Effects of RNA interference of the enol1 gene on the malignant biological behaviors of gastric cell lines

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Abstract: To observe the expression of α-Enolase (ENO1) correlated with the pathological progression of gastric cancer and to investigate the effects of ENO1 down-regulation on the proliferative and migratory abilities of gastric cell lines in vitro. The expression of ENO1 was detected in 22 cases of gastric cancer, 20 cases of atypical hyperplasia and 18 cases of normal gastric tissues by immunohistochemistry. The expression level of ENO1 mRNA and protein was determined in AGS cell lines by quantitative real-time reverse transcription PCR (qRT-PCR) and Western blot, respectively. The effects of down-regulation of ENO1 on proliferative and migratory abilities of AGS cells were detected by the experiments of CCK-8, colony formation and wound healing assays. The expression level of ENO1 protein in gastric cancer tissues was higher than that of normal gastric tissues and atypical hyperplasia in the immunohistochemical study (HC = 22.70, P < 0.05). It was shown from the cell proliferation curves that the proliferative ability of AGS-ENO1-siRNA transfected group was slower than that of the AGS-NC-siRNA over 72 h (t = 2.50, P = 0.03), meanwhile, the number of the cell-colonies of the AGS-ENO1-siRNA group was less than that of the control group after 10 days (t = 22.05, P = 0.01). For the ability of migration, it was significantly decreased in the down-regulation ENO1 cells than in the negative cells after 48 h (t = 8.54, P = 0.01). The results indicate that ENO1 protein may be an important potential tumor-marker associated with the development of gastric cancer.

Keywords: α-Enolase; gastric cancer; proliferation; migration

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1. Introduction

Gastric cancer is one of the most common cancers and the second leading cause of cancer-related deaths worldwide [1]. Although some progress in early diagnosis and therapeutic strategies has been made in recent years, the prognosis for gastric cancer patients remains poor, which is associated with the invasion and metastasis of gastric cancer [2]. Thus, identification of the novel cancer-related marker and then investigations into the molecular mechanisms of invasion and metastasis of gastric cancer are of great significance for early diagnosis, treatment and prognosis of gastric cancer.

It is well known that during the process of tumor formation and metastasis, tumor cells need increase glucose metabolism to support the increased demand of energy necessary for unrestricted growth [3]. Recent studies have revealed that α-Enolase (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 [4]. In hypoxic situations, it acts as a stress protein that promotes hypoxic tolerance in tumor cells by increasing anaerobic metabolism (Warburg effect) [5]. Furthermore, recent evidences have shown that, in addition to its central role in glycolysis, ENO1 has been related to tumorigenesis of several human cancers [6,7]. In particular, 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 tissues (for example: breast, cholangiocarcinoma, small-cell lung cancer, pancreatic cancer, prostate cancer and head and neck tumors) [6,8,9]. Therefore, ENO1 has been considered to be a potential tumor-marker associated with the development and progression of many tumors.

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2. Materials and methods

2.1. Materials

2.1.1 Tissue collection

Frozen tissues of surgically resected tumors, atypical hyperplasia and adjacent normal tissues obtained from Dongguan People’s Hospital. These tissue samples included 22 cases of gastric cancers tissues, 18 cases of normal gastric tissues and 20 cases of atypical hyperplasia. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Affiliated Hospital of Guangdong Medical University. Informed consent was attained from all patients.

2.1.2 Cell line and reagents

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). siRNA targeting human ENO1 and control siRNA were obtained from RiboBio Co., Ltd (Guangzhou, China). TRNZol Reagent and TIANScript cDNA were purchased from TIANGEN BIOTECH Co., Ltd (Beijing, China). Cell Counting Kit-8 (CCK8) was purchased from DOJINDO Co., Ltd (Shanghai, 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).

2.2 Methods

2.2.1. Immunohistochemical staining and scoring

IHC assays were performed as previously described [12]. Firstly, the paraffin sections were deparaffinized in xylene twice and then put them into an ethanol gradient to rehydrate. Using 3% H2O2 blocked endogenous peroxidase for 20 minutes, washed by phosphate-buffered saline (PBS) for three times. Then the sections were performed antigen retrieval in 10 mM citrate buffer (pH6.0) for 5 minutes at boiling water, after that, washed by PBS for three times. Then the sections were incubated with the primary anti-TPI antibody (dilution of 1:100, Abcam) at 4°C overnight. In the next day, the section was washed by PBS for three times and then incubated with secondary antibodies for 30 minutes. Finally, slides were colorized with 3,3-diaminobenzidine (DAB) for 3 minutes, restrained with hematoxylin.

The results of IHC staining were analyzed by two pathologists independently to avoid the interobserver variability. ENO1 staining scores were calculated as follows: combined with the proportion of positive cells (none=0, <10%=1; 10%-50%=2; 51%-80%=3; >80%=4) and staining intensity (none=0; weak=1; moderate=2; strong=3) of the reaction product of immunization rates (immunoreactive score, IRS). The scores were divided into four groups: low expression (1–3), moderate expression (4–8) and high expression (9–12).

2.2.2. Cell culture and tissue collection

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). siRNA targeting human ENO1 and control siRNA were obtained from RiboBio Co., Ltd (Guangzhou, China). TRNZol Reagent and TIANScript cDNA were purchased from TIANGEN BIOTECH Co., Ltd (Beijing, China). Cell Counting Kit-8 (CCK8) was purchased from DOJINDO Co., Ltd (Shanghai, 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). Frozen tissues of surgically resected tumors, atypical hyperplasia and adjacent normal tissues obtained from Dongguan People’s Hospital.

2.2.3. Cell transfection

The AGS cells cultured in RPMI-1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO2, and they were inoculated in a 6-well cell culture plate with a density of 0.2 × 104 per well for 24 hours before transfection (at 50% confluence were used). They were transiently transfected with the ENO1
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small interfering RNA (ENO1-siRNA) (50 nM) and noncontrolled siRNA (NC-siRNA) by mixing 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.

2.2.4. Real-time reverse transcription PCR (qRT-PCR)

The total RNA was extracted separately from the ENO1-siRNA AGS cells and NC-siRNA cells, and were used for the synthesis of first strand cDNA using Super M-MLV Reverse transcriptase. Subsequently, the ENO1-siRNA and NC-siRNA mRNA was examined in by real-time PCR analysis. The primer sequences for ENO1 were follows:

Forward: 5′-CCGGAAATTCGACCACATGCTATTCTCAAG ATCCA-3′
Reverse: 5′-GCTCTAGATTACTTGGCCAAGGGGTTC-3′.

The PCR reaction was carried out using SYBR Green Mix reagent and the experiment was repeated three times.

2.2.5. Cell proliferation assay

AGS cells were collected 24h after transient transfection and cultured in 96-well plates (1.0 × 10⁴ per well) with RPMI- 1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO₂. The proliferative effect of ENO1 on AGS cells was determined by the CCK-8 assay after 4 hours (as 0 day), and a total cell number was detected every 24 hours. At each time course, 100μl of CCK-8 diluent (up to 10μl CCK-8 was added to 100μl RPMI-1640) was added to each well, and incubated at 37°C for 2 hours. Optical density (OD) value at 450 nm was measured using a 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).

2.2.6. 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% CO₂. The medium supplemented with Neomycin was changed every 3 days. The medium was removed after 10 days and the cells were washed three times with 1xPBS. They were fixed with cold methanol for 30min and stained with 0.2% crystal at room temperature for 30min, 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.

2.2.7. Wound healing assay

AGS cells were transiently transfected and cultured in 6-well plates (0.3 × 10⁵ per well) with RPMI- 1640 containing 10% Neonatal Bovine Serum at 37°C, 5% CO₂ until the cells were confluent. The monolayers were wounded by sterile 20μl 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.

2.2.8. Western blot

The total protein was obtained and quantified. Then, the equivalent of 30μg of protein was boiled in a sodium dodecyl sulfate (SDS) with a 5x loading buffer. The bands of the protein separated by sodium dodecyl sulfate (SDS) with a 5× loadingbuffer. The bands of the protein separated by sodium dodecyl sulfate (SDS) PAGE were transferred onto PVDF membranes. After 1 hour incubation with PBS solution containing 5% skim milk powder, the membrane was probed with corresponding primary antibodies including ENO1 and β-actin at 4°C overnight with gentle rocking. After PBST washing and incubation with secondary antibodies conjugated with HRP (1:5000) for 1 hour at 37°C. Signals were detected using ECL reagents.

2.2.9. Statistical analysis

All statistical analyses were carried out using SPSS software (version 15.0). Quantitative data and qualitative data are described as mean ± standard deviation (SD) and frequency, respectively. The statistical methods of Student’s t test, analysis of variance (ANOVA), least significant difference (LSD) and rank sum test were used according to the application condition. A two-sided probability value <0.05 was considered statistically significant.

3. Results

3.1. Identification the associations between ENO1 expression and clinicopathological features of gastric cancer.

The expression of ENO1 protein in gastric cancers tissues was detected by immunohistochemistry. The result of immunohistochemical analyses revealed that 28% (6/22) of the gastric cancers tissue samples expressed ENO1, higher than 0% (0/18) cases of normal gastric tissues and 0%(0/20) cases of atypical hyperplasia, the difference has the statistically significance (HC =22.70, P<0.05) (Table 1, Figure 1).
Figure 1. The expression of α-enolase in different tissues (×200) A: Normal ovarian tissue, B: Atypical hyperplasia, C: Moderately differentiated adenocarcinoma (IRS = 0), D: Moderately differentiated adenocarcinoma (IRS = 1), E: Moderately differentiated adenocarcinoma (IRS = 9), F: Moderately differentiated adenocarcinoma (IRS = 12), G: Poorly differentiated adenocarcinoma (IRS = 2), H: Poorly differentiated adenocarcinoma (IRS = 9), I: Poorly differentiated adenocarcinoma (IRS = 12).

Table 1 The expression of α-enolase in different tissues

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Total</th>
<th>Expression of α-enolase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>negative</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>18</td>
<td>11 (61.6%)</td>
</tr>
<tr>
<td>Atypical hyperplasia</td>
<td>20</td>
<td>10 (50.0%)</td>
</tr>
<tr>
<td>Gastric carcinoma*</td>
<td>22</td>
<td>2 (9.1%)</td>
</tr>
</tbody>
</table>

Normal tissue and Atypical hyperplasia vs. Gastric carcinoma; * indicates statistical significance.
3.2. mRNA and protein expressions of ENO1-siRNA in AGS cells

After 24 hours transfection, the expression of ENO1-siRNA and NC-siRNA 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 ENO1 small interfering RNA-1 (ENO1-siRNA-1) was significantly lower than that of the control group (Figure 2).

![Graph showing mRNA and protein expressions of ENO1-siRNA in AGS cells]

Figure 2 Quantitative expression of eno1 gene transfection cells. ENO1 mRNA was measured by quantitative real-time RT-PCR and western blot, *: AGS-ENO1-siRNA-1+ Lipofectamine™ 2000 compared with the control groups (F=789.37, P=0.00).

3.3. The down-regulation of ENO1 led to slowly down proliferation and reduced colony-forming potential of AGS cells in vitro

The proliferative abilities of AGS cells influenced by down-regulation 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 3 A), which indicated that the group transfected with ENO1-siRNA grew slower than the control group transfected with the NC-siRNA (t=2.50, p=0.03). 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 ENO1-siRNA was less than those in the control group. (t=22.05, P=0.01) (Figure 3B).

3.4. The down-regulation of ENO1 showed the decreased migratory properties of AGS cells in vitro

The effects of down-regulation 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% CO2, the group transfected with the ENO1-siRNA-1 had a slower healing speed than the NC-siRNA (t=8.54,P=0.01) (Figure 4).
4. Discussion

ENO1, a multifunctional protein, is involved in many different physiological and pathophysiological processes [13], such as wound healing, tissue remodeling, embryogenesis and the process of cancer cell invasion and metastasis [14]. Recent studies have showed that ENO1 took part in tumor cells proliferation, apoptosis, invasion, migration and angiogenesis of tumors [15]. The latest research indicated that ENO1 displayed high expressions in many tumors including breast, cholangiocarcinoma, small-cell lung cancer, pancreatic cancer, prostate cancer and head and neck tumors [6, 8, 9]. Ito et al [16] found that the over-expression of ENO1 could significantly promote the ability of proliferation and migration of oral cancer cells. However, it is rare to see the precise role of ENO1 correlated with the pathological progression of gastric cancer and mechanism of the down-regulation of ENO1 in cancer metastasis. In this study, we planned to confirm the effects on the biological behaviors of gastric cell line AGS by knockdown enol gene.

Compared to normal gastric tissues and atypical hyperplasia, this study demonstrates that the expression of ENO1 is unregulated in gastric cancer; it was showed that the expression of ENO1 significantly associated with the pathological progression of gastric cancer. Meanwhile, some researchers have founded that the levels of ENO1 protein were significantly increased in gastric cancer [17]. Furthermore, Liu 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 [18]. This is consistent with our finding that ENO1 was an important potential tumor-marker associated
with the development of gastric cancer.

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[19]. 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[18]. Consistent with the previous studies, it was showed by the CCK-8 cell proliferation assay that ENO1-siRNA can decrease the abilities of proliferation of AGS cells \((P=0.03)\). 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 [3] 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. Hamaguchi et al [20] reported that the knockdown of ENO1 by small-interfering RNA significantly inhibited the proliferation of a hepatocellular carcinoma cell line (HLE cells).

The migration and invasion of the tumor cells determine the aggressive potential of the cancer [21]. In order to confirm the relation between the expressions of ENO1 and the invasion and metastasis of gastric cancer, the wound healing assay experiments were performed. Our study have found that ENO1-siRNA gene transfer contributed to a wound healing slower than the NC-siRNA \((P=0.01)\), indicating a migration ability effect of ENO1 knockdown. Consistent with the notion, More reports have showed that ENO1 as a plasminogen receptor expressed 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 [22]. Hsiao et al [10] found that down-regulation of ENO1 decreased extracellular matrix degradation and the invasion capacity of lung cancer cells. Moreover, over-expression of ENO1 is associated with glioma progression, knockdown of ENO1 expression led to suppressed cell growth, migration and invasion progression [23]. Principe et al [24] indicated that ENO1 is involved in Pancreatic Ductal Adenocarcinoma (PDAC) cell invasion, blockade of ENO1 expression reduced the migration and invasion capacity of PDAC cells in vitro and in vivo.

In summary, the above results implied that the expression of ENO1 significantly associated with the pathological progression of gastric cancer. Moreover, ENO1 played an important role in the carcinogenesis and invasion of gastric cancer. Knocking down the expression of ENO1 significantly reduced the abilities of proliferation and migration in AGS cells. Further research is needed for elucidating the specific mechanism of ENO1 as a potential tumor-marker associated with the development of gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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