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Inhibition of α-enolase affects the biological activity of breast cancer cells by attenuating PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: This study aimed at exploring the role of α-enolase (ENO1) in proliferation, invasion, and cell apoptosis in MDA-MB-231 and MCF-7 breast cancer human cells, to provide a theoretical basis for the clinical treatment of breast cancer.

MATERIALS AND METHODS: MDA-MB-231 and MCF-7 cells were randomly divided into five groups: normal control group (Control group), negative control group (negative control virus, NC group), and shENO1 (sh1, sh2, and sh3) group, respectively. The expressions of ENO1 mRNA and protein were measured by Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Cell proliferation, cell invasion ability, and cell apoptosis rate were detected by methyl thiazolyl tetrazolium (MTT) assay, transwell invasion assay, and flow cytometer, respectively. The expressions of the proteins correlated with phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway were analyzed by Western blot.

RESULTS: In MDA-MB-231 and MCF-7 cells, the gene and protein expressions of ENO1 in the three sh groups in MDA-MB-231 and MCF-7 cells were significantly lower than those in control group and NC group. In MDA-MB-231 and MCF-7 cells, the gene and protein expressions of ENO1 in the three sh groups were significantly lower than those in control group and NC group. Compared with NC group, the proliferation activity, invasion ability, and apoptosis rate of shENO1 group were significantly decreased ($p < 0.01$). PI3K and Akt protein levels in shENO1 group were significantly downregulated ($p < 0.01$). Bcl-2 protein expression was markedly upregulated ($p < 0.01$), meanwhile Bax protein revealed a significant reduction ($p < 0.01$).

CONCLUSIONS: The results revealed that silencing ENO1 reduced proliferation activity, invasion ability, and apoptosis rate of breast cancer cells by decreasing the phosphorylation of PI3K and Akt pathway. Our results suggested that ENO1 may be a potential therapeutic target in breast cancer.

Key Words:

α-enolase, Breast cancer cells, Proliferation activity, Phosphatidilinositol 3-kinase/protein kinase B signaling pathway.

Introduction

Breast cancer, the leading cause of cancer-related death in women, is the second most common type of cancer around the world comprising approximately 11.6% of new cancer cases up to 2018[1,2]. In the past two decades, there was an increasing incidence of breast cancer reported in China, which grew twice as fast as the global proportion[3]. Treatment for breast cancer usually includes a combination of surgery, radiation therapy, and chemotherapy. Early detection and surgical intervention are essential to successfully treat and prevent the spread of breast cancer, but breast cancer was more likely to spread in patients with regional lymph node metastases. Also, the survival rate of patients with breast cancer remains dismal[4]. Significant progress also has been made in the identification of the genetic markers and cellular pathways involved with the development of breast cancer. However, breast cancer remains a lethal disease, and new diagnostic and treatment options are needed to improve patient outcomes in the clinic.

From the perspective of tumor microenvironment, the lack of local blood supply in breast cancer cells can result in hypoxia with breast cancer cells growing constantly[5]. The possible mechanism was declared to be the enhancement of glucose uptake and aerobic oxidation capacity. Recent studies revealed that some enzymes related to glycolysis were kinds of functionally complex and multi-layered proteins, rather than simple components of the glycolysis pathway. Enolase is a key enzyme in the glycolysis process, which could promote converting phosphoglycerate to phosphoenolpyruvate. There are three different forms of enolases, including α-enolase, β-enolase, and γ-enolase. They are encoded by separate genes[6].

α-enolase (ENO1), as a key enzyme in the glycolysis process, plays an important role in many

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physiological processes. It was reported that the transcription activity and protein expression of ENO1 were upregulated in many tumors[7]. Since the glycolytic activity is related to the upregulation of adenosine triphosphate (ATP) citrate lyase activity, ENO1 may be a promoter of tumor metabolism in tumour cells[8,9]. Several reports[10,11] investigated that ENO1 mainly played a role of tumor promotion gene in cancers, such as pancreatic cancer, prostate cancer, and cholangiocarcinoma. Most researchers pointed out that ENO1 thrived in energy metabolism during the growth of tumor cells. In particular, current research[10] largely focused on ENO1 growth dependence on glycolysis in high metabolic conditions in most tumors.

PI3K/Akt pathway is one of the most important intracellular signal transduction pathways and it can regulate cell cycle and apoptosis by activating the downstream multiple effector molecules. PI3K/Akt pathway enhanced the activity in breast cancer, associating with tumor angiogenesis, invasion, metastasis, drug resistance, and radiation resistance[12]. Song et al[13] indicated that the downregulation of ENO1 expression led to the suppressed glioma cells growth, migration, and invasion progression by inactivating the PI3K/Akt pathway. Kabbage et al[14] reported that ENO1 was overexpressed in tumors when compared with normal tissues, in infiltrating ductal carcinomas of the breast. However, there is less evidence of ENO1 that attenuate cell growth and invasion by inactivating the PI3K/Akt pathway in breast cancer.

In this study, we investigated the role and mechanism of ENO1 in the growth, invasion, and apoptosis of breast cancer cells using silencing ENO1 strategies, as well as providing a theoretical basis for exploring the molecular mechanisms of breast cancer.

Materials and Methods

Cell Cultures

MDA-MB-231 and MCF-7 (American Type Culture Collection; ATCC, Manassas, VA, USA) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) contained 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibodies (100 U/ml penicillin G and 100 μg/ml streptomycin, Hyclone, South Logan, UT, USA) in an incubator containing 5% CO_2 at 37°C. The cells were digested with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and then passaged after the cell fusion rate reached 80% to 90%.

Lentiviral Vector Interfering ENO1 Expression

MDA-MB-231 and MCF-7 cells were randomly divided into five groups: normal control group (Control group), negative control group (negative control virus, NC group), and shENO1 (sh1, sh2, and sh3) group, respectively. The specific lentiviral vector and negative control virus for ENO1 interference were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). MDA-MB-231 and MCF-7 cells were cultured when the cell fusion rate had reached 80%-90%, washed with D-hanks solution (Beijing Solaibao Technology Co., Ltd., Beijing, China) twice. Cultured cells were digested and centrifugated, of which the supernatant was re-suspended with the medium into a single cell suspension. Cell numbers were counted. Then, 1×10^5 cells were inoculated in a 24-well plate, 500 μl culture medium was added. After the cell wall adhesion, the lentiviral particles were added according to the required virus volume based on the MOI value of 1. Finally, 0.5 μl polylysine (10 μg/ml) was injected to the 24-well plate and uniformly mixed with the liquid 8 h once a time. The culture medium was replaced after every 24-36 h. When the cell growth density reached to more than 80%, repeated screening was performed until no dead cells were observed. QRT-PCR and Western blot were used to detect the expression of ENO1.

Quantitative Real Time-Polymerase Chain Reaction

The total cellular RNA was isolated with TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The first strand cDNA was synthesized using Super Script II First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). PCR assay was performed by the SYBR Premix Ex Taq system (TaKaRa, Otsu, Shiga, Japan) and the reaction entailed 95°C for 30 s, 95°C for 10 s, 60°C for10 s, 72°C for 20 s (45 cycles), 72°C extension of 1 min. The primers for shENO1: sh1, sense, 5′-CCGGAATGTCATCAAGGAGAAATATCTCGAGATATTTCTCCTTGATGACAT TTTG-3′; antisense, 5′-AATTCAAAAAAATGTCATCAAGGAGAAATATCTCGAGATATTTCTCCTTGATGACATT-3′; sh2, sense, 5′-CCGGCGTGAACGAGAAGTCCTGCAACTCGAGTTGCAG-

GACTTCTCGTTCACGTTTTG-3′, antisense, 5′-AATTCAAAACGTGAACGAGAAGTCCTGCAACTCGAGTTGCAGGACTTCTCGTTCACG-3′; sh3, sense, 5′-CCGGCCACTGTTGAGGTTGATCTCTCTCGAGAGAGATCAACCTCAACAGTGGTTTTTG-3′; antisense, 5′-AATTCAAAAACCACTGTTGAGGTTGATCTCTCTCGAGAGAGATCAACCTCAACAGTGG-3′. The primer sequence for β-actin: forward, 5′-TTGCCGACAGGATGCAGAA-3′; reverse, 5′-GCCGATCCACACGGA

GTACT-3′. Relative gene expression was obtained after normalization with endogenous β-actin and determination of the difference in threshold cycle (Ct) using the $2^{-\Delta\Delta Ct}$ method.

MTT Assay

4×10^3 cells were seeded into each well of a 96 well plate. After 24 h, 48 h, 72 h incubation, 25 μl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added, 37°C, 5% CO_2 incubator incubated for another 4 h. Then, the solution was aspirated, finally 125 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was injected to dissolve the crystal. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The cell proliferation activity was calculated using the following equation: cell proliferation activity = $(OD_{treated} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$.

Transwell Invasion Assay

50 μl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was added on the membrane of the upper chamber and solidified at 37°C for 30 min. 100 μl cell suspension (containing 1×10^5 cells) was added into the upper chamber of transwell (Millipore, Billerica, MA, USA). The lower chamber injecting with 600 μl DMEM medium containing 10% fetal bovine serum, then incubated for 24 h in the incubator. Then, we removed the chamber and discarded the upper chamber medium with 4% paraformaldehyde fixed for 10 min, 1% crystal violet staining solution for 3 min and using the cotton swab gently wiping the cells that failed to pass on the upper surface of the chamber, and the number of invasion cells were counted by an inverted microscope (Olympus, Tokyo, Japan).

Apoptosis Detection

Cells were trypsinized, collected, and washed with cold phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) twice and centrifuged at 1500 rpm for 3 min. 5×10^5 cells were re-suspended with 1 × Annexin V solution. 100 μl cell suspension was added into a flow tube, then added 5 μl of Annexin V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI), mixed gently, incubated at room temperature for 15 min. Each tube was added with 1 × Annexin V (400 μl) working solution and mixed, the cell suspension was detected by flow cytometry (Bio-Rad, Hercules, CA, USA).

Western Blot Analysis

Breast cancer cells were collected as indicated and lysed in a radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) buffer with proteinase inhibitors. The protein concentration was determined by the bicinchoninic acid kit (BCA; Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Then, 30 μg protein sample was loaded in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were transferred to polyvinylidene difluoride (PVDF) membrane (Merck, Darmstadt, Germany) under 20 V semi-dry conditions for 50 min, then the membrane was blocked for 1 h with 5% skimmed milk in a Tris-Buffered Saline and Tween-20 solution (TBST; Sigma-Aldrich, St. Louis, MO, USA). ENO1 (1:1000, ab155102, Abcam, Cambridge, MA, USA), PI3K (1:1000, #4292, Cell Signaling Technology, Boston, MA, USA), p-Akt antibody (1:1000, #5012, Cell Signaling Technology, Boston, MA, USA), Akt antibody (1:1000, #9272, Cell Signaling Technology, Boston, MA, USA), Bcl-2 (1:1000, ab196495, Abcam, Cambridge, MA, USA) and Bax antibody (1:500, ab53154 , Abcam, Cambridge, MA, USA) were added with the concentration of 1:1000, overnight at 4°C washing with TBST in triplicates, then incubated with second antibody horseradish peroxidase (HRP) goat anti-rabbit IgG (1:3000, ab6721, Abcam, Cambridge, MA, USA) for 1 h. Finally, the membrane was washed with TBST three times. The enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) system was used to detect the signal. The protein expression level was normalized by β-actin and quantified by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

SPSS 19.0 statistical software (IBM, Armonk, NY, USA) was used to analyze our data. All the data results were expressed as mean

± standard deviation. Multiple comparisons were evaluated by repeated measures analysis of variance (ANOVA). One-way ANOVA was used to compare the mean of multiple samples. The comparison between any two means was performed by the LSD method. The LSD method was applied in the comparison between the two groups. $p < 0.05$ was considered significant.

Results

Gene and Protein Expression of ENO1 After Lentivirus Interference

As shown in Figure 1, in MDA-MB-231 and MCF-7 cells, the gene and protein expressions of ENO1 in three sh groups were significantly lower than those in control group and NC group ($p < 0.01$). There was no significant difference

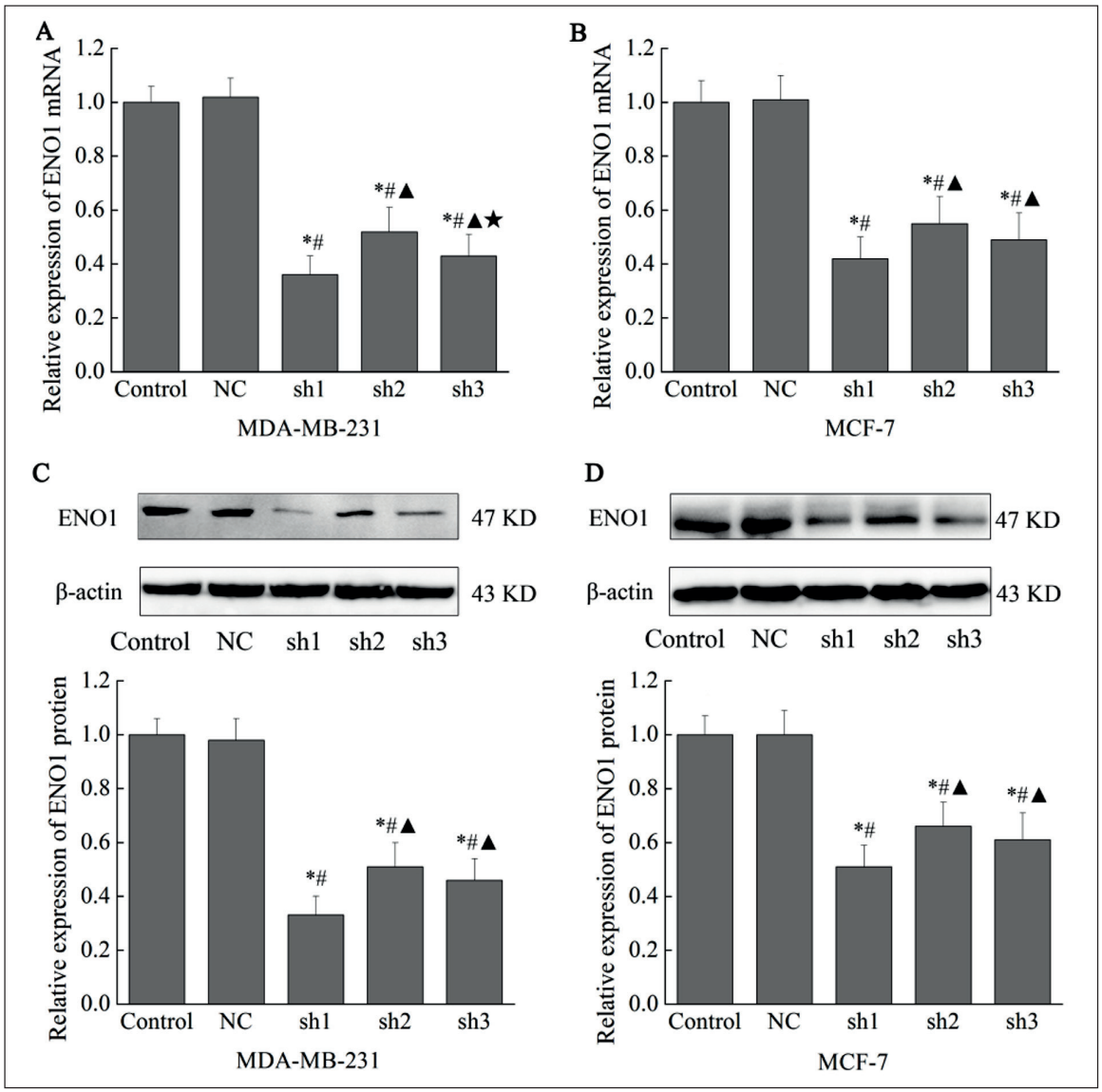

Figure 1. Relative mRNA and protein expressions of ENO1. **A,** Relative mRNA expression of ENO1 in MDA-MB-231 cells was detected by qRT-PCR. **B,** Relative mRNA expression of ENO1 in MCF-7 cells was detected by qRT-PCR. **C,** Relative protein expression of ENO1 in MDA-MB-231 cell was detected by Western blot. **D,** Relative protein expression of ENO1 in MCF-7 cell was detected by Western blot. *p<0.01 *vs.* Control group; #p<0.01 *vs.* NC group; ▲p<0.01 *vs.* sh1 group (p<0.01); ★p<0.01 *vs.* sh2 group (p<0.01).

between control group and NC group, which indicated that the negative control vector had no effect on ENO1 expression. ENO1 gene and protein expression in sh1 group was lower than that in sh2 group and sh3 group. Therefore, sh1 group performed in the follow-up experiments. Furthermore, qRT-PCR and Western blot results suggested that the negative control virus had no effect on ENO1 expression in breast cancer cells, thus NC group was only used to conduct in the follow-up experiments.

Silencing ENO1 Enhanced the Proliferative Activity of Breast Cancer Cells

To verify the effects of ENO1 on the proliferation of breast cancer cells, we detected whether ENO1 inhibition was able to decrease the proliferative activity of MDA-MB-231 and MCF-7 cells. The MTT assay results revealed that MDA-MB-231 and MCF-7 cells proliferative activity in shENO1 group was significantly decreased compared to NC group ($p < 0.01$; Figure 2).

Silencing ENO1 Reduced the Invasion Ability of Breast Cancer Cells

We used transwell assay to investigate the changes of cell invasion capacity, the result was shown in Figure 3. Compared with NC group, the number of invading cells in shENO1 group was significantly decreased ($p < 0.01$). The results indicated that ENO1 could promote the invasion ability of MDA-MB-231 and MCF-7 cells. However, silencing of ENO1 significantly inhibited the cell invasion ability.

Silencing ENO1 Promoted the Apoptosis of Breast Cancer Cells

It was revealed in Figure 4, the apoptosis rate of MDA-MB-231 cells in shENO1 group was 27.28 ± 1.12, which was significantly higher than that in NC group (1.25 ±0.27, $p < 0.01$). The apoptosis rate of MCF-7 cells in shENO1 group was 23.64 ± 2.09, it was also significantly higher than that in NC group (1.92 ±0.36, $p < 0.01$). The results showed that the inhibition of ENO1 expression could promote the apoptosis of breast cancer cells.

Silencing ENO1 Inhibited the Cell Proliferation Via PI3K/Akt Signaling Pathway

To determine whether PI3K/Akt signaling pathway is essential for ENO1-mediated cells hyper-proliferation, we estimated the protein expression of PI3K and Akt. Meanwhile, we also detected the protein expression of the Bcl-2 and Bax. As shown in Figure 5, there was no difference of the expressions of T-PI3K and T-Akt in MDA-MB-231 cells and MCF-7 cells. Compared with NC group, the phosphorylation levels of PI3K and Akt in shENO1 group were significantly decreased ($p < 0.01$), the expression of Bcl-2 was significantly higher ($p < 0.01$) and the expression of antiapoptotic protein Bax was significant-

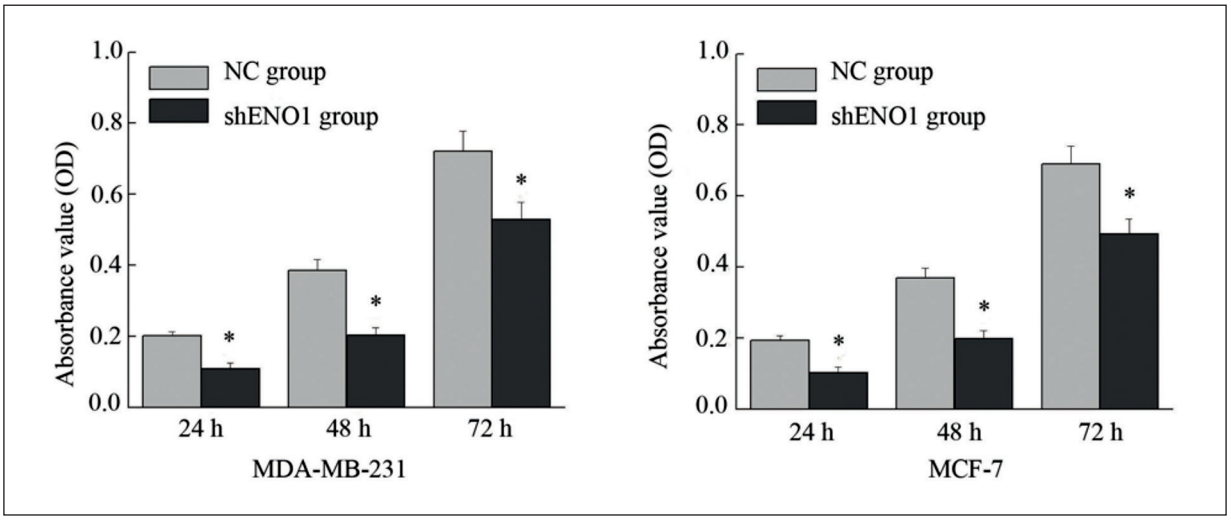

Figure 2. Changes of cell viability. The viability of MDA-MB-231 and MCF-7 cells was determined by MTT assay. *p<0.01 *vs.* NC group.

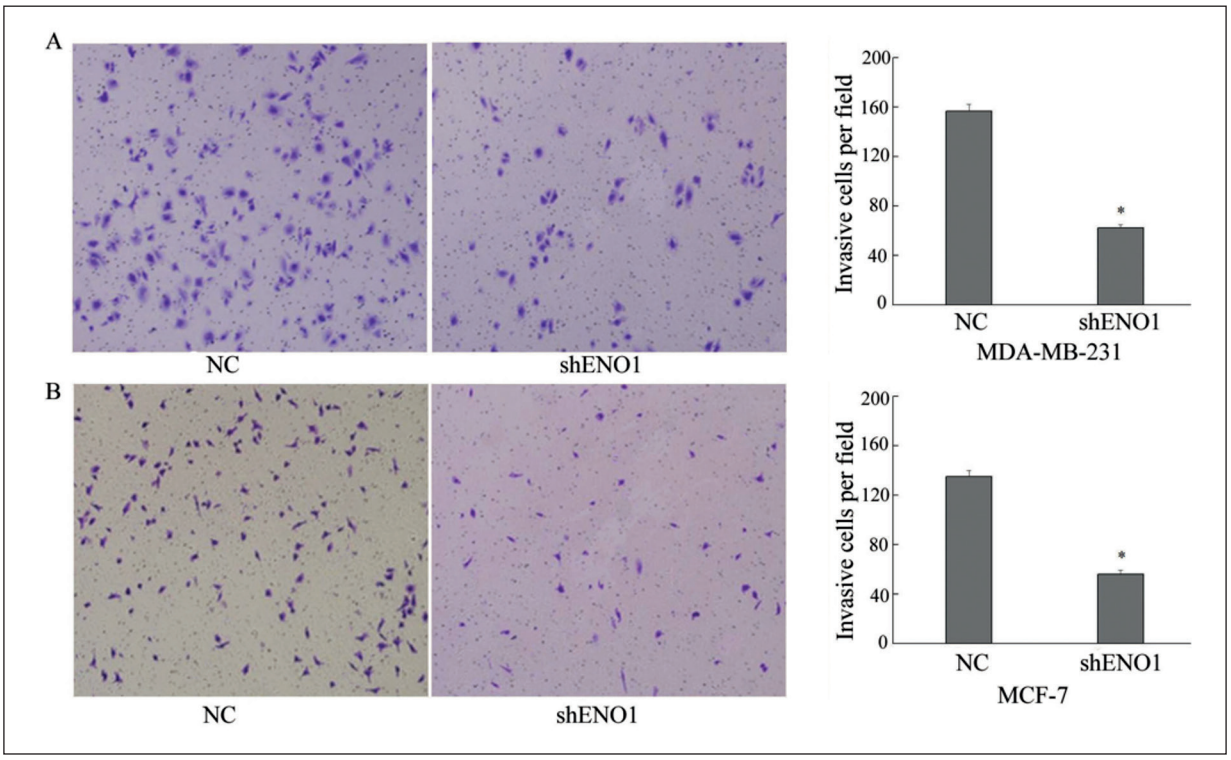

Figure 3. Changes in cell invasion capacity (Magnification: 200×). **A,** Number of MDA-MB-231 cells in NC groups and shENO1 groups. **B,** Number of MCF-7 cells in NC groups and shENO1 groups. *p<0.01 *vs.* NC group.

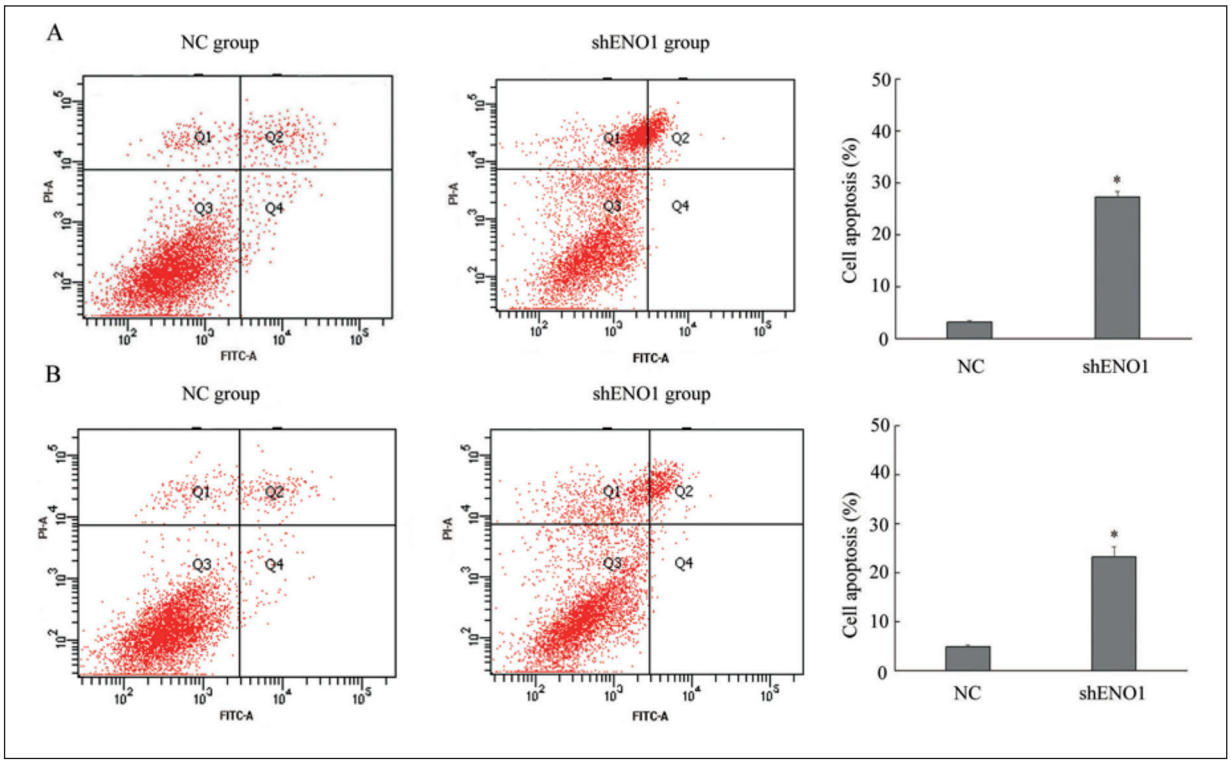

Figure 4. Changes in cell apoptosis rate. **A**, Apoptosis rate of MDA-MB-231 cells. **B,** Apoptosis rate of MCF-7cells. *p<0.01 *vs.* NC group.

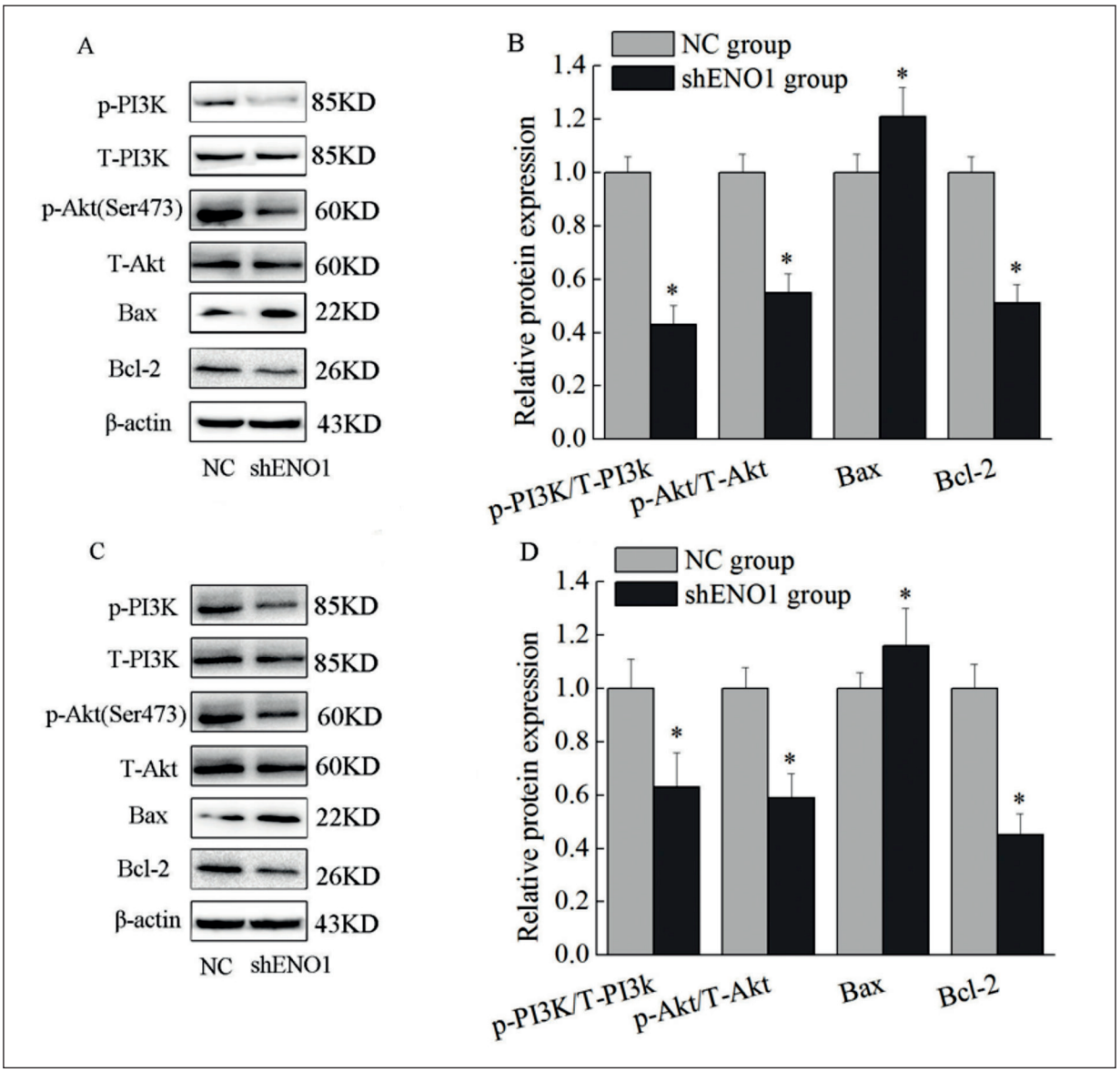

Figure 5. Relative protein expression in cells. **A,** MDA-MB-231 Western blot assay. **B,** Relative expression of MDA-MB-231 cells. **C,** MCF-7 Western blot assay. **D,** Relative expression of MCF-7 cells. **p*<0.01 *vs.* NC group.

ly lower ($p < 0.01$). The results showed that the inhibition of ENO1 could inhibit cell proliferation *via* attenuating the phosphorylation of PI3K/Akt signaling pathway.

Discussion

The proliferation, migration, and invasion of cancer cells require numerous energy supplies. Some researches pointed out that interfering with cell glycolysis to cut off the generation of energy could be a therapeutic way to cure breast cancer[9,15]. Gao et al[9] used MDA-MB-231 breast cancer cells and they revealed that ENO1 expression significantly decreases the response to hypoxia and enhances the sensitivity of the breast cells to radiation therapy. In our study, we estimated the ENO1 expression in MDA-MB-231 and MCF-7 cells. The results revealed that the proliferation and invasion activity of breast cancer cells was significantly reduced while the cell apoptosis rate significantly increased after the inhibition of ENO1. Many enzymes involved in glycolysis are expected to be the targets of cancer therapy[16], such as enolase, hexokinase, phosphoglucose

isomerase, phosphofructokinase, acetal, phosphoglyceraldehyde dehydrogenase, and pyruvate kinase, etc[17,18].

Therapies of breast cancer have made significant advances in recent years, nevertheless chemo-resistance to chemotherapeutic agents has become a major obstacle in treating breast cancer[19]. Previous studies[20] demonstrated that down-regulated ENO1 could overcome the tamoxifen resistance in estrogen receptor-positive (ER+) breast cancer therapy. This treatment only aimed to cure ER+ breast cancer, however, with a poor prognosis in triple-negative breast cancer[21]. In this study, ER+ breast cancer cell MCF-7 and triple-negative breast cancer cell MDA-MB-231 were selected to evaluate the effects of ENO1 on proliferation and invasion abilities. The results revealed that the effective of silencing ENO1 was similar to MCF-7 and MDA-MB-231 cells.

Song et al[22] pointed out that that differentially expressed proteins in breast cancer were associated with glycolysis/gluconeogenesis, including the overexpression of GAPDH, ENO1, lactate dehydrogenase (LDH), pyruvate kinase isozyme type M2 (PKM2) and aldolase A (ALDOA). Indeed, these proteins were up-regulated in invasive cancer cells supports the concept that fast growing tumor cells exhibited higher glucose metabolic levels than normal cells[23]. ENO1 mRNA expression level is elevated in a variety of tumors, which is consistent with our investigation. It was revealed that ENO1 expressed on the surface of the tumor cells, acting as a plasminogen receptor for cell invasion, metastasis, and inflammatory response[23-25].

In recent years, researches focused on evaluating the role of ENO1 acting on breast cancer cells or the expression of ENO1 in clinical breast cancer tissues. We found that there is few systematic research studying biological activity of breast cancer cells and the possible mechanism after inhibiting the ENO1 expression. PI3K/Akt signaling pathway is a classical pathway, which is associated with a series of life activities, the activation of PI3K/Akt signaling pathway induces glycolysis[26]. We then addressed whether PI3K/Akt contributes to the ENO1-mediated in breast cancer cells biological activity. The results suggested that inhibiting ENO1 expression could attenuate the phosphorylation of PI3K and Akt proteins, promote the expression of Bcl-2, and inhibit the expression of Bax protein.

Although we performed the experiment of inhibiting ENO1 expression on breast cancer cells, we should perform a further study to analyze the molecular mechanism. Furthermore, we would conduct a clinical investigation to examine the expression of α-enolase in breast tumor and normal adjacent control samples, thus determining the clinical significance.

Conclusions

Taken together, the inhibition of ENO1 expression decreased the biological activity in breast cancer cells *via* PI3K/Akt signaling pathway. Our study provided a more theoretical basis for scientific research on breast cancer. The aerobic glycolysis pathway and the corresponding mechanisms in breast cancer will be subject to further study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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