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Original Article

S100A16 overexpression reduces sensibility of MCF-7 cells to tamoxifen via downregulation of ERα and PI3K/AKT-mediated activation of ERα

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Abstract: Our previous study has indicated that overexpression of S100A16 promoted epithelial-mesenchymal transition (EMT) via Notch1 pathway in breast cancer. It is reported that EMT accelerated tamoxifen resistance by certain ways. However, the role of S100A16 in tamoxifen resistance of breast cancer cells remains to be clarified. In our study, we demonstrated that overexpression of S100A16 reduced the growth inhibition rate of tamoxifen to MCF-7 cells by cytotoxicity assays and colony formation assays. In flow cytometry analysis, we found that both apoptosis and necrosis of MCF-7 cells induced by tamoxifen decreased due to overexpression of S100A16. Increasing evidence has indicated that PI3K/AKT-mediated activation of ERα and the low expression level of ERα contributed to tamoxifen resistance. In our study, we found that overexpression of S100A16 facilitated the downregulation of ERα in mRNA level and protein level by western blotting and quantitative real time PCR respectively and upregulation of phosphorylated AKT (Ser473), phosphorylated mTOR (Ser2448), phosphorylated P70S6K (Thr389), phosphorylated ERα (Ser167) by western blotting. Moreover, PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin could downregulate the expression of p-ERα (Ser167) and partly revese the tamoxifen resistance caused by overexpression of S100A16 in cytotoxicity assays and flow cytometry assays, respectively. Taken together, our study demonstrated that overexpression of S100A16 promoted the resistance of MCF-7 cells to tamoxifen via downregulation of ERα and the activation of ERα via PI3K/AKT pathway.

Keywords: Breast cancer, MCF-7, S100A16, tamoxifen, sensibility, PI3K/AKT, ERα

Introduction

Breast cancer is a common malignant disease in women worldwide. Up to 75% breast cancers express estrogen receptor (ER) α and/or progesterone receptor (PR) [1]. Endocrinotherapy is an important therapy for hormone receptor-positive breast cancer. Tamoxifen is still widely used in endocrine therapy, especially in premenopausal patients. Nevertheless, the resistance of tamoxifen has been a vital clinical problem. Therefore, it is essential to determine the underlying mechanisms of reduced sensibility of tamoxifen in hormone receptor-positive breast cancer. Multi-factors are relevant to tamoxifen resistance including alteration of components of ERα pathway itself, cell cycle and cell apoptosis signal channel. In addition, the activation of escape pathways contributes to alternative survival and proliferative biological behavior of tumor cells [2]. Among these factors, low expression level of ERα is the dominating account for de novo and intrinsic resistance of tamoxifen [3-5]. PI3K is the most commonly altered pathway in breast cancer, which consists of catalytic subunits p110α and p110β and regulatory subunits p85α. PI3K effector AKT is activated by phosphorylation at Ser473 and Thr308 [3]. The PI3K pathway correlates with ERα directly and indirectly in addition to its role of survival stimuli and growth-promoting [1]. ERα phosphorylation at Ser167 by AKT or P70S6K augment ERα transcriptional activity, which lowers the sensibility of breast cancer cells to tamoxifen [6, 7]. So the resistance of tamoxifen resulted from activation of AKT or P70S6K signaling pathway could be reversed by pathway inhibitors.

S100A16 is low-affinity calcium-binding protein of EF-hand superfamily [8-10]. We have demonstrated that S100A16 is overexpressed in human breast cancer tissues and upregulation of S100A16 promoted epithelial-mesenchymal transition (EMT) via Nocth1 pathway in breast cancer [11]. Previous researchers have proposed EMT facilitate resistance of chemotherapy and endocrinotherapy via certain mechanisms [12-14]. All these findings suggest that S100A16 may be related to tamoxifen resistance. This study indicated that overexpression of S100A16 reduced the sensibility of MCF-7 breast cancer cells to tamoxifen. These findings implied that S100A16 might play an important role in tamoxifen resistance of human breast cancer and possibly provided a potential molecular target in endocrine therapy of breast cancer.

Materials and methods

Drug preparation

Tamoxifen citrate (Sigma) was prepared in DMSO (Biosharp) and stored at a stock concentration of 0.2 M in -20°C.

Plasmid construction and lentivirus packaging

The human breast cancer cell line MCF-7 was obtained from American Tissue Culture Collection (ATCC). Plasmid construction and lentivirus packaging were conducted as previously described [11]. The lentivirus was transduced into MCF-7 cells. The new cell line which overexpressed S100A16 was named as MCF-7/S100A16 and the control was named as MCF-7/GFP.

Cell culture

All cells used in this study were cultured in complete medium containing DMEM, 10% fetal bovine serum and 100 IU/ml penicillin, 100 ug/ml streptomycin in a 37°C, 5% CO_2 incubator.

Cytotoxicity assay

The Cell Counting Kit-8 (CCK-8) was applied to measure the growth inhibition ratio of tamoxifen to breast cancer cells. In brief, 5000 cells were seeded in 96-well plate quadruple. After 24 hours, cells were treated with different concentration of tamoxifen (0, 2 uM, 4 uM, 8 uM, 16 uM, and 32 uM). After treatment for 48 hours, cell count solution (10 ul) were added to each well and incubation for 2 hours in incubator prior to measurement at 490 nm microplate reader (5082 Grodig, TECAN, Austria). For the PI3K inhibitor and mTOR inhibitor assay, cells were pre-incubated with PI3K inhibitor LY-294002 (10 uM) and mTOR inhibitor Rapamycin (10 nM) 1 hour before tamoxifen treatment. The inhibition ratio of cell proliferation was calculated as (average OD of the control group-average OD of the treatment group/average OD of the control group-average OD of the blank group) × 100%.

Colony formation assay

MCF-7/S100A16 cells and MCF-7/GFP cells were seeded in six-well plates at the density of 1500/well. After cultured for 24 h, cells were treated with different concentration of tamoxifen (0 uM, 10 uM, 20 uM) for 48 h and incubation for 2 weeks. The colonies were fixed in paraform and stained with Giemsa solution after washed with PBS twice. Then the number of colonies were counted.

Apoptosis analysis of flow cytometry

3×10^5 cells were seeded in 6-well plate and treated with different concentration of tamoxifen (0 uM, 10 uM, 20 uM) after cultured for 24 hours. After exposure to tamoxifen for 48 hours, cells were harvested and washed with cold PBS (HyClone), and resuspended in binding buffer (BD Pharmingen). Predicted stain containing 7-AAD and APC-AnnexinV were added to cell suspensions. The samples were detected with FACSAria flow cytometry (Becton Dickinson, San Jose, CA, USA) after incubation at room temperature for 15 min in dark. The data was analyzed with FACSDiva software. The PI3K and mTOR inhibition assays were performed as above described.

Western blotting

Western blotting assays were performed as previously described [15]. The primary antibody used were anti-rabbit AKT (CST), p-AKT (Ser473) (CST), mTOR (CST), p-mTOR (Ser2448) (CST), P70S6K (CST), p-P70S6K (Thr389) (CST), ERα (CST), p-ERα (Ser167) (CST), anti-sheep S100A16 (bioworld). GAPDH (Beyotime) was used to normalize for protein loading.

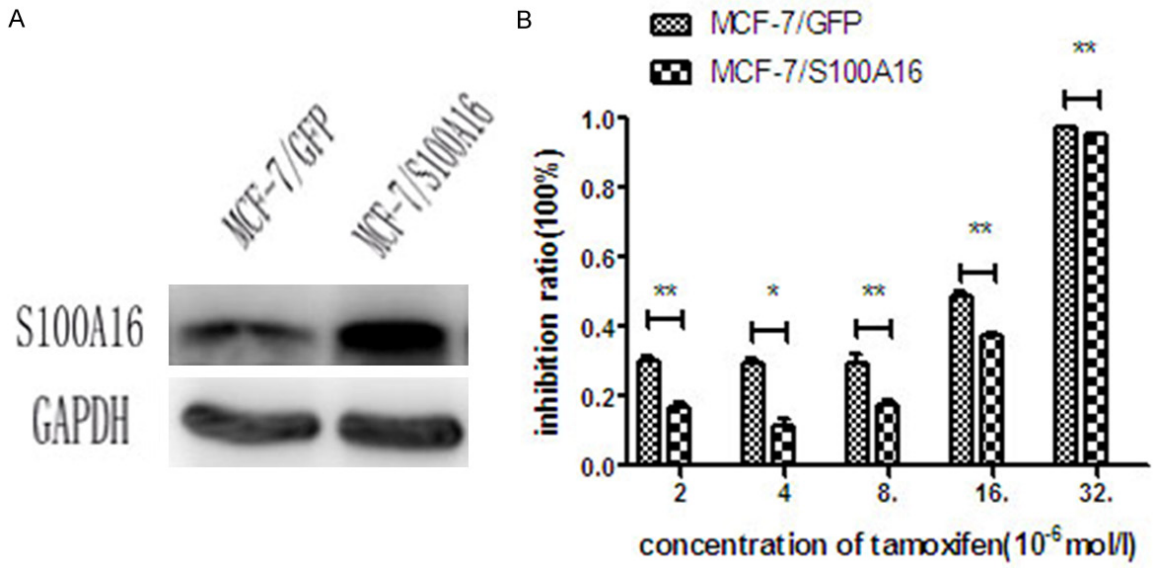

Figure 1. Overexpression of S100A16 reduced the growth inhibition of tamoxifen to MCF-7 cells. A. S100A16 was transduced in MCF-7 cells. Western blot was used to measure S100A16 protein expression in control cells (MCF7-GFP) and S100A16 overexpressed cells (MCF7-S100A16). B. The short-term inhibition rates of tamoxifen to MCF-7/GFP and MCF-7/S100A16 cells determined by CCK-8. Cells were treated with increasing concentration of tamoxifen for 48 h. *P<0.05, **P<0.01.

RNA extraction, reverse transcription and quantitative real time PCR

RNA extraction, reverse transcription and quantitative real time PCR were implemented as previously described [11]. GAPDH was used as loading control.

Statistical analysis

All experiments were conducted in the study triply. The statistic difference between control and treatment group was analyzed by independent-samples t tests. P<0.05 was considered to manifest a statistically significant difference.

Results

Overexpression of S100A16 reduced the growth inhibition of tamoxifen to MCF-7 cells

The results of cytotoxicity assays indicated that the inhibition ratio of tamoxifen to MCF-7/S100A16 cells was markedly lower than that of MCF-7/GFP cells in different concentration (0, 2 uM, 4 uM, 8 uM, 16 uM, and 32 uM) (P<0.05, **Figure 1B**). Similarly, the colony formation assays implied that the long-term inhibition of tamoxifen to MCF-7/S100A16 cells was significantly lower than that to MCF-7/GFP in dose-dependent manner (P<0.05, **Figure 2**).

Overexpression of S100A16 decreased apoptosis and necrosis of MCF-7 cells induced by tamoxifen

To understand whether over-expression of S100A16 strengthened the resistance of MCF-7 to tamoxifen was on account of reducing in apoptosis or necrosis, flow cytometry analysis of APC-annexinV/7-AAD was used. As shown in **Figure 3A3**, **3A4**, remarkable reduction in early apoptotic population (APC-annexinV+/7-AAD- fraction) of MCF-7/S100A16 cells was found compared to that of MCF-7/GFP cells induced by different concentrations of tamoxifen (10 uM, 20 uM), so did in necrotic rate (APC-annexinV+/7-AAD+ fraction) (p<0.05). Moreover, the difference of early apoptotic and necrotic ratios induced by tamoxifen at concentration of 20 uM between MCF-7/S100A16 cells and MCF-7/GFP cells were much more obvious than that at the concentration of 10 uM (**Figure 3A3**, **3A4**).

Overexpression of S100A16 lowered the expression of ERα

We measured the expression of ERα in MCF-7/GFP cells and MCF-7/S100A16 cells by western blot and real time quantitative PCR. The results showed the expression of ERα was inhibited in MCF-7/S100A16 cells compared to MCF-7/GFP

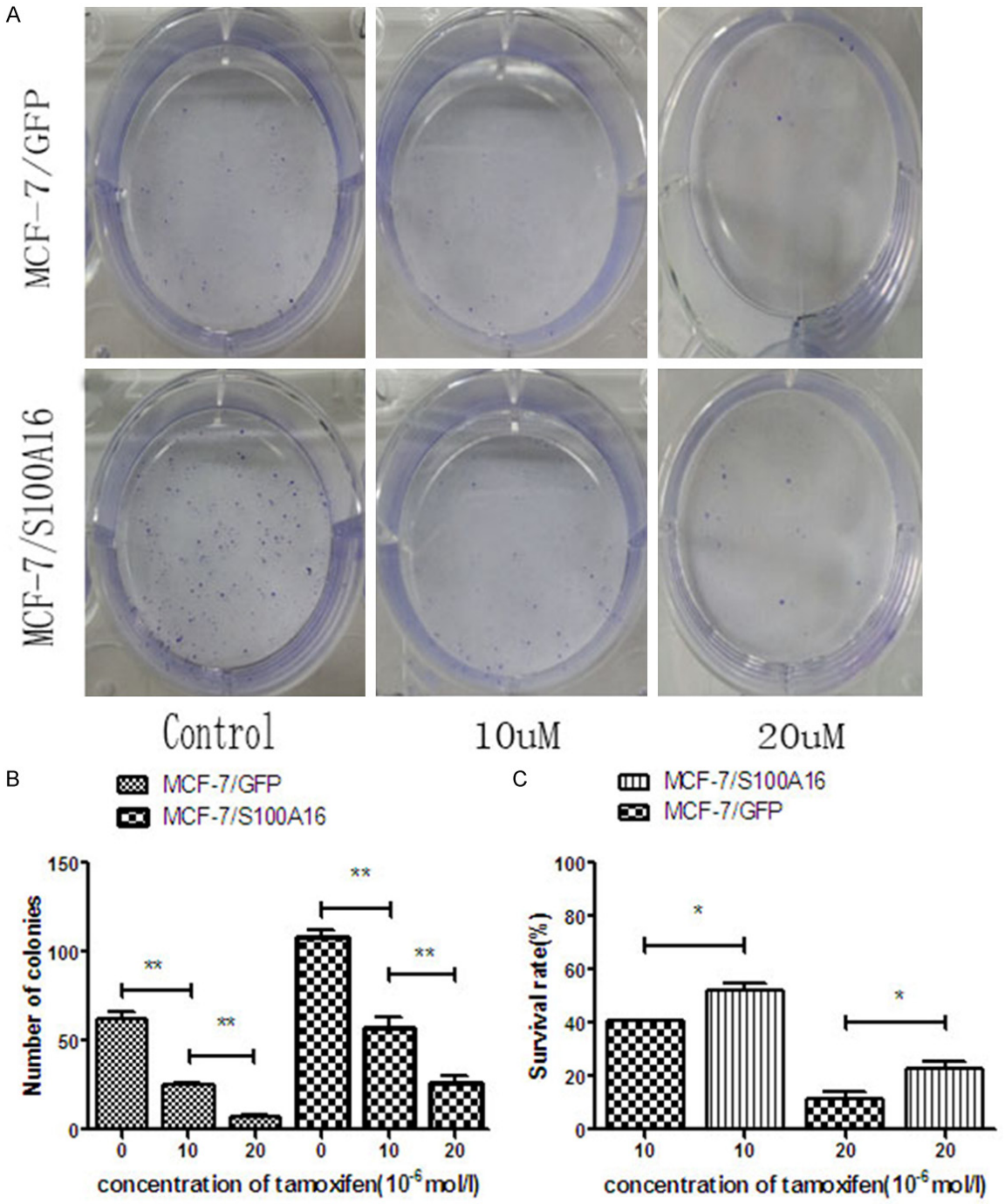

Figure 2. Overexpression of S100A16 decreased the long-term effect of tamoxifen on MCF-7. A. Colony formation assays of MCF-7/S100A16 cells and MCF-7/GFP cells following treatment with tamoxifen for two weeks. B. MCF-7/S100A16 cells and MCF-7/GFP cells was obviously inhibited by tamoxifen on dose-depended manner. C. Overexpression of S100A16 reduced the long-term inhibition of tamoxifen to MCF-7 cells (survival rate: number of colonies in tamoxifen-treated group/number of colonies in control group). *P<0.05, **P<0.01.

cells in both protein level (P<0.05, **Figure 4A**) and mRNA level (P<0.001, **Figure 4B**).

Overexpression of S100A16 induced PI3K/AKT mediated activation of ERα in MCF-7 cells

Western blot revealed that the expression of phosphorylated AKT (Ser473), phosphorylated mTOR (Ser2448), phosphorylated P70S6K (Thr389), phosphorylated ERα (Ser167) were elevated in MCF-7/S100A16 cells in contrast

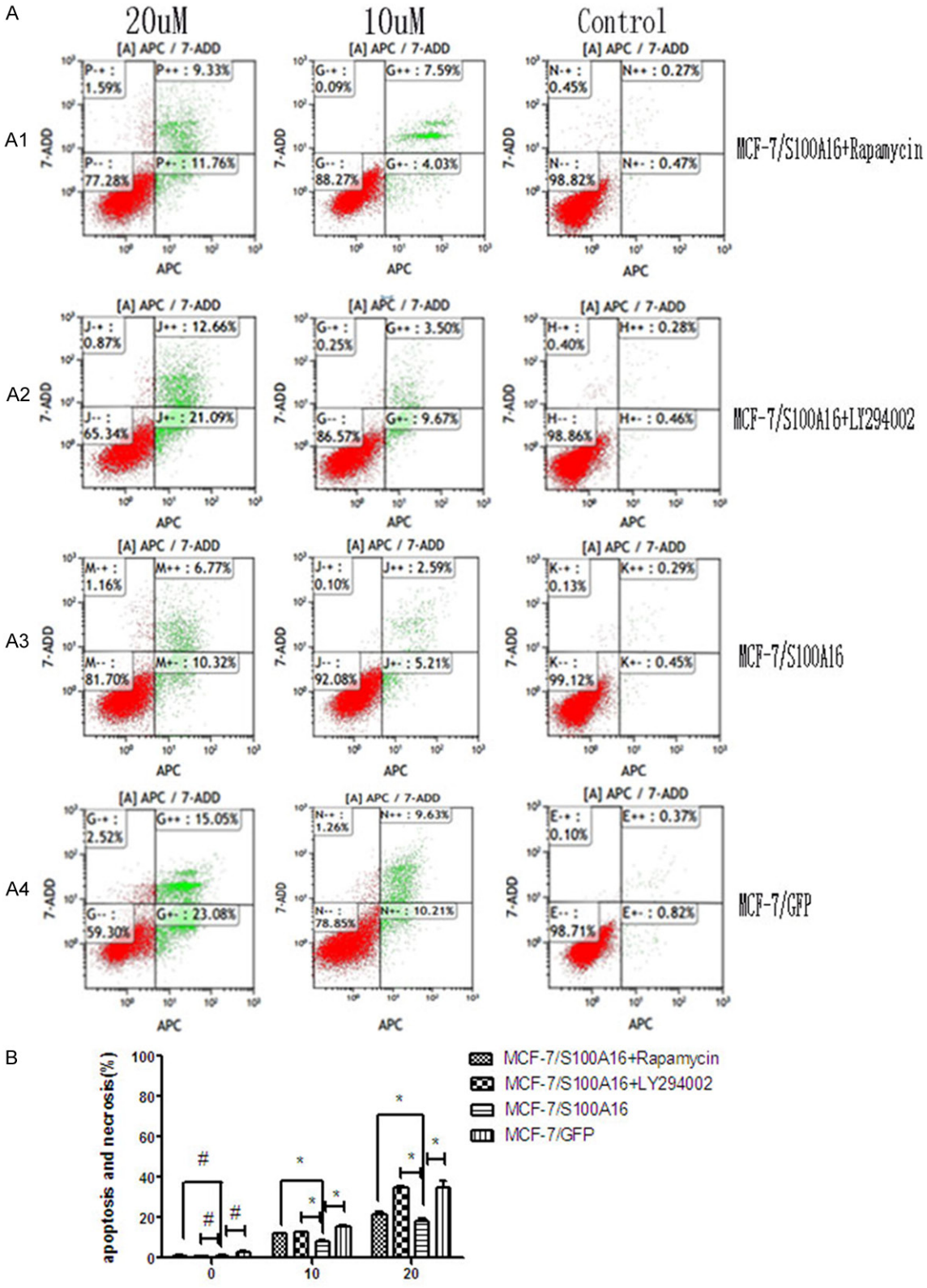

Figure 3. Overexpression of S100A16 decreased the necrosis and apoptosis of MCF-7 cells induced by tamoxifen and LY294002 and Rapmycin can partly reverse the tamoxifen resistance of MCF-7/S100A16. A1, A2. Represented MCF-7/S100A16 cells pretreated with mTOR inhibitor Rapmycin and PI3K inhibitor LY294002 for 1 h before the treatment with different concentration of tamoxifen for 48 h, respectively. A3, A4. Represented MCF-7/S100A16 and MCF-7/GFP cells treated with different concentration of tamoxifen for 48 h, respectively. B. The rate of apopto-

sis and necrosis of three independent experiments were shown in column statistics. #represents two groups had no statistically significant difference. *P<0.05, **P<0.01.

with those in MCF-7/GFP cells (P<0.05, **Figure 4A**) and phosphorylated ERα (Ser167) could be lowered by PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin (P<0.05, **Figure 4C**).

PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin increased growth inhibition effect of tamoxifen to MCF-7/S100A16 cells

To understand the role of activation of AKT and mTOR signaling pathway in sensibility of MCF-7/S100A16 cells to tamoxifen, we further evaluated the inhibitory effect of tamoxifen on MCF-7/S100A16 cells by the use of PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin. As shown in **Figure 4D**, with the treatment of LY294002 and Rapamycin, the growth inhibition ratio of tamoxifen to MCF-7/S100A16 significantly increased (P<0.05).

PI3K inhibitor LY294002 and mTOR inhibitor Rapamycin promoted apoptosis and necrosis of MCF-7/S100A16 cells induced by tamoxifen

In apoptosis analysis of flow cytometry, We also found that the apoptosis and necrosis of MCF-7/S100A16 cells induced by different concentration of tamoxifen (10 uM, 20 uM) was elevated obviously under the application of the two inhibitors (P<0.05, **Figure 3A1**, **3A2**, **3A3**).

Discussion

Endocrinotherapy is an essential therapeutic strategy in hormone receptor-positive breast cancer. Tamoxifen is an usual endocrine (antiestrogen) therapy inducing objective response or disease stabilization in breast cancer patients with ER+ tumors [14]. However, the previous study has showed that one-third of women treated with tamoxifen for 5 years would have recurrent disease within 15 years [16], and most relapse is attributed to tamoxifen resistance. Therefore, identification of more sensitive biomarkers that estimate therapeutic response to endocrine treatment and development of targeted therapy in hormone resistant disease are urgent.

S100A16 is low-affinity calcium-binding protein of EF-hand superfamily [8], and we have found its upregulation in human breast cancer tissue compared to paired adjacent non-cancerous and its acceleration in EMT via notch1 pathway in breast cancer cells [11]. The previous study has indicated that EMT promoted tamoxifen resistance in breast cancer [14]. In this study, we demonstrated that overexpression of S100A16 reduced the sensibility of MCF-7 cells to tamoxifen in dose-dependent manner by Cytotoxicity assay and colony formation assay. The mechanism of the tamoxifen-resistant phenomenon was connected with the downregulation of ERα and the activation of ERα via PI3K/AKT pathway induced by the overexpression of S100A16 gene. Researchers have proposed that the reduced expression of ERα lead to endocrine resistance [3, 17], and it has been reported that low expression of ERα promoted EMT in human breast cancer cells [17, 18], which further explained our previous findings. Moreover, it has been reported that Notch pathway plays an important role in EMT induction [19, 20], and the previous study has indicated that down-regulation of Notch1 potentiated the effect of tamoxifen in breast cancer cells [21]. In consideration of upregulation of Notch1 pathway caused by overexpression of S100A16 [11], the connection of Notch1 with tamoxifen-resistance caused by overexpression of S100A16 also need further investigation.

In our study, we found that downregulation of ERα may be due to the overexpression of S100A16, while we didn't make clear that by which way the overexpression of S100A16 reduced the expression of ERα, which prompted us to conduct a thoroughly study. Increasing experimental and clinical evidence has indicated that activation of PI3K/AKT pathway [1, 22] and PI3K/AKT-mediated activation of ERα facilitated tamoxifen resistance. In our study, we found the activation of AKT/mTOR/P70S6K pathway induced by overexpression of S100A16, and the use of PI3K inhibitor and mTOR inhibitor could obviously inhibited phosphorylation of ERα, which is consistent with the viewpoint mentioned above, that is activation of ERα could result from AKT or P70S6K. What's more, the inhibitors could significantly increase the sensibility of MCF-7/S100A16 cells to tamoxi-

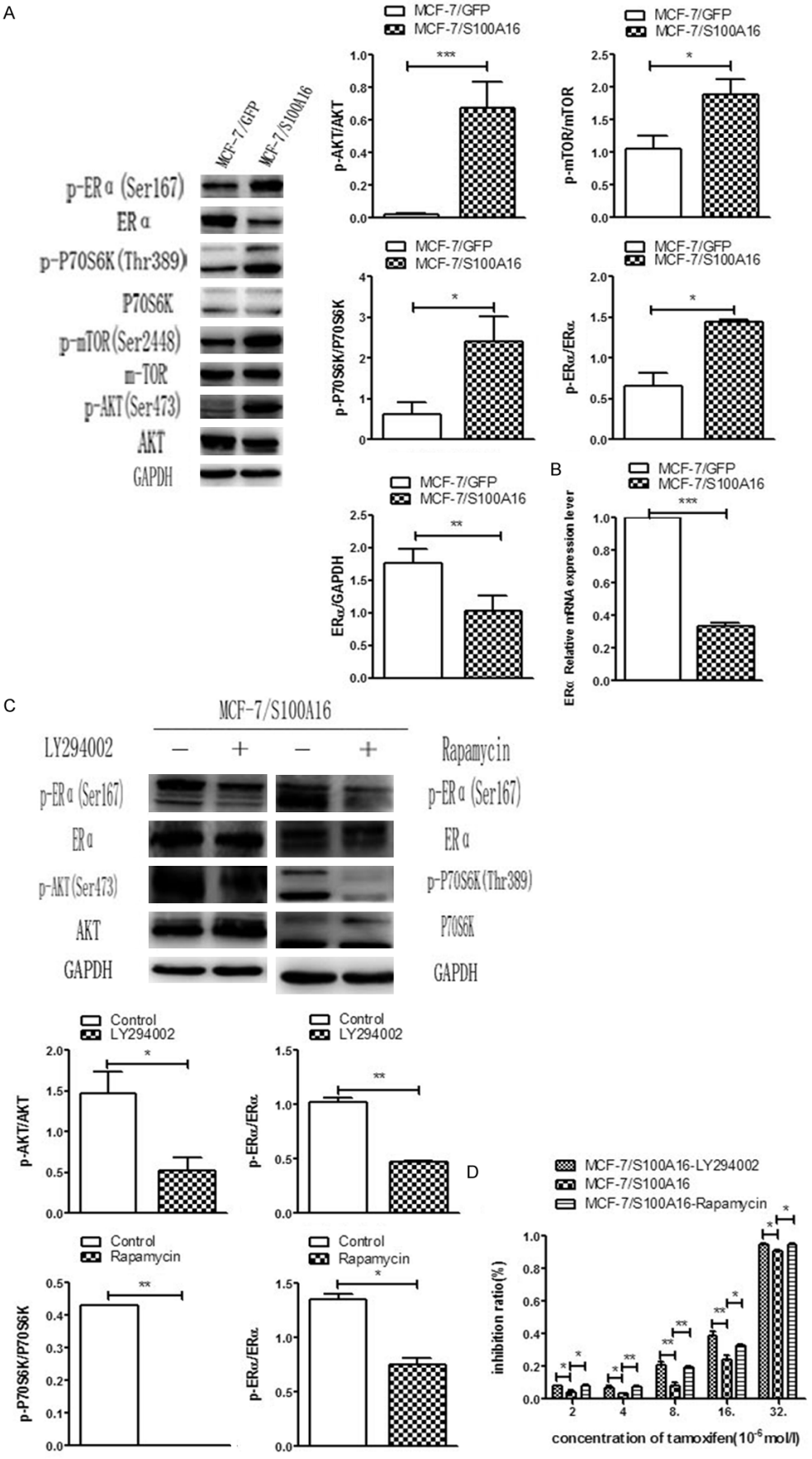
A
MCF-7/GFP
MCF-7/S100A16
p-ERα (Ser167)
ERα
p-P70S6K (Thr389)
P70S6K
p-mTOR (Ser2448)
m-TOR
p-AKT (Ser473)
AKT
GAPDH
p-AKT/AKT
p-mTOR/mTOR
p-P70S6K/P70S6K
p-ERα/ERα
ERα/GAPDH
B
ERα Relative mRNA expression lever
C
LY294002
Rapamycin
Control
D
MCF-7/S100A16-LY294002
MCF-7/S100A16-Rapamycin
inhibition ratio(%)
concentration of tamoxifen(10-6 mol/l)

Figure 4. S100A16 reduced the sensibility of tamoxifen to MCF-7 by downregulation of ERα and AKT/mTOR/P70S6K-mediated activation of ERα. A. S100A16 activated ERα by phosphorylation at Ser167 via AKT/mTOR/P70S6K pathway and lower the expression of ERα in protein lever. B. S100A16 decreased the expression of ERα in mRNA level. C. ERα phosphorylation at Ser167 could be inhibited by LY294002 and Rapamycin, respectively. *P<0.05, **P<0.01, ***P<0.001. D. The PI3K inhibitor and mTOR inhibitor decreased tamoxifen resistance of MCF-7/S100A16 by Cytotoxicity assay, respectively. MCF-7/S100A16 cells were pre-incubated with LY294002 and Rapamycin for 1 h before the treatment of tamoxifen. *P<0.05, **P<0.01, ***P<0.001.

fen, which demonstrated that PI3K/AKT-mediated activation of ERα play an important role in tamoxifen resistance caused by overexpression of S100A16. Our findings indicated that overexpression of S100A16 promoted tamoxifen resistance and the possible mechanisms were determined; however, only one human breast cancer cell line was involved in our study, so the relationship between overexpression of S100A16 and tamoxifen resistance should be explored in many more hormone receptor-positive breast cancer cell lines in future study.

Conclusion

In conclusion, our study demonstrated that overexpression of S100A16 gene increased the resistance of MCF-7 cells to tamoxifen via the downregulation of ERα and the activation of ERα by PI3K/AKT pathway. These findings indicated that S100A16 could be a new marker of endocrinotherapy responsiveness and a candidate for endocrinotherapy targets in human breast cancer.

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Disclosure of conflict of interest

None.

Authors' contribution

SW has contributed to the conception and design of the study, the analysis and interpretation of data, the revision of the article as well as final approval of the version to be submitted. Chenghao Liu, WZ and LL participated in the design of the study, performed the statistical analysis, drafted and revised the article. Chenghao Liu, WZ, LL, GM, ML, TX, and YL performed the experimental study. All authors read and approved the final version of the manuscript.

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