ENO1 Inhibits Apoptosis and Promotes Cell Invasion and Proliferation in Malignant Melanoma

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Abstract

Introduction

The glycolytic enzyme, α-Enolase (ENO1), catalyzes the production of phosphoenolpyruvate from 2-phosphoglycerate, which enhances glycolysis, and thus contributes to tumor progression. In the present study, we aimed to determine ENO1's role in malignant melanoma (MM) and the potential underlying mechanism.

Methods

Western blotting was used to assess the levels of ENO1, c-Myc, β-catenin, MMP-9, PGAM1, and MMP-13 in MM-derived cell lines or tumor tissues from patients with MM. Plasmids pCMV-SPORT6-ENO1 and pET-28a-ENO1siRNA plasmids were used to overexpress and knockdown *ENO1* in MM cells, respectively. To determine the function of ENO1 in the malignant behavior of MM cells, we performed wound healing, Cell counting kit 8, Transwell chamber, and flow cytometry analyses. Pyruvate determination and lactic acid kits were used to evaluate the production of pyruvate and lactic acid in tumor cells.

Results

The protein levels of ENO1 and PGAM1 in MM tissue were significantly higher than that in mole tissue. In MM cells, *ENO1* overexpression inhibited apoptosis; promoted invasion, migration, and proliferation; increased pyruvate and lactate production; and increased in β-catenin, MMP-9, MMP-13, and c-Myc levels. The opposite effects were observed in MM cells silenced for *ENO1*.

Conclusion

These results indicated that ENO1 is involved in MM progression by enhancing invasion and proliferation, while inhibiting apoptosis. In addition, ENO1 might have an important function in tumor cell glycolysis. Therefore, ENO1 represents a potential therapeutic target to treat MM.

1 Introduction

Malignant melanoma (MM) is the most malignant skin cancer. In the past 30 years, its incidence has increased steadily, and causes the highest skin cancer-related mortality worldwide. [1] At present, there are no effective drugs to treat patients with advanced stages of MM, and most patients with distant metastases die soon after diagnosis. [2] Increasing evidence shows that epigenetic changes are important in MM tumorigenesis and development. Therefore, there is an urgent need for molecular biological research to provide a high-value experimental basis for the treatment of MM.

α-Enolase (ENO1, or 2-phospho-D-glycerate hydrolase) mainly catalyzes the production of phosphoenolpyruvate from 2-phosphoglycerate in glycolysis. ENO1 is multifunctional, participating in biological growth, development, and reproduction; parasitic infections; cancer occurrence and metastasis; autoantigen activity; bacterial and fungal infections; and cell stress. [3] Recent studies have found that ENO1 is highly expressed in lung adenocarcinoma, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, and other types of tumor tissues. [4–8] The aim of the present study was to investigate ENO1's function and mechanism in MM.

2 Materials And Methods

2.1 Tissue Specimens from Patients with MM

From January 2016 to January 2020 at the Fourth Hospital of Hebei Medical University, we collected 52 MM tissues from patients diagnosed with MM and 52 mole tissues from healthy controls. The hospital human tissue research committee approved and supervised all the procedures. The study was also approved by the Ethics Committee of Tianjin Medical University General Hospital (Ethical number: 2019016).

2.2 Cell Lines and Culture

The American Type Culture Collection (ATCC, Manassas, VA, USA) provided four MM cell lines (SK-MEL-28, HMCB, A375, and SK-MEL-19, which were grown in Dulbecco's modified Eagle's medium (Gibco® BRL; Thermo Fisher Scientific, Gaithersburg, MD, USA) added with 10% fetal bovine serum (Gibco® BRL), penicillin and streptomycin (both 100 U/mL) at 37°C in a 5% CO_2 humidified incubator.

2.3 Western Blotting Analysis

Total proteins were extracted from cells and tissues using Radioimmunoprecipitation assay lysis buffer (Roche, Basel, Switzerland). A bicinchoninic acid (BCA) kit (Sigma-Aldrich Co., St. Louis, MO, USA) was used to determine the total protein concentration. Equal amounts of protein (50 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrotransferred onto a polyvinylidene fluoride membrane (Roche). Five percent skim milk was used to block the membranes for 2 h at 26°C, which were then incubated with mouse monoclonal antibodies recognizing human ENO1 (1:2000; Origene Technologies, Rockville, MD, USA), phosphoglycerate mutase 1 (PGAM1) (1:2000; Santa Cruz Technology, Santa Cruz, CA, USA), matrix metalloproteinase (MMP)-9 (1: 2000; Santa Cruz Technology), MMP-13 (1: 2000; Santa Cruz Technology), β-catenin (1: 2000; Santa Cruz Technology), c-Myc (1:2000; Santa Cruz Technology), or β-actin (1:10,000; Abcam, Cambridge, MA, USA), at 4°C overnight. The membranes were then incubated with secondary antibodies (conjugated to horseradish peroxidase), comprising anti-mouse immunoglobulin IgG, for 2 h at room temperature (1:5000; Thermo Fisher Scientific, Waltham, MA, USA). We applied the Enhanced Chemiluminescence reagent (Thermo Fisher Scientific) to the membrane, which was then exposed to the FluorChem® HD2 Western Blot Imaging System (Alpha INNOTEC, San Leonardo, CA, USA).

2.4 Transfection of cells

MM cells were grown to the logarithmic phase and then seeded into six-well plates. We transfected A375 cells with plasmids pCMV-SPORT6-ENO1 (for *ENO1* overexpression) and pLENTI-CMV-GFP/Puro (vector control) (GeneCopoeia, Rockville, MD, USA) with the aid of Lipofectamine 2000 (Thermo Fisher Scientific), following to the manufacturer's protocol (GeneCopoeia). We transfected SK-MEL-19 cells with plasmids pET-28a-ENO1siRNA (to silence *ENO1*) and pET-28a (GeneCopoeia). Western blotting analysis was used to confirm the successful knockdown and overexpression of *ENO1*. Clone selection was performed at 72 h after transfection using 0.6 μg/mL puromycin (Sigma-Aldrich Co.). For weeks later, we harvested stably transfected cells for subsequent analysis.

2.5 Assay of Cell Proliferation

To assess the proliferation of A375 and SK-MEL-19 cells, we used a Cell Counting Kit (CCK-8) (Dojindo Laboratories, Kumamoto City, Japan). Harvested stably transfected cells were seeded into 96-well microplates (Corning Incorporated, Corning, NY, USA) at 1×10^3 cells per well. For each group, six duplicate wells were set. At various time points (0, 12, 24, 48, and 72 h), CCK-8 solution (10 μL) was added to each well and incubated for 2 h at 37°C with 5% humidified CO_2. A Microplate Autoreader (Bio-Tek Instruments, Winooski, VT, USA) was then used to measure the absorbance of the cells at 450 nm.

2.6 Assay of Wound-Healing

From each group, stably transfected cells in the logarithmic phase were selected and inoculated into a six-well plate. The cells were grown to 100% confluence and then a scratch was generated in the cell monolayer using a 200-μL sterile micropipette tip. The cells were then cultured for 24 h in serum-free medium. Wound closure was assessed by photographing the wound under a microscope at 0 and 24 h. For each wound, at least five fields were assessed. The migration rate was calculated as the width of the scratch at 24 h divided by the width of the same scratch at 0 h.

2.7 Transwell Chamber Assay

Cells of each group were collected and combined into a single cell suspension. The cells were suspended in serum-free medium and 200 μL of the suspension (containing 2×10^4 cells) was transferred to the upper chamber of a Transwell chamber (8 μm, Corning, Inc.) that contained Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Five hundred microliters of complete medium were placed in the lower chamber. The chambers were cultured in an incubator for 24 h, and then the medium and cells in the upper chamber were removed using a cotton swab, while the cells on the lower surface of the membrane were fixed using with methanol, and stained using 0.1% crystal violet. The Transwell chamber was placed under an inverted microscope and the cells in five fields of view were observed and counted.

2.8 Flow Cytometry

To detect cellular apoptosis, we used flow cytometry. We collected each group of cells, washed them twice using cold phosphate-buffered saline, and resuspended them in binding buffer. PE Annexin V and 7-

AAD (BD Biosciences, Pharmingen, San Diego, CA, USA) were added to the cells, which were then incubated at 25°C in the dark for 15 Minutes. Cell apoptosis was then analyzed using a fluorescence activated cell sorting (FACS) Aria II flow cytometer (BD Biosciences).

2.9 Measurement of Pyruvate and Lactic Acid

Twenty-four hours after the transfection of A375 and SK-MEL-19 cells, the production of pyruvate and lactic acid was examined using a Micro pyruvate assay kit and lactic acid kit (Sigma-Aldrich Co.), respectively.

2.10 Statistical Analysis

All data are shown as the mean ± the standard deviation. Student's t-test was used to analyze the statistical differences between two groups, whereas one-way analysis of variance (ANOVA) was used to compare three or more groups. Statistical significance was accepted at $P < 0.05$. SPSS version 19.0 software (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses.

3 Results

3.1 Levels of ENO1 and PGAM1 are Increased in MM cell lines tissues

The levels of ENO1 and PGAM1 in 52 tumor samples and mole (nevus) tissues were determined using western blotting. The results showed that compared with that in normal benign nevus tissue samples, the levels of ENO1 and PGAM1 in MM tissue samples and cell lines were significantly higher (Fig. 1A-C). The ENO1 level was highest in SK-MEL-19 cells and lowest in A375 cells (Fig. 1D and E). These two types of cells were used for knockdown and overexpression analysis, respectively. Western blotting was used to verify the successful knockdown or overexpression of *ENO1* (Fig. 1F-I).

3.2 Overexpression of *ENO1* inhibits apoptosis and promotes proliferation in A375 cells

PCMV-Sport6-ENO1 was transfected into A375 cells to overexpress *ENO1* because of the low level of ENO1 in these cells. Plentiv-CMV-GFP/Puro (vector) was also transfected into these cells as a control. CCK-8 analysis demonstrated that compared with that in the control and blank control groups, after 36 hours of culture, *ENO1* overexpression stimulated the proliferation of A375 cells significantly (Fig. 2A and B). Subsequently, flow cytometry showed that in the *ENO1* overexpressing cells, the proportion of apoptotic cells was significantly lower compared with that that in the blank control and control groups (Fig. 2G and H). Thus, overexpression of *ENO1* could promote the proliferation of A375 cells and inhibit their apoptosis.

3.4 Overexpression of *ENO1* promotes A375 cell invasion and migration

Next, we used a wound-healing assay and Transwell chamber analysis to determine the effect of overexpressing *ENO1* on A375 cell migration and invasion. *ENO1* overexpression increased the migration and invasion abilities of A375 cells significantly compared with that of the control cells (Fig. 2C-F). Thus, *ENO1* overexpression in MM cells enhanced their invasion and migration capabilities.

3.5 Silencing of *ENO1* in SK-MEL-19 cells induces apoptosis and inhibits invasion, migration, and proliferation

The level of *ENO1* in SK-MEL-19 cells transfected with the pET-28a-ENO1siRNA lentiviral plasmid was reduced significantly. CCK-8 analysis showed that the proliferation ability of *ENO1*- silenced SK-MEL-19 cells was reduced significantly at 36 h after transfection (Fig. 3A and B). Silencing *ENO1* resulted in significant reductions in the invasion and migration abilities of SK-MEL-19 cells (Fig. 3C-F). Flow cytometry analysis showed that silencing of *ENO1* in SK-MEL-19 cells increased the rate of apoptosis significantly compared with that in the control group (Fig. 3G and H). These data indicated that silencing *ENO1* expression promoted MM cell apoptosis and inhibited their invasion, migration, and proliferation.

3.6 ENO1 enhances glycolysis and increases the levels of MMP-13, MMP-9, c-Myc, and β-catenin in MM cells

ENO1 is one of the key enzymes in glycolysis, affecting the formation of key glycolytic products. Therefore, we assessed the impact of altered *ENO1* expression on MM cell glycolysis. *ENO1* overexpression increased the levels of lactate and pyruvate in A375 cells significantly (Fig. 4A and B). By contrast, silencing of *ENO1* reduced the formation of lactate and pyruvate in SK-MEL-19 cells significantly (Fig. 4C and D). To determine the signaling pathway and mechanism of ENO1, the impact of altered *ENO1* expression on the activity of the Wnt/β-Catenin signaling pathway and MMP-9 and MMP-13 levels were evaluated. *ENO1* overexpression enhanced the levels of β-catenin and c-Myc significantly. These proteins are target of the Wnt/β-catenin signaling pathway. *ENO1* overexpression also increased the levels of MMP-9 and MMP-13 significantly (Fig. 4E and F). Silencing *ENO1* showed the opposite effects (Fig. 4E and G). Taken together, our results demonstrated that ENO1 affects the activity of the Wnt/β-catenin signaling pathway, regulates MM cell proliferation, and promotes invasion and migration by increasing the levels of MMP-9 and MMP-13.

4 Discussion

ENO1 mainly exists in the cytoplasm, and has an important function in tumor cell glycolysis. Thus ENO1 expression might be related to malignant tumor development. [4–8] Recently, Chinese scholars have reported the production of superparamagnetic iron oxide nanoparticles that target the highly expressed ENO1 in pancreatic cancer tissues, which significantly increased the diagnostic rate of pancreatic cancer

by magnetic resonance imaging. [9] To the best of our knowledge, this is the first report that ENO1 and PGAM1 levels are high in MM cell lines and tumor tissues. Functional experiments showed that *ENO1* overexpression promoted the invasion, migration, and proliferation, and inhibited apoptosis, in MM cells

Several studies have shown that the Warburg effect is one of the important signs of cancer. Warburg hypothesized that differences in energy sources are the main reason why tumor cells have a higher growth rate than normal cells. Tumor cells mainly use glucose generated through glycolysis and reduce aerobic phosphorylation in mitochondria, which is considered the most important cause of tumor development. [10]

The high expression of glycolytic enzymes might be a key factor leading to the excessive tumor cell proliferation. [11] In addition to high expression of ENO1, PGAM1 is also highly expressed in melanoma tissues. PGAM1 is also an important glycolytic enzyme. Its main function is to catalyze the reversible production of 2-phosphoglycerate from 3-phosphoglycerate. [12] The combination of ENO1 and PGAM1 regulates multiple cell functions. [13] PGAM1 is highly expressed in various tumors, such as oral squamous cell carcinoma, prostate cancer, non-small cell lung cancer, renal clear cell carcinoma, pancreatic ductal carcinoma, and colorectal cancer, and is associated with tumor invasion, migration, and proliferation. [14–19] The present study showed that ENO1 and PGAM1 are highly expressed in MM. Understanding the cooperative mechanism by which ENO1 and PGAM1 affect MM development is our next research direction.

Wnt/β-catenin canonical signaling has a vital function melanoma apoptosis, invasion, migration, proliferation, and differentiation. [20–23] The core component of the Wnt/β-catenin pathway, β-catenin, has an important function in tumor progression. [24] c-Myc can induce tumor cells to transition from the G1 phase to the S phase, thereby promoting cell proliferation. [25] Our study revealed that *ENO1* overexpression could promote the expression of β-catenin and c-Myc significantly in MM cells. *ENO1* knockdown had the opposite result. These results indicated that the development of MM involves Wnt /β-catenin signaling.

We also found that *ENO1* overexpression increased the formation of lactate and pyruvate in MM cells. During tumor growth, tumor cells must increase their glucose metabolism, and excessive proliferation under hypoxic conditions is the main feature of solid tumors. [26] The acidification of the cellular environment provides a favorable microenvironment for the activation of MMPs. [27] In almost all human malignancies, the expression levels of MMPs, a class of zinc-dependent proteases, are increased, and are associated with tumor migration and invasion. The extracellular matrix (ECM), which forms a natural physical barrier that prevents tumor cells spread, is degraded an recombined by MMPs. [28–30] *In vivo*, aggressive MM cells show higher expression of MMPs (especially MMP-9, MMP-10, and MMP-13) compared with the mesenchymal phenotype. MMP-9 has an important function in ECM degradation. MMP-9 activation benefits the infiltration of melanoma cells into surrounding tissues and the transfer of melanoma cells. The expression of MMP-9 is related to prognosis. [31–33] The present study found *ENO1*

overexpression promoted MM cell invasion and proliferation by altering the levels of Wnt/β-catenin target proteins MMP-13, MMP-9, and c-Myc. These alterations might be related to the development of MM caused by ENO1. Other related mechanisms and signal pathways of involving ENO1 in MM require further exploration. This study provided only a partial theoretical basis for the treatment of MM.

Declarations

Disclosure

The author reports no conflicts of interest in this work.

Ethics approval and consent to participate

The study was also approved by the Ethics Committee of Tianjin Medical University General Hospital (Ethical number: 2019016).

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Figures

Figures 1-4 can be found in the supplementary files section

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