Downregulation of $\alpha$-enolase (ENO1) Inhibits Growth, Invasion, and Metastasis of Human Cervical Cancer Cells

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Summary

Objectives: The aerobic glycolysis is a characteristic of cancer. $\alpha$-enolase (ENO1) is an important glycolytic enzyme and is suggested to play a role in the development of tumor. The purpose of this study was to investigate the effects of ENO1 on growth, invasion, and metastasis of human cervical cancer cells. Methods: We knocked down gene encoding ENO1 in Hela and SiHa cells with lentivirus, which was confirmed by western-blot. Following downregulation of ENO1, the drug sensitivity, invasion, metastasis, tumourigenic abilities of ENO1-altered cells were investigated. Results: Our studies demonstrated that downregulation of ENO1 enhanced the sensitivity of Hela and SiHa cells to paclitaxel and cisplatin and decreased their tumorigenicity. In addition, the abilities of invasion, metastasis, and tumourigenesis were decreased in ENO1-silenced Hela and SiHa cells. Conclusions: ENO1 is involved in the tumorigenicity, invasion, metastasis, and drug sensitivity of Hela and SiHa cells.

Key words: $\alpha$-enolase; Drug sensitivity; Cell tumorigenicity; Invasion and metastasis.

Introduction

Cervical cancer is one of the most common gynecologic malignant tumors and accounted for more than half of the malignant tumors of female reproductive system. The patients often have no obvious symptoms at early stage. When the patients go to the hospital for vaginal bleeding, it has been at the advanced stage. High-risk human papillomavirus (HPV) infection is the main cause of cervical cancer [1-3] and its oncogenes E6 and E7 play an important role in this disease. With the development of thinprep cytologic test and detection of HPV infection, the early detection rate of cervical cancer has been increased significantly. However, the mortality rate of cervical cancer is still high in developing countries due to the failure of early diagnosis of HPV infection [4, 5].

It has been reported that cervical cancer development relates to cancer cell metabolism and the rate of glycolysis is higher in cancer cells which is caused by up-regulation of glycolytic enzymes [6-9]. Although recent efforts have been made to decrease tumor growth and alter behavior by changing the glycolytic metabolism [10, 11], the relationship between glycolysis and cancer progression is still unclear.

Alpha-enolase1 (ENO1) is a glycolytic enzyme which catalyzes 2-phosphoglycerate to phosphoenolpyruvate. Besides, ENO1 plays important roles in various pathophysiological processes [12, 13]. The action of ENO1 in tumorigenesis has caught much attention of researchers. The tumour suppressor function of ENO1 has been indicated in non-small lung cancer cells [14]. Compared to metastatic head and neck cancer cells, ENO1 is lowly expressed in non-metastatic counterparts [15]. The expression of ENO1 was higher in breast cancer patients with estrogen receptor positive than that in patients with estrogen receptor negative [16]. We have identified ENO1 was down-regulated in cervical cancer tissues after treatment with paclitaxel (T) and cisplatin (P). However, the role of ENO1 in human cervical cancer cells remains unclear. The aim of this study was to evaluate its effects on growth, invasion and metastasis of human cervical cancer cells [17].

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Materials and methods
Preparation of Lentivirus
The cell lines used in this manuscript were originally from National biomedical experimental cell resource bank. ENO1 was downregulated by using the pLKO.1 puro vector. The 293T cells were incubated in complete DMEM medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2. 293T cells were seeded in 6-well plates. The cells were transfected with pLKO.1 puro vector containing shRNA against ENO1 or control vector using FuGENE® 6 Transfection Reagent (Promega), and the information of the vectors were mentioned in previous study [18]. Cell were harvested after 48 h and 72 h and filtered with a 0.45 μm cellulose acetate filter to remove cellular debris and stored at -80 °C, which were used as lentivirus stocks.

Down regulation of ENO1
The Hela and SiHa cells were all incubated in complete DMEM medium. Hela and SiHa cells were grown up to 70~80% confluence in 6-well culture plates and infected with pLKO.1 shENO1 lentivirus or control lentivirus. Polybrene (8 μg/ml, Sigma) was added to screen infected cells. After over-night incubation, the modified Hela and SiHa cells were washed with PBS and infected by lentivirus. The successful transfected cells were selected by puromycin (3 μg/ml, Invitrogen) after second infection, and the cells carried pLKO.1 shENO1 or scrambled shRNA sequences are named as pLKO.1 shENO1 and scr.

Verification of ENO1 expression level using western blot analysis
After lentivirus infection, cells were washed with PBS and carefully harvested. After being washed twice, the cells were lysed in 200 μl lysis buffer with 1% protease inhibitor cocktail (Gibco) on ice for 30 min. The protein was harvested by centrifugation (12,000 g, 4 °C, 20 min) and the concentration of protein was measured using the BCA Kit (Beyotime Biotechnology, China). Equal amounts of 20 μg protein were loaded on 12% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane was blocked with 5% milk/TBST and incubated with anti-ENO1 rabbit antibody (1 : 1000) (Cell Signaling Technology, USA) and anti-GAPDH antibodies (1 : 4000) (Merck, Germany) at 4 °C overnight. The membranes were washed 3 times with TBST and incubated with the secondary antibody (HRP-conjugated anti-IgG, ZSGB-BIO, China) for 2 h at room temperature. The membranes were performed with SuperSignal West Femto Max Sensitivity Substrate. The intensity of sample’s bands was determined by ImageJ software (https://imagej.nih.gov/ij/download.html).

Analysis of drug sensitivity of tumor cells using MTT assay
The cells were seeded in 6-well plates (2 × 10^5/well). When the cells reached 80%-90% confluence, they were treated with different concentrations of paclitaxel (5, 10, 20 μg/ml) and cisplatin (0.5, 1, 2 μmol/L) for 48h. Then 20 μl MTT (5 mg/ml) was added and incubated for 4 h at 37 °C. The culture medium was removed and 150 μL DMSO was added to each well for 30 min. The absorbance (A) was read by a microplate reader at 490 nm. Inhibition percentage (%) = (1 - experimental group A value / A value of the control group) × 100% was calculated.

Cloning formation assay
The cells were seeded into 6-well plates (200 cells to each well) and cultured in DMEM with 10% fetal bovine serum and under 5% CO2, 37 °C. After 2 weeks, cells were stained with 1% crystal violet for 20min and washed by PBS. The number of colonies was counted under a microscope.

Wound healing assay
The cells were seeded into 6-well culture plates (1 × 10^5 cells/each well) overnight. Three parallel wounds were created with a 10 μl pipette tip. After 0 h, 24 h, 48 h the appropriate pictures were taken and analyzed by ImageJ software (https://imagej.nih.gov/ij/download.html).

Trans-well migration and invasion assay
The migration and invasion assays were implemented using 24-well plates with Transwell (8 μm pore size) insert. DMEM 300 μL with 10% fetal bovine serum were added to the lower chambers. About 1 × 10^5 cells were added to upper chambers with 200 μL DMEM with 10% fetal bovine serum. After 24 h incubation, cells in the lower side of the membrane were stained with 1% crystal violet for 20 min, and counted using microscope. The upper chambers were coated with extracellular 1 mg/ml matrixgel for the invasion assay.

Immunocytochemistry
There were 2 × 10^4 cells seeded on 19 mm circular coverslips into 6-well plates, and fixed with paraformaldehyde and rinsed with PBS. The cells were permeabilized with 1% Triton X-100 for 10 minutes at room temperature, and then rinsed with PBS. The circular coverslips were incubated with anti-VEGF rabbit antibody (1 : 200), anti-EGFR rabbit antibody (1 : 200), anti-MMP9 rabbit antibody (1 :
Figure 2. — The knock-down of ENO1 reduced tumorigenicity. The inhibition effect on Hela (A, C) and SiHa (B, D) cells' tumorigenicity was detected at 2 weeks after shRNA interference. * Hela pLKO.1 shENO1 vs. scr cells group or Hela cells $p < 0.05$. # Hela pLKO.1 shENO1 vs. scr cells group or Hela cells $p > 0.05$. * SiHa pLKO.1 shENO1 vs. scr cells group or SiHa cells $p < 0.05$. △ SiHa pLKO.1 shENO1 vs. scr cells group or SiHa cells $p > 0.05$.

200), anti-Notch2 rabbit antibody (1 : 200) and anti-COX2 antibodies (1 : 200) at 4 °C overnight. All these antibodies mentioned above were purchased from Cell Signaling Technology (USA). The circular coverslips were then washed 3 times by PBS and incubated with the secondary antibody (HRP-conjugated anti-IgG) for 2 h at 37 °C. The results were visualized by DAB staining and photographed using fluorescence microscopy.

**Statistical analysis**

The results were expressed as mean ± SD of three separate experiments. The statistical significance was assessed by two-tailed unpaired t-test. Differences with $p < 0.05$ were considered statistically significant.

**Results**

The expression of ENO1 in Hela and SiHa cells was knocked down by lentivirus-mediated RNA interference

The expression of ENO1 in Hela and SiHa cells was silenced through lentivirus carrying pLKO.1 shENO1 and detected by western blot. Compared with control group, the expression level of ENO1 in Hela and SiHa cells infected with pLKO.1 shENO1 lentivirus was lower than the detection limit (Figure 1). These results suggested that the experimental model was useful for further investigation of the effects of ENO1 on human cervical cancer cells.

**Downregulation of ENO1 expression increased sensitivity to cisplatin and paclitaxel**

The sensitivity of cells with knock-down ENO1 to cisplatin and paclitaxel was assessed by MTT assay. Hela cells treated with pLKO.1 shENO1 were more sensitive to cisplatin (Table 1) and paclitaxel (Table 2) than cells treated with scr ($p < 0.05$). Similar results were obtained in SiHa cells ($p < 0.05$).

**ENO1-knock-down inhibited cloning formation**

The effect of ENO1-knock-down on tumorigenicity was observed by cloning formation assay. Significantly less numbers of clones were formed in Hela pLKO.1 shENO1 cells and SiHa pLKO.1 shENO1 cells compared to control groups (Figure 2). The knock-down of ENO1 reduced tumorigenicity of Hela and SiHa cells, which suggested that ENO1 would affect the tumorigenicity of human cervical cancer cells.
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Figure 3. — The knock-down of ENO1 reduced cell migration. The effects of ENO1-knock-down on the migration ability of Hela cells (A) and SiHa cells (B) were quantified by wound healing assay and taken pictures at 0 h, 24 h, 48 h. The effect of ENO1-knock-down on the migration ability of cells was quantified by trans-well migration assay at 24 h (C). The number of Hela pLKO.1 shENO1 cells and SiHa pLKO.1 shENO1 cells that migrated the membrane were less than that of the scr groups (D and E). * Hela pLKO.1 shENO1 vs. scr cells group or Hela cells \( p < 0.05 \). # Hela pLKO.1 shENO1 vs. scr cells group or Hela cells \( p > 0.05 \). \( \star \) SiHa pLKO.1 shENO1 vs. scr cells group or SiHa cells \( p < 0.05 \). \( \triangle \) SiHa pLKO.1 shENO1 vs. scr cells group or SiHa cells \( p > 0.05 \).

ENO1-knock-down inhibited metastasis of Hela and SiHa cells

We performed wound healing and trans-well migration assays to test the effect of ENO1-knock-down on cell migration ability. The migration distance of ENO1-knock-down cells were significantly shorter than that of normal and scr control cells, but no significant differences were observed between normal and scr control cells (Figure S 3A and 3B). The result of trans-well migration assay was similar to the wound healing assay. The number of cells migrated the membrane from Hela pLKO.1 shENO1 cells and SiHa pLKO.1 shENO1 cells was less than that from scr groups (Figure S 3C and 3D). Taken together, ENO1 played a role in cell migration ability.

ENO1-knock-down inhibited the invasion of Hela and SiHa cells

The trans-well migration assays with matrigel was used to observe the effect of ENO1-knock-down on cell invasion ability. Matrigel is a soluble basement membrane matrix. The number of cells that passed the basement membrane could reflect cell invasion ability. The numbers of Hela pLKO.1 shENO1 cells and SiHa pLKO.1 shENO1 cells that passed the basement membrane were significantly less than the scr and normal groups while there was no obvious difference between scr and normal cells (Figure 4).
eno1-knock-down inhibited the expression of proteins related to invasion and metastasis of siha cells

To further confirm the effect of eno1 on invasion and metastasis of siha cells, the expression of vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), matrix metallo proteinases 9 (MMP9), Notch2, and Cyclooxygenase-2 (COX2) which were related to invasion and metastasis were analyzed by immunocytochemistry. The expression of these genes significantly decreased in siha plko.1 sheno1 cells than in control scr cells (Figure 5).

Discussion

Aerobic glycolysis has been regarded as an important metabolic hallmark of cancer. The investigation of whether
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Table 2. — The inhibition ratio of paclitaxel on each group of cells.

| group                  | Inhibition ratio (%) |
|------------------------|----------------------|
|                        | 20 μg/ml Taxo | 10 μg/ml Taxo | 5 μg/ml Taxo |
| Hela                   | 0.379 ± 0.056 | 0.283 ± 0.048 | 0.240 ± 0.035 |
| Hela scr               | 0.381 ± 0.056# | 0.291 ± 0.040# | 0.250 ± 0.030# |
| Hela pLKO.1shENO1      | 0.491 ± 0.060* | 0.375 ± 0.050* | 0.328 ± 0.060* |
| SiHa                   | 0.352 ± 0.068 | 0.294 ± 0.066 | 0.262 ± 0.081 |
| SiHa scr               | 0.369 ± 0.037* | 0.303 ± 0.036* | 0.250 ± 0.028* |
| SiHa pLKO.1shENO1      | 0.452 ± 0.052△ | 0.409 ± 0.060△ | 0.379 ± 0.123△ |

*Hela pLKO.1 shENO1 vs. Hela or Hela scr p < 0.05, # Hela scr vs. Hela p > 0.05
△SiHa pLKO.1 shENO1 vs. SiHa or SiHa scr p < 0.05, * SiHa scr vs. SiHa p > 0.05

Figure 5. — The knock-down of ENO1 inhibited the expression of COX2, EGFR, MMP9, VEGF, and Notch2 in SiHa pLKO.1 shENO1 cells.

Silence of ENO1 in Hela and SiHa cells led to the decline of the abilities of invasion, metastasis, and tumorigenesis of these cells. Since ENO1 has different actions in various physiological and pathological process, we supposed that knock-down ENO1 might inhibit cell viability through the inhibition of glycolysis pathway or may involve in the process of growth and proliferation of cervical cancer cells directly as a multifunctional protein. To further explore the role of ENO1 in cervical cancer cell lines, the expression of VEGF, EGFR, MMP9, Notch2, and COX2 was analyzed through immunocytochemistry and we found that ENO1-knock-down could inhibit the expression of these genes.

VEGF is a class of glycoproteins separated and purified from the culture medium of bovine pituitary follicular stellate cells [24]. EGFR is a member of epidermal growth factor receptor family and involved in regulating cellular proliferation, differentiation, and survival [25]. MMPs is a group of endopeptidases and MMP9, an important member of Type IV collagenases, can degrade various proteins of the extracellular matrix [26]. COX-2 is an important rate-limiting enzyme in metabolism of arachidonic acid. COX-2 expression is closely related with tumor angiogenesis [27]. VEGF, EGFR, COX2 are important molecular markers related to tumor angiogenesis [28, 29]. The decreased expression of these genes in Hela pLKO.1 shENO1 cells and SiHa pLKO.1 shENO1 cells indicated that the inhibition of invasion and metastasis of cancer cells by ENO1 through multiple molecular targets. The interaction of VEGF and EGFR in the ERK/COX-2/VEGF signaling pathway would further inhibit tumor angiogenesis [28].

In summary, this study presented that ENO1 knockdown inhibited growth, invasion and metastasis of human cervical cancer cells and enhanced Hela and SiHa cells sensitivity to paclitaxel and cisplatin. The mechanism of ENO1 inhibiting cervical cancer cell growth should be further an-
alyzed.

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Conflict of Interest

The authors report no conflicts of interest in this work.

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