNA; NC – negative control; BL – control siRNA; TAM – treated with tamoxifen.

Small interfering RNA (siRNA)-mediated RNA interference is currently the most effective method for specific gene silencing [25]. The application of siRNA technology *in vivo* is a rapidly development field with many challenges, including the development of transfection systems, instability and adverse reactions *in vivo*. siRNA plasmids can be integrated into the host genome to achieve long-term gene silencing. In the present study, siRNA plasmids were designed to target CD59. The siRNAs downregulated CD59 at mRNA and protein levels, and significantly increased the resistance to TAM in TAMR-MCF-7 cells.

CD59 is overexpressed in the breast cancer cells when compared to adjacent normal cells, and inhibiting CD59 function can improve breast cancer treatment [24]. Our results were similar to the study results of Ouyang et al. [15]. We found that CD59 was aberrantly upregulated in the ER $\alpha$ -negative breast cancer MCF-10A cells when compared to the ER $\alpha$ -positive breast cancer MCF-7 cells, but the expression of CD59 was elevated in the TAM-resistant breast cancer MCF-7 (TAMR-MCF-7) cells when compared to the sensitive MCF-7 cells. Therefore, CD59 plays a pivotal role in raising the resistance of MCF-7 cells to TAM. In addition, the downregulation of CD59 promotes TAMR-MCF-7 cell proliferation with improving Bax expression and inhibiting BCL-2 expression; conversely, CD59 knockdown upregulates

cleaved-caspase3, cleaved-caspase6, and cleaved-caspase8 to induce TAMR-MCF-7 cell apoptosis.

TAM remains an important drug for adjuvant therapy particularly for premenopausal women, which was first used in the clinics over 40 years ago [26,27]. Furthermore, recent clinical trials showed TAM treatment for 10 years for patients with early stage ER $\alpha$ -positive breast cancer [28], but approximately 30% of patients treated with adjuvant TAM develop resistance [29]. CD59 downregulation is likely a causative and sufficient contributor to TAM resistance. To verify our hypothesis, we first evaluated TAM-induced apoptosis of TAMR-MCF-7 cell in the CD59 siRNA group and BL group. We found CD59 downregulation in TAMR-MCF-7 cells notably increased TAM-induced cell death, which suggested that CD59 could reverse the TAM resistance in TAMR-MCF-7 cells. As expected, TAMR-MCF-7 cells in the BL group were resistant to TAM significantly, in contrast to TAMR-MCF-7 cells in the CD59 siRNA group. Notably, we found that cleaved-caspase3 and Bax were increased after TAM treatment, while Bcl-2 was decreased following TAM treatment, in the TAMR-MCF-7 cells transfected with CD59 siRNA. This indicated that the downregulation of CD59 may result in the resistance of TAMR-MCF-7 cells to TAM.

However, the molecular mechanism of CD59 in the drug resistance of breast cancer cells is still poorly understood. Few investigations have focused on the relationship between CD59 and the drug resistance, so we studied its role in the resistance of breast cancer cells to TAM and found that downregulation of CD59 can significantly improve the effect. Unfortunately, the role of CD59 in breast cancer resistance to other chemotherapeutic agents and the role of CD59 in other human cancers in the resistance to TAM is still unclear. Further research should study this point, expand the scope of application of CD59, and provide valuable research basis for the full application of CD59 in other human diseases.

## Conclusions

This study contributing to TAM resistance is an important direction of breast cancer research. In this regard, this research

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## Conflict of interests

None.