Silencing of ENO1 inhibits the proliferation, migration and invasion of human breast cancer cells

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Summary

Purpose: Studies have shown that α-enolase ENO1 is involved in the regulation of cancer cell proliferation and metastasis. However, the role of ENO1 is yet to be explored in breast cancer. This study was undertaken to explore the role and therapeutic potential of ENO1 in breast cancer.

Methods: Expression analysis was carried out by qRT-PCR. Transfections were performed by Lipofectamine 2000 reagent. WST-1 assay was used for cell viability. Wound healing assay was used for cell migration analysis. Western blot analysis was used to determine protein expression.

Results: The results showed that the expression of ENO1 was significantly upregulated in breast cancer by up to 4-fold. Silencing of ENO1 caused significant decline in the proliferation rate and colony formation of the SK-BR-3 breast cancer cells. The decrease in the proliferation rate of the ENO1 cells was due to the induction of apoptosis as revealed by DAPI staining. Annexin V/propidium iodide (PI) showed a significant increase in the apoptotic SK-BR-3 cells. The apoptosis percentage was 2.17 in si-NC and 23.1% in si-ENO1 transfected SK-BR-3 cells. The apoptosis induction was also accompanied by increase in Bax and decrease in Bcl-2 expression. ENO1 silencing also resulted in the arrest of the SK-BR-3 cells in the G2/M phase of the cell cycle which was also associated with depletion of Cdc2, Cdc25 and cyclin B1 expression levels. ENO1 silencing also caused decrease in the migration and invasion of the SK-BR-3 cells as revealed by the wound healing and transwell assays.

Conclusion: These findings suggest that ENO1 has oncogenic properties in breast cancer which can be exploited in breast cancer treatment.

Key words: ENO1, breast cancer, apoptosis, cell cycle arrest, invasion

Introduction

The α-enolase (ENO1) plays different roles in human cells depending on its localization [1]. Apart from its well established role in the process of glycolysis, it has also been reported to act as a plasminogen receptor on the surface of cells [2]. ENO1 has been shown to be dysregulated in cancer tissues and has been shown to act as a biomarker for the progression of different cancers [3]. ENO1 has also been shown to be a potential prognostic biomarker for head and neck cancer [4]. It has been reported to regulate several processes in cancer cells such as proliferation and metastasis [5]. The invasiveness of the pancreatic cancer cells decreases significantly if the expression of ENO1 is silenced [6]. Given the role of ENO1 in different cancer related processes, it is believed that ENO1 may be used as potential therapeutic target for the treatment of cancer. This study was designed to investigate the role and therapeutic implications of ENO1 in breast cancer. Accounting for 14% of all the cancer-related deaths, breast cancer is one of the destructive malignancies world over. Annu-
ally, 0.4 million deaths are caused by breast cancer alone [7]. Despite the treatment of early-stage breast cancer with surgery and/or chemotherapy which offers satisfactory results, the survival rate of advanced stage breast cancer is still very poor. The late diagnosis of breast cancer hinders its efficient treatment [8]. Additionally, the efficiency of the currently available chemotherapy is low and the side effects can be very severe. The development of drug resistance among breast cancer cells further increases the problem [9]. The detection of novel therapeutic targets or development of highly effective and safer chemotherapeutic agents may prove beneficial in the breast cancer treatment [10]. Herein, we examined the expression of ENO1 in four different breast cancer lines and one normal cell line and the expression of ENO1 was found to be significantly upregulated. Silencing of ENO1 resulted in significant decline in the proliferation of SK-BR-3 cells via induction of apoptosis and arrest of the SK-BR-3 cells at the G2/M check point of the cell cycle. ENO1 silencing also caused a remarkable decrease in the migration and the invasion of the SK-BR-3 breast cancer cells via downregulation of metalloproteinase (MMP-2) and MMP-9. Taken together, ENO1 may prove of therapeutic potential in breast cancer and merits further investigation.

Methods

Cell lines and culture conditions

The breast cancer cell lines MDA-MB-231, MDA-MB-436, SK-BR-3, and CAMA-1 and the normal breast cell line Hs859.T were procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin and a humidified atmosphere containing 5% CO₂. Since SK-BR-3 cells exhibited the highest expression of ENO1, only this cell line was used for further experiments.

Cell transfection

The transfection of the si-NC and si-ENO1 into the SK-BR-3 cells was carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. As the SK-BR-3 cells reached 80% confluence, the appropriate concentrations of si-NC and si-ENO1 were transfected into these cells.

Analysis of cell proliferation and colony formation

The proliferation rate of SK-BR-3 cells was monitored by WST-1 assay. In brief, SK-BR-3 cells were cultured in 96-well plates at a density of 2×10⁶ cells/well. The cells were then transfected with miR-NC or miR-181 mimics and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for another 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals. Colony formation assay was performed as described previously [11].

Analysis of cell death

After transfection with si-NC and si-ENO1, the SK-BR-3 cells were cultured in 24 well plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with phosphate buffered saline (PBS). After this, the cells were stained with DAPI. The SK-BR-3 cells were then washed with PBS and observed both by fluorescence and phase contrast microscopy. For annexin V/PI assay, the si-NC and si-ENO1 transfected SK-BR-3 cells (5×10⁴ cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic SK-BR-3 cells was determined by flow cytometry.

Cell cycle analysis

The cultured human breast cancer SK-BR-3 cells were transfected with si-NC or si-ENO1 and then cultured for 24 h at 37°C. The cells were then washed with PBS and stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Wound healing assay

The transfected SK-BR-3 cells were cultured till 80% confluence. This was followed by removal of the Dulbecco’s modified Eagle’s medium (DMEM) and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope.

Western blotting

The normal and the breast cancer cell lines were cultured at 37°C for 24 h and then centrifuged at high speed. The cell pellet was washed with PBS and then suspended again in RIPA lysis buffer. Thereafter, the concentrations of the proteins were determined and equal concentrations of the proteins were loaded on 12% SDS-PAGE gel. The samples were transferred to polyvinylidene fluoride membranes and blocking was done using 5% skimmed milk powder. This was followed by membrane incubation with primary antibodies at 4°C for 24 h. Next, the membranes were incubated with horseradish peroxidase-linked secondary biotinylated antibodies for 2 h. The membranes were washed with tris buffered saline and immunoreactive bands observed by ECL-PLUS/Kit as per the manufacturer’s guidelines.

Statistics

The experiments were done in triplicate and the values represent the mean ± standard deviation (SD). P<0.05 was considered as significant difference. Student’s t-test using Graph Pad prism 7 software were used for the statistical analyses.
Results

ENO1 is aberrantly upregulated in breast cancer cells

The transcript analysis of ENO1 was examined in one normal and five different breast cancer cells with quantitative RT-PCR (Figure 1). It was found that relative to the normal cells, the expression of ENO1 was significantly enhanced in breast cancer cells. The expression of ENO1 was found to be up to 4.2-folds higher than in the normal cells. The highest fold upregulation of ENO1 was found in the SK-BR-3 cells and therefore this cell line was taken forward for further experimentation.

ENO1 silencing inhibits proliferation by promoting apoptosis in SK-BR-3 cells

In order to unveil the role of ENO1 in SK-BR-3 cells, the expression of ENO1 was silenced (Figure 2A). It was found that silencing of ENO1 causes significant decrease in the proliferation as well as the colony formation of the SK-BR-3 cells (Figure 2B and 2C). To elucidate the underlying mechanism for the inhibition of cell proliferation, DAPI staining was performed. The DAPI staining assay showed that ENO1 silencing caused nuclear fragmentation of the SK-BR-3 cells (Figure 3A). Annexin V/PI staining revealed that the apoptotic cell percentage increased from 2.17% in si-NC to 23.1% Si-ENO1 cells (Figure 3B). The induction of apoptosis was further confirmed by expression analysis of Bax and Bcl-2 proteins by western blotting. The results showed that ENO1 silencing caused increased expression of Bax, while the expression of Bcl-2 was decreased (Figure 3C).

Silencing of ENO1 triggers G2/M arrest of SK-BR-3 breast cancer cells

The effects of ENO1 silencing were also inves-

Figure 1. Expression of ENO1 in normal Hs 849.T and breast cancer cell lines. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Figure 2. A: Expression of ENO1 in si-NC and si-ENO1 transfected SK-BR-3 breast cancer cell line. B: Cell viability of si-NC and si-ENO1 transfected SK-BR-3 breast cancer cell line. C: Colony formation of the si-NC and si-ENO1 transfected SK-BR-3 breast cancer cell lines. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
Enolase 1 has anticancer activity in breast cancer

The distribution of the SK-BR-3 cells in different phases of cell cycle. The results revealed that ENO1 caused a remarkable increase in the G2/M phase of the cell cycle. The G2/M phase cells increased from 11.21% in si-NC to around 44.62% in the Si-ENO1 cells, indicative of G2/M arrest (Figure 4A). The G2/M arrest was further confirmed by examining the expression of the cell cycle-related proteins. It was found that ENO1 silencing caused significant decrease in the Cdc25, Cdc2 and cyclin B1 (Figure 4B).

Silencing of ENO1 inhibits migration and invasion of SK-BR-3 cells

The effects of ENO1 silencing were also examined on the migration of the SK-BR-3 cells by wound healing assay. The results showed that ENO1 silencing caused significant decline in the migration of the SK-BR-3 cells as evidenced from the wound width (Figure 5). The effects of ENO1 silencing on the migration of the SK-BR-3 cells are shown in Figure 5. The experiments were performed in triplicate.
silencing were examined on the invasion of the SK-BR-3 cells by transwell assay and the results showed that upon silencing of ENO1, the invasion of SK-BR-3 cells decreased significantly. The cell invasion was decreased by 66% upon ENO1 silencing.

**Discussion**

Breast carcinoma is one of the prevalent types of malignancies in women across the world [12]. The late diagnosis, lack of effective and safer drugs and lack of novel therapeutic targets are the main obstacles that limit its treatment [9,10]. The present study was designed to investigate the role and therapeutic potential of ENO1 in breast cancer. ENO1 has been reported to regulate tumorigenesis of different types of cancers [3]. Herein, we observed that ENO1 is significantly overexpressed in breast cancer and suppression of ENO1 could decrease the proliferation and colony formation of the breast SK-BR-3 cells. These results are in concordance with previous studies wherein ENO1 has been shown to play a role in the proliferation of cancer cells, for example, ENO1 regulates the proliferation of Non-Hodgkin’s Lymphoma cells [13]. Similarly, the proliferation of the rheumatoid arthritis fibroblasts-like synoviocytes is also suppressed upon decrease in the expression of ENO1 [14]. In yet another study, MiR-22-3p inhibits the proliferation of the retinoblastoma cells by suppressing the expression of ENO1 [15]. To study the molecular mechanisms underlying the decrease in the proliferation rate of the SK-BR-3 cells, we carried out DAPI staining and the results showed that ENO1 silencing triggered apoptotic cell death of the SK-BR-3 cells as evidenced from the nuclear fragmentation. Bax and Bcl-2 are important marker proteins for apoptosis. The increase in the Bax/Bcl-2 expression ratio has been shown to favor apoptosis [16]. Herein, we observed that silencing of ENO1 enhanced the expression of Bax and decreased the expression of Bcl-2, favoring apoptosis. The ENO1 silencing has also been shown to cause cycle arrest of the cancer cells [17]. Therefore, we also examined the effects of ENO1 silencing on the cell cycle distribution of SK-BR-3 cells. The results showed that ENO1 silencing caused an increase in the G2/M phase cells, indicative of G2/M cell cycle arrest. This was also concomitant with depletion of Cdc2, Cdc25 and cyclin B1 expression. Studies have also shown the role of ENO1 in the metastasis of cancer cells, for example the ENO1 has been shown to play a role in the migration and invasion of glioma cells [18]. Herein we examined the effects of ENO1 on the migration and invasion the SK-BR-3 breast cancer cells and the results showed that ENO1 silencing caused significant decline in the migration and invasion of these cells, indicative of the role of ENO1 in breast cancer cell metastasis.

**Conclusion**

The findings of this study revealed that ENO1 is upregulated in breast cancer cells and silencing of ENO1 decreases the proliferation and colony formation of SK-BR-3 breast cancer cells via induction of apoptosis and cell cycle arrest. In addition, ENO1 silencing also reduces the migration and invasion of the SK-BR-3 cells. Taken together, ENO1 may have important therapeutic implications in the treatment of breast cancer.

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**Conflict of interests**

The authors declare no conflict of interests.

**References**

Enolase 1 has anticancer activity in breast cancer


