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Original Article

Effects of α-enolase (ENO1) over-expression on malignant biological behaviors of AGS cells

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Abstract: Objective: To investigate the effects of α-Enolase (ENO1) over-expression on the proliferative and migratory abilities of AGS cells. Methods: The target gene was cloned and mounted to the eukaryotic expression vector pcDNA3.1(+), then was transfected into gastric cancer cell lines AGS. mRNA and protein level of ENO1 in AGS cells were verified by real-time quantitative RT-PCR and Western Blot, respectively. The effects of over-expression of ENO1 on proliferative and migratory abilities of AGS cells were detected by the experiments of CCK-8, colony formation and wound healing assays. Results: The eukaryotic expression vector pcDNA3.1(+)/*eno1* was successfully constructed, and verified by sequencing. It was shown from the cell proliferation curves that the proliferative ability of AGS-ENO1 transfected group was higher than that of the control group after 72 hours ($t = 3.44$, $P = 0.04$), meanwhile, the number of the cell-colonies of the AGS-ENO1 group were significantly greater than that of the control group ($t = 5.26$, $P = 0.01$). For the ability of migration, it was significantly enhanced in the over-expression ENO1 cells than in the negative cells ($t = 7.35$, $P < 0.001$). Conclusion: The over-expression of ENO1 protein can enhance the abilities of proliferation and migration in gastric cancer cells of AGS, which indicates that ENO1 may be an important potential tumor-marker associated with the development of gastric cancer.

Keywords: α-enolase, gastric cancer, proliferation, migration

Introduction

ENO1 is one of the key enzymes which can catalyze the conversion of glycerol phosphate to phosphoenolpyruvate in the glycolytic process and plays an important role in energy metabolism [1]. Recent studies have revealed that ENO1, a multifunctional protein, is involved in many different physiological and pathophysiological processes [2]. In particular, ENO1 expressed on the cell surface can promote metastasis and migration of tumor cells by accelerating plasminogen activation and degrading extracellular matrix as a plasminogen receptor [3]. In hypoxic situations, it may acts as a stress protein that promotes hypoxic tolerance in tumor cells by increasing anaerobic metabolism [4]. Shih et al [5] showed that ENO1 as an autoantigen and it has been recognized as common markers of systemic autoimmune diseases and inflammatory, degenerative and other pathological disorders. Furthermore, the over-expression of ENO1 correlated with tumor size, the degree of malignancy and accumulation of blood vessels, tumor metastasis and prognosis in many tumor tissue [6-9] (for example, breast, cholangiocarcinoma, small-cell lung cancer, pancreatic cancer, prostate cancer and head and neck tumors). Takashima et al [10] showed that an significantly increase of ENO1 expression in hepatitis C virus-related hepatocellular carcinoma of poorly differentiated and vein invasion. Tsai et al [11] found that the over-expression of ENO1 could significantly promote the ability of proliferation and migration of oral cancer cells. Therefore, ENO1 has been considered to be a potential tumor-marker associated with the development and progression of many tumors besides its innate glycolytic function. Some reports have discovered that high expression ENO1 in gastric cancer tissue compared with normal mucosa by proteomic analysis [12], nevertheless, the study about the effects of ENO1 on the gastric cells in vitro is still limited. In view of mentioned above, our study focused on the effects of ENO1 over-expression on the

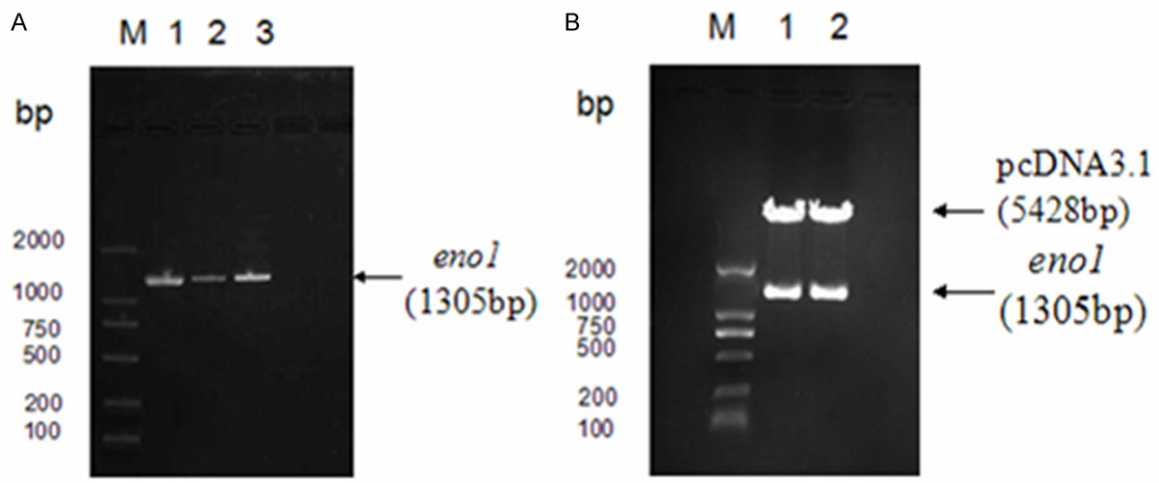


Figure 1. Assessment of *eno1*. (A) Electrophoresis of full-length *eno1* gene PCR product, M-DL2000 DNA Marker; 1, 2, 3-*eno1* gene PCR product (B) EcoR1/Xba1 double enzyme digestion analysis of pcDNA3.1/*eno1* recombinant plasmid, M-DL2000 DNA Marker; 1, 2-pcDNA3.1/*eno1* double enzyme digestion.

proliferative and migratory abilities of AGS cells, in order to provide the experimental evidences for clarifying the function of ENO1 in vitro.

Materials and methods

Materials

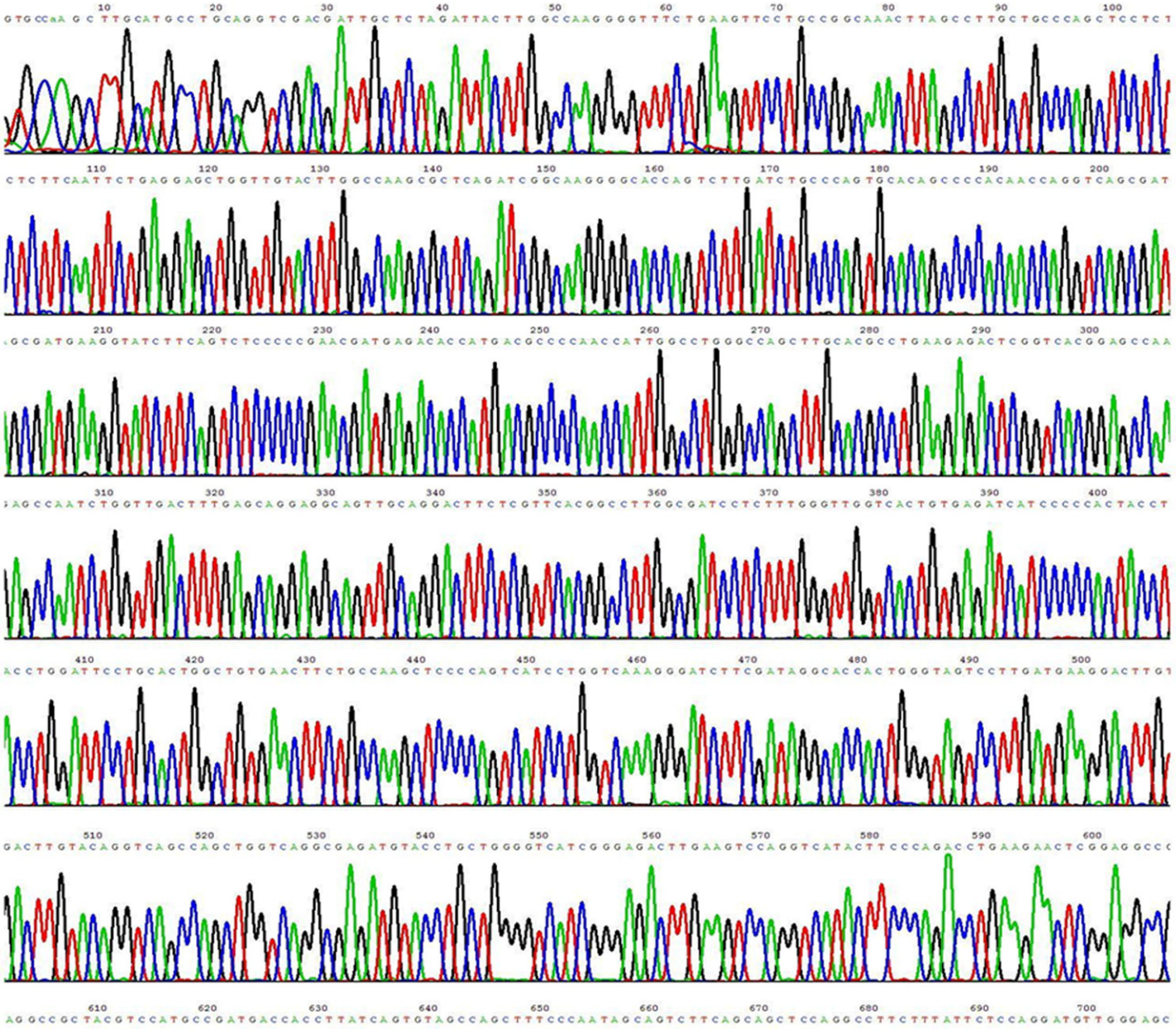
Plasmid pcDNA3.1(+) was obtained from the Central Laboratory of Affiliated Hospital of Guangdong Medical College (Guangdong, China). Human gastric cancer cell line AGS was purchased from Cell Bank of Institute of Life Science, Chinese Academy of Science, Shanghai and obtained from the Chinese and American Institute for Cancer Research Guangdong Medical College (Guangdong, China). Lipofectamine™ 2000 was purchased from Invitrogen Biotechnology (USA). Restriction endonuclease EcoR1, Xba1, DNA Ligation Kit Ver.2.1, pMD18-T Simple Vector were purchased from TAKARA Biotechnology Inc (Dalian, China). E. coli DH5α, TIANprep Mini Plasmid Kit, TINgel Midi Purification Kit, TIANquick Midi Purification Kit, TRNzol Reagent and TIANScript cDNA were purchased from TIANGEN BIOTECH Co., Ltd (Beijing, China). Primary rabbit polyclonal anti-ENO1 antibody was purchased from Abcam trading Co., Ltd (Shanghai, China). Primary rabbit polyclonal β-actin antibody was purchased from Cell Signaling Technology, Inc (Shanghai, China). Primer synthesis and DNA sequencing were acquired from Invitrogen Trading Co., Ltd (Shanghai, China). Primers synthesis and DNA sequencing were acquired from Invitrogen Trading Co., Ltd (Shanghai, China).

ENO1 cDNA synthesis, purification and ligation with pMD18-T simple vector

The human *eno1* gene cDNA sequence data (NM_001428.3) was accessed from the database of GenBank, and was used in design the primer to amplify the target gene with primer design software Premier 5.0 The primers containing EcoR1/Xba1 restriction enzyme cutting sites ,were designed as follow: forward: 5'-TCACCGGTCCTATC TGGGG-3'; reverse: 5'-GAGAGCCGTCACTCACTCATTCCC-3'. The length of PCR amplification product is 1305 bp. PCR products were detected by 1.5% agarose gel electrophoresis (80 V, 45 min). The target fragments were retrieved and purified by TIANquick Midi Purification Kit, which were polyadenylated by using DNA A-Tailing Kit, then were ligated into pMD18-T Simple Vector at 16°C for overnight by using DNA Ligation Kit Ver.2.1 (TA Clone). The recombinant pMD18-T/*eno1* was transformed into competent *E. coli* DH5*α* cells, which inoculated in LB solid medium plates containing Ampicillin (final concentration of 100 μg/ml) at 37°C for overnight. The positive clones were isolated from transformants by TIANprep Mini Plasmid Kit and the pMD18-T/*eno1* was sequenced by Invitrogen Trading Co., Ltd (Shanghai, China).

Construction of recombinant pcDNA3.1(+)/eno1 eukaryotic expression vector

The pMD18-T/*eno1* plasmids were digested by restriction enzymes *EcoR*1 and *Xba*1 at 37°C for 3 h and the target fragments containing the full length cDNA of *eno1* were isolated and puri-



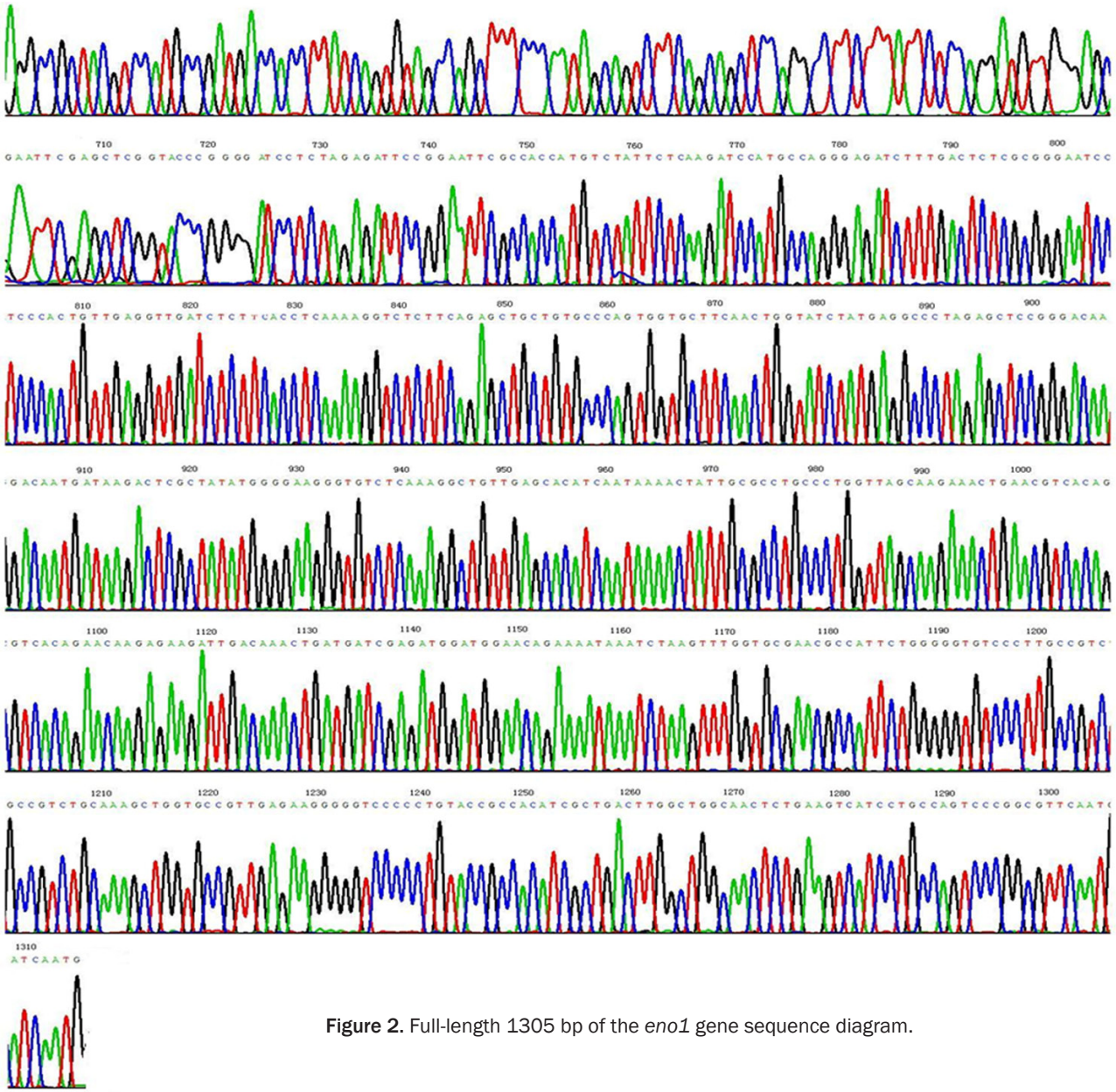


Figure 2. Full-length 1305 bp of the *eno1* gene sequence diagram.

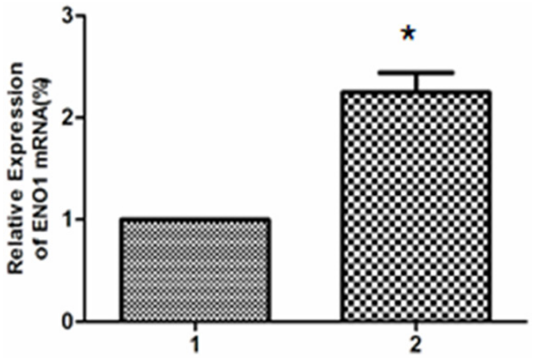


Figure 3. Quantitative expression of *eno1* gene transfection cells, ENO1 mRNA was measured by quantitative real-time RT-PCR, 1-pcDNA3.1 + Lipofectamine™ 2000, 2-pcDNA3.1/ENO1 + Lipofectamine™ 2000, *: compared with the control groups (t = 6.39, $P < 0.05$).

fied. The pcDNA 3.1(+) vectors were also digested by *EcoR*1 and *Xba*1 and *eno1* cDNA fragment was mounted to the cloning site by using DNA Ligation Kit v.2.0. The reaction system was as follow: ligation buffer 1 μl, pcDNA3.1(+) 1 μl, *eno1* gene fragment 2 μl, T_4 DNA ligase (400 U/μl) 0.5 μl, ddH_2O 5.5 μl. The ligation products were transformed into competent *E. coli* DH5α cells, and the positive clones were cultured and isolated by using the same methods mentioned above, then were identified by restriction enzyme mapping and DNA sequencing.

Expression of the recombinant pcDNA3.1(+)/eno1 vector in AGS cells

Cell transfection: The AGS cells cultured in RPMI-1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO_2, at 80% confluence were used, and they were inoculated in a 6-well cell culture plate with a density of 0.3×10^6 per well for 24 hours before transfection. They were transfected with pcDNA3.1(+) and pcDNA3.1(+)/*eno1* by mixing the plasmids with Lipofectamine™ 2000 with the proportion of 1:3 according to the Lipofectamine™ 2000 instructions. Twenty-four hours after transfection, cells were collected to perform subsequent assays.

Cell proliferation assay

AGS cells were collected 24 hours after transient transfection and cultured in 96-well plates (1.0×10^4 per well) with RPMI- 1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO_2. The proliferative effect of ENO1 on AGS cells was determined by the CCK-8 assay after 5 hours (as 0 day), and a total cell number was detected every 24 hours. At each time course, 100 μl of CCK-8 diluent (up to 10 μl CCK-8 was added to 100 μl RPMI-1640) was added to each well, and incubated at 37°C for 2 hours. Optical density (OD) value at 490 nm was measured using an micro plate reader (Thermo, USA). This procedure was repeated three times. Mean values were calculated using registered figures derived from at three independent tests, and the result was presented by mean ± standard deviation (SD).

Colony formation assay

AGS cells were collected 24 hours after transient transfection and cultured in 6-well plates (3000 per well) with RPMI- 1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO_2. The medium supplemented with Neomycin was changed every three days. The medium was removed after 10 days and the cells were washed three times with 1 × PBS. They were fixed with cold methanol for 30 min and stained with 0.2% crystal at room temperature for 30 min, following by wash, dry and photograph. Colonies containing more than 50 cells were counted, this procedure was repeated three times and each time 3 wells.

Wound healing assay

AGS cells were transiently transfected and cultured in 6-well plates (0.3×10^6 per well) with RPMI- 1640 containing 10% Neonatal Bovine Serum at 37°C ,5% CO_2 until the cells were confluent. The monolayers were wounded by sterile 20 μl tips washed, and fresh RPMI- 1640 containing 5% Neonatal Bovine Serum was added (at 0 hours). Then they were observed every 24 hours and photographed. This procedure was repeated three times and each time 3 wells.

Statistical analysis

All data are described as mean ± standard deviation ($\bar{x}$ ± SD) based on three independent experiments. The statistical methods of Student's t test, analysis of variance (ANOVA) and least significant difference (LSD) were used according to the application condition. Data were analyzed by using SPSS 15.0 software. Statistical significance was defined as $P < 0.05$.

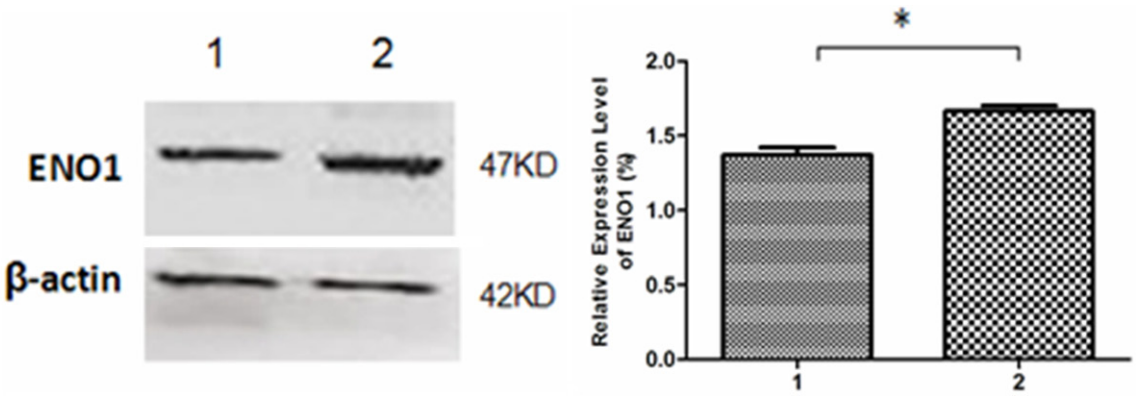


Figure 4. Quantitative expression of *eno1* gene transfection cells, ENO1 protein was analyzed by western blot, 1-pcDNA3.1 + Lipofectamine™ 2000, 2-pcDNA3.1/ENO1 + Lipofectamine™ 2000, *: compared with the control groups (t = 4.70, $P < 0.05$).

Results

Amplification of eno1 gene and construction of recombinant eukaryotic expression vector pcDNA3.1(+)/eno1

The total RNA extracted from human immortal gastric mucous epithelial cells (GES-1) was reversely transcribed to cDNA as a template for PCR, and the full length of 1305 bp of *eno1* was amplified (**Figure 1A**). The recombinant pMD18-T/*eno1* was then constructed by using the method of T-A cloning, and was sequenced, as shown in **Figure 2**. The recombinant vector of pcDNA3.1(+)/*eno1* was constructed by mounting the *eno1* cDNA fragment to the cloning site of the pcDNA 3.1(+) plasmid, which was digested by restriction enzymes *EcoR1* and *Xba*1 and identified by 1.0% agarose gel electrophoresis (**Figure 1B**).

RNA and protein expressions of ENO1 in AGS cells

After 24 hours transfection, the expression of ENO1 in AGS cells was assessed by quantitative real-time RT-PCR and western blot, respectively. The results showed that the expression level of ENO1 in AGS cells transfected with the recombinant vector pcDNA3.1(+)/*eno1* was significantly higher than that of the control group (**Figures 3**, **4**).

Effect of over-expression of ENO1 on proliferation of AGS cells

The proliferative abilities of AGS cells influenced by over-expression of ENO1 were detected by using the methods of CCK-8 test and colony formation assay, respectively cell proliferation curves were depicted with mean OD values of each time point (**Figure 5A**), which indicated that the group transfected with the recombinant vector pcDNA3.1(+)/*eno1* grew faster than the control group transfected with the mock pcDNA3.1(+) after 72 hours (t = 3.44, $P = 0.04$). Being in coincidence with the results of CCK-8 test, the colony formation assay results showed that the amount of formed colonies in the group transfected with the recombinant vector pcDNA3.1(+)/*eno1* were more than those in the control group. (t = 5.26, $P = 0.01$) (**Figure 5B**).

Wound healing assay of ENO1 transfected AGS cells

The effects of over-expression of ENO1 on cell mobility of AGS cells were measured by wound healing assay. Forty eight hours after transfection with RPMI-1640 containing 5% Neonatal Bovine Serum at 37°C, 5% CO_2, the group transfected with the recombinant vector pcDNA3.1(+)/*eno1* had a faster healing speed than the control group (t = 7.35, $P < 0.001$) (**Figure 6**).

Discussion

It is widely accepted that ENO1 is a highly conserved glycolytic enzymes playing an important role in cellular energy metabolism [13]. Accumulation of recent evidences has shown that, in addition to its central role in glycolysis, ENO1 has been related to tumorigenesis of several human cancers [14]. ENO1 can acts as plas-

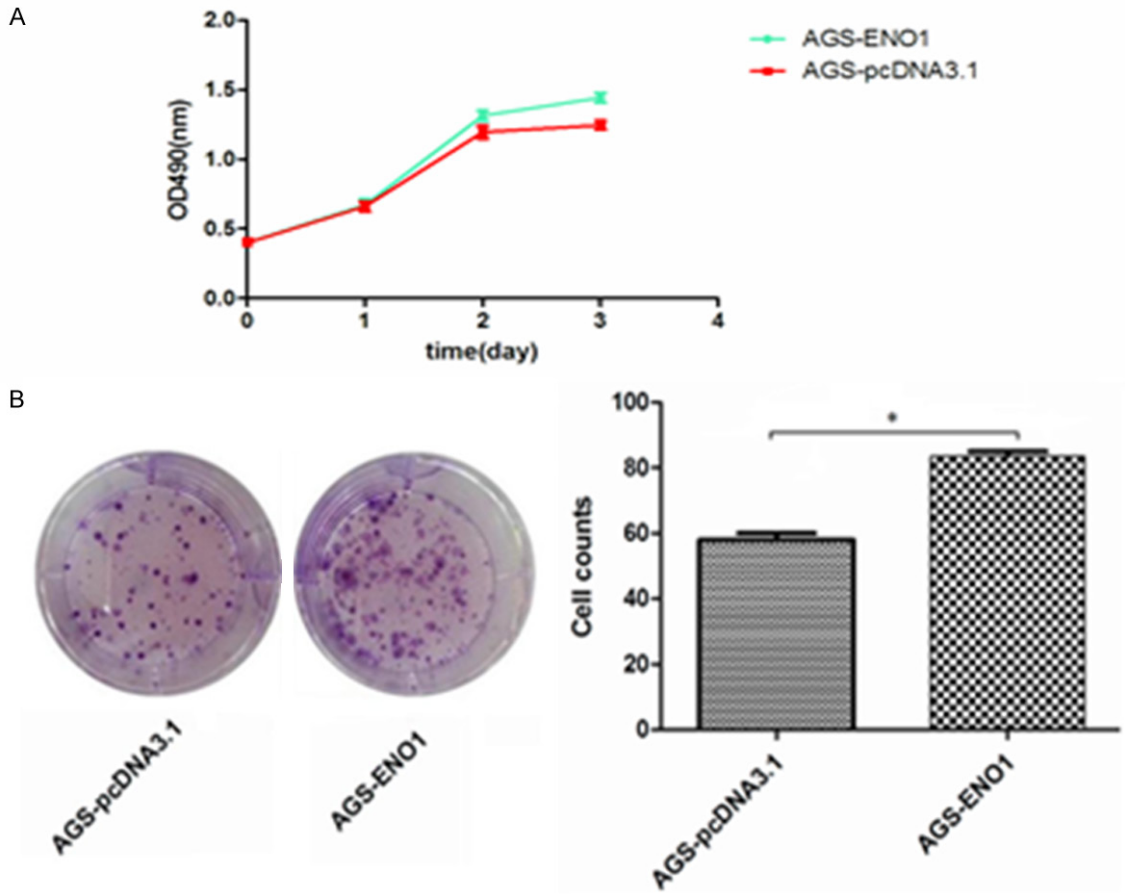


Figure 5. Effect of ENO1 over-expression on proliferation of AGS cells, compared with the control groups. A. AGS cell growth curves; B. The number of formed colonies and cell clone histogram (n = 3, **P* < 0.05).

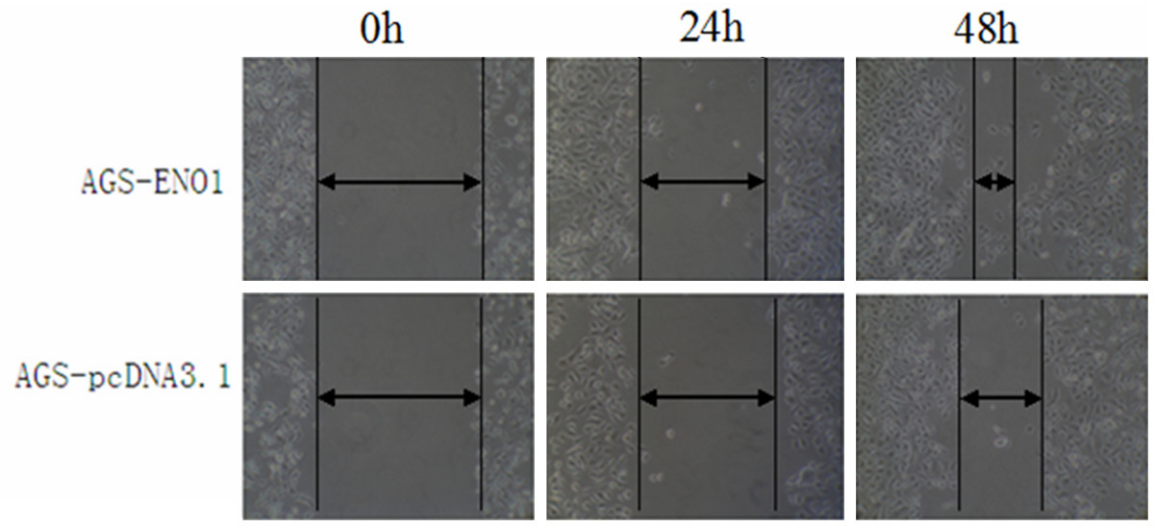


Figure 6. Effect of ENO1 on migratory of AGS cells, the results of cell scarification assay.

minogen-binding receptor and autoantigen. Ejeskar et al [13] have shown that *eno1* gene is located in chromosome region 1p36.2, commonly deleted in advanced neuroblastoma tumors, can act as a strong tumor suppressor by slowing down the cell growth and inducing

apoptosis. However, more and more studies have demonstrated that ENO1 is highly expressed in many kinds of cancers and correlated with tumor size and venous invasion [15]. Ni et al [16] have reported that the over-expression of ENO1 was significantly related to differentiation grade, depth of invasion, lymph node metastasis and TNM staging in gastric cancer tissue compared with normal mucosa. ENO1 also used as a diagnostic marker for human lung cancer and a high expression has been implicated in human tumorigenesis [17]. To date, the exact mechanism between ENO1 and occurrence of gastric cancer is still unclear. To clarify the effects of ENO1 on gastric cancer, we constructed an eukaryotic expression vector pcDNA3.1(+)/eno1 and detected the effects of ENO1 over-expression on the malignant biological behaviors of AGS cells.

As a key enzyme of glycolysis, ENO1 participates in the process of energy metabolism to meet energy demand of the rapid growth of tumor cells [18]. A high level of ENO1 leads to rapidly proliferation of tumor cells, conversely, significantly decreased synthesis of ENO1 has been observed in the non-proliferation of keratinocytes [19]. Consistent with the previous studies, it was showed by the CCK-8 cell proliferation assay that ENO1 can enhance the abilities of proliferation of AGS cells ($P = 0.04$). At the same time, the colony formation assay also showed that ENO1 can significantly promote proliferation of AGS cells ($P = 0.01$). In line with our observations, Tsai et al [20] found that ectopic ENO1 expression in head and neck cancer cells could enhance cell proliferation and colony formation abilities, and ENO1 knockdown obviously attenuated the accelerating ability of ENO1 expression on cell proliferation.

In this study, we have found that ENO1 gene transfer contributed to a wound healing faster than the mock pcDNA3.1 by cell scratch assay ($P < 0.001$), indicating a migration ability effect of ENO1 over-expression. Consistent with the notion, an increase of ENO1 expression on the surface of monocytes promotes their migratory and lung-infiltrating ability [21]. Moreover, high expression of ENO1 can promote tumor cell invasion through Matrigel and regional lymphatic invasion in vivo [20]. Recent studies have shown that ENO1 acts as a plasminogen-binding receptor besides of playing an important role in glycolysis in cytoplasm, which may involve in wound healing, tissue remodeling, embryogenesis and the process of cancer cell invasion and metastasis [15]. Liu et al [3] have shown that ENO1 on the cell membrane consists of a cross cytoplasm COOH-terminal (composed of 16 amino acids). Tumor cells could concentrate and activate plasminogen that involves in remodeling of extracellular matrix and basement membrane which results in the invasion and metastasis.

In conclusion, we constructed a recombinant eukaryotic expression vector pcDNA3.1(+)/*eno1* and observed that ENO1 over-expression could enhance the abilities of proliferation and migration in AGS cells, which indicates that ENO1 may be an important responsible factor associated with the development of gastric cancer. In addition, it should be aware that the transient transfection in the current study could impact the results to some extent. So, the effects of ENO1 on malignant biological behaviors of gastric cell lines warrant further studies. For example, knockdown of *eno1* gene and carrying out the experiments in vivo or in vitro, which would shed some light on the association between ENO1 and gastric cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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