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# Knockdown of α-Enolase (ENO1) to Suppress Glycolytic Pathway in Human Hepatocellular Carcinoma Cell Line (HepG2)

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## Abstract

α-Enolase is an important enolase isoenzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate; it is one of the leading regulators of the Warburg effect, so may play an important role in carcinogenesis and tumor maintenance. α-Enolase has been noticed to be over-expressed in tumors including hepatocellular carcinoma. This study aimed to analyze the effect of α-enolase knockdown on pyruvate , the final product of glycolysis , level and on the proliferation and progression of human hepatocellular carcinoma cell line (HepG2). α-Enolase in the cell line was knockeddown by successful construction of the corresponding short interfering RNA (siRNA). Pyruvate level was measured using a colorimetric assay kit; it was significantly lower in siRNA HepG2 cells compared to HepG2 control cells (7.54 ± 1.06 nmol/$10^6$ cells and 28.4 ± 3.12 nmol/$10^6$ cells; respectively ($p<0.005$) . The proliferation ability in siRNA HepG2 cells was significantly suppressed ($p<0.005$) as measured by WST-8 assay. In conclusion, α-enolase knockdown by siRNA can efficiently suppress glycolysis and has significant antiproliferative effect in HepG2 cell line.
**Keywords: α-enolase, Warburg effect, Hepatocellular carcinoma**.

# تعطيل إنزيم الألفا إنوليز(ENO1) عبر الإسكات الجيني لتثبيط مسلك تحلل الكلوكوز في الخلايا السرطانية الكبدية HepG2

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## الخلاصة

الألفا إنوليز هو نظير هام لإنزيم الإنوليز و الذي يحفز تحويل ٢-فوسفوكليسيريت إلى فوسفوإنول بايروفات ؛ إنه أحد المنظمين الرئيسيين لتأثير واربورغ ، لذلك يمكن أن يلعب دورًا مهمًا في التسرطن والحفاظ على الورم. لوحظ أن الألفا إنوليز يتم التعبير عنه بشكل مفرط في الأورام بما في ذلك سرطان الخلايا الكبدية. هدفت هذه الدراسة إلى تحليل تأثير الإسكات الجيني للألفا إنوليز على مستوى البيروفات , المنتج النهائي لتحلل السكر, وعلى تكاثر وتطور خط خلايا سرطان الكبد البشري. تمت إزالة الألفا إنوليز في خط الخلية من خلال البناء الناجح للحمض النووي الريبي المتداخل القصير المقابل (سيرنا). تم قياس مستوى البيروفات باستخدام مجموعة المقايسة اللونية. كان أقل بشكل ملحوظ في خلايا الإعاقة القصير للحامض النووي الريبي لإنزيم الألفا إنوليز مقارنة بخلايا الكبد السرطانية± 1.06 7.54) نانومول / ١٠٦ خلية و ٢٨,٤ ٣,١٢ ± نانومول / ١٠٦ خلية على التوالي .(($p<0.005$)تم قمع قدرة التكاثر في خلايا الإعاقة القصير للحامض النووي الريبي لإنزيم الألفا إنوليز في خلايا الكبد السرطانية بشكل ملحوظ ($p <0.005$) كما تم قياسها بواسطة مقايسة صبغة التترازوليوم الذائبة في الماء-٨. في الختام ، يمكن الإسكات الجيني للألفا إنوليز بواسطة الإعاقة القصير للحامض النووي الريبي لإنزيم الألفا إنوليز بكفاءة من قمع تحلل السكر ولها تأثير كبير مضاد للتكاثر في خط خلايا الكبد السرطانية .
**الكلمات المفتاحية: إنزيم الألفا إنوليز، تأثير واربورغ ، سرطان الخلايا الكبدية.**

## Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer [(1)]. Intrinsically, liver cancer is chemoresistance and has limited molecular targets for treatment. Tumor resection or liver transplantation is the only curative treatment which is only applicable for early stages of the disease. The urgent demand for new therapies for HCC is needed because of the limited treatment outcomes of the current chemotherapies [(2-3)]. Overexpression of α-enolase, which is also known as (ENO1), was related with tumor development mediated by the Warburg effect [(4)]. Warburg hypothesized that the difference in energy source was the major cause for the higher growth rate of tumor cells as compared with that of normal cells. Warburg observed that tumor cells take up enormous amount of glucose, compared to normal cells, and glucose is fermented into lactate

even in the presence of sufficient oxygen, thus the term aerobic glycolysis. Later on Warburg proposed that mitochondrial dysfunction is the cause of aerobic glycolysis, this effect can be the primary cause of cancer [5]. A study by Altenberg and Greulich also showed that the genes of glycolysis enzymes are overexpressed in several tumor cells. Altenberg and Greulich regarded that the overexpression of glycolysis enzymes might be a crucial factor causing excessive tumor cell proliferation [6]. As an important glycolytic enzyme, ENO1 might have an important part in the development, progression and metastasis of malignant tumors [4].

In this study, we constructed a small interfering RNA (siRNA) that specifically targeting ENO1 to downregulate its expression in human hepatocellular carcinoma cell line (HepG2); to investigate its effect on pyruvate level and cellular proliferation which may provide a new basis for hepatic cancer gene treatment.

## Materials and Methods

### *Cells and cell culture*

The human hepatic cancer HepG2 cell line was provided by Sigma-Aldrich (Merck, USA). The HepG2 was cultured in complete Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented by 1% penicillin/streptomycin and 10% fetal bovine serum in an incubator at 37°C, with 5% carbon dioxide.

### *siRNAs and siRNA transfection*

In this experiment, the small fragment small interfering RNA (siRNA) against human ENO1 mRNA and the scrambled (negative control) siRNA were purchased from Integrated DNA Technologies (Inc., USA) for silencing ENO1. Scrambled sequence as a negative control for this experiment is 5′-GGGTGAACTCACGTCAGAA-3′ and this scrambled siRNA sequence does not target any known human gene, the sequences of scrambled designed by this website: https://www.genscript.com/tools/create-scrambled-sequence. The siRNA ENO1 sequences were as follows: forward, 5' GCAUUGGAGCAGAGGUUUATT 3' and reverse, 5' UAAACCUCUGCUCCAAUGCTT3'. The siRNAs transfection experiment was conducted using Lipofectamine 3000 reagent according to the manufacturer's protocol [7-8]. HepG2 cell lines were incubated in RPMI 1640 and assigned to three groups: HepG2 group, 50 nM scrambled siRNA group, and ENO1-knockdown group that transfected with 50 nM siRNA against ENO1.

### *RNA Extraction*

A TRIZOL reagent Kit (Invitrogen, Germany) was used to extract the total RNA according to the manufacturer's instructions [7-8]. The extracted RNA was quantified using NanoDrop Microvolume Spectrophotometer (Invitrogen, Germany) [9].

### *Quantitative real-time PCR Detection of the ENO1 mRNA Expression level in ENO1 siRNA/HepG2 Cells*

To determine the interference efficiency of siRNA-ENO1 and to validate the *ENO1* gene result, *ENO1* gene was selected for RT qPCR analysis using SYBER-Green qPCR supermix kit (Qiagen, Germany). Reverse transcription was used to obtain cDNA from the isolated mRNA according to the Reverse transcriptase PCR Kit, Bioneer. At 48 h post transfection, the interference effect was measured. The interference effect of ENO1siRNA was evaluated by determining the down regulation of the ENO1 gene. Reverse transcription quantitative PCR (RTqPCR) were performed 3 times. The mean value of the experimental results was used as the relative expression level of ENO1. The primer sequences were as follows: ENO1 forward, 5'-GGG AATCCCACTGTTGAGGT-3' and reverse, 5'-CGGAGCTCT AGGGCCTCATA-3'; β-actin forward, 5'-GGGAAATCGTGC GTGACATTAAGG-3' and reverse, 5'-CAGGAAGGAAGG CTGGAAGAGTG-3' [10].

### *Determination of pyruvate level*

Cellular pyruvate levels were detected by using Pyruvate Colorimetric assay kit (Sigma-Aldrich Ltd. USA). Pyruvate concentration was determined by a microplate reader (Promega, USA) at wave length 570nm according to the manufacturer's protocol [11].

### *Detection of the proliferative activity of ENO1 siRNA/HepG2 cells by WST-8*

The water soluble tetrazolium (WST) assay was used for assessing the effects of ENO1 knockdown on HepG2 viability. In 96-well plates, $5\times10^3$ (cell/well) from each HepG2 (blank control group), ENO1 siRNA/HepG2 (experimental group) and Scramble siRNA/HepG2 (negative control group) were seeded in 100 µl of complete media for each well with three replicate wells for each group after 24 h of transfection. Cells were cultured for 24 , 48, and 72 hours. One plate was evaluated each day; 10 µl of WST-8 reagent was added and incubated at 37°C with 5% CO2 for three hours. Then, optical density (OD) of each well was measured using a microplate reader at 450 nm, with the OD value on the y-axis and the number of days (d) on the x-axis the growth curve was plotted, with triplicate assays and 5 independent experiments [12-13].

### *Statistical analysis*

All statistical analyses were performed using GraphPad prism 8. All results (the fold change in *ENO1* expression, pyruvate level, and the proliferation of HepG2, Scrambled siRNA/HepG2 and ENO1 siRNA/HepG2 cells) are presented as the mean ± standard error of the mean. A comparison between HepG2 (blank control group), ENO1 siRNA/HepG2 (experimental group) was performed using an independent sample t-test. Comparisons of

the fold change in ENO1 expression and of cellular proliferation of the HepG2, Scrambled siRNA/HepG2 and ENO1 siRNA/HepG2 was performed using one way analysis of variance (ANOVA) test. P<0.05 was considered to indicate a statistically significant result.

## Results

***Decreased mRNA expression level of ENO1 in HepG2 cells after ENO1 siRNA transfection***

Quantitative real-time PCR results revealed that ENO1 mRNA expression in the ENO1 siRNA/HepG2 group was significantly lower compared to that in the blank and negative control groups p<0.05 as shown in Table 1 and Figure 1.

**Table 1.Fold change of ENO1 expression for untransfected group, negative control group (Scrambled siRNA), ENO1 transfected group.**

| Untransfected HepG2 cell line | Transfected with 50 nM scrambled siRNA | Transfected with 50 nM siRNA against ENO1 |
|---|---|---|
| 1±0.21 | 1.09±0.13 | 0.33±0.09 |

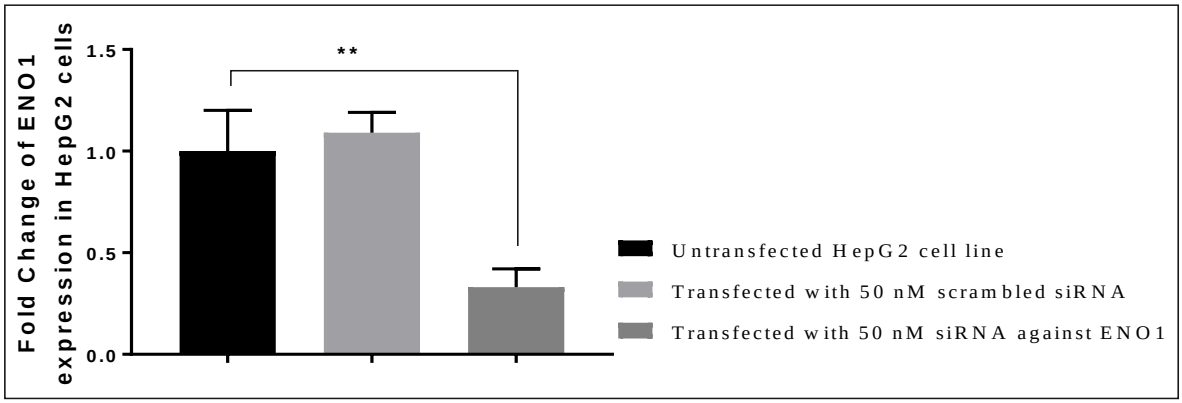


**Figure 1. Relative expression change of ENO1 level. **p<0.05 .**

***Decreased pyruvate level after ENO1 siRNA transfection***

After ENO1 siRNA transfection the pyruvate level, in experimental group (ENO1 siRNA/HepG2) was significantly lower as compared to control group (HepG2); as presented in Figure 2. The pyruvate level in experimental group was 7.54 ±1.06 nmol/$10^6$ cells while in control group was 28.4 ± 3.12 nmol/$10^6$ cells.

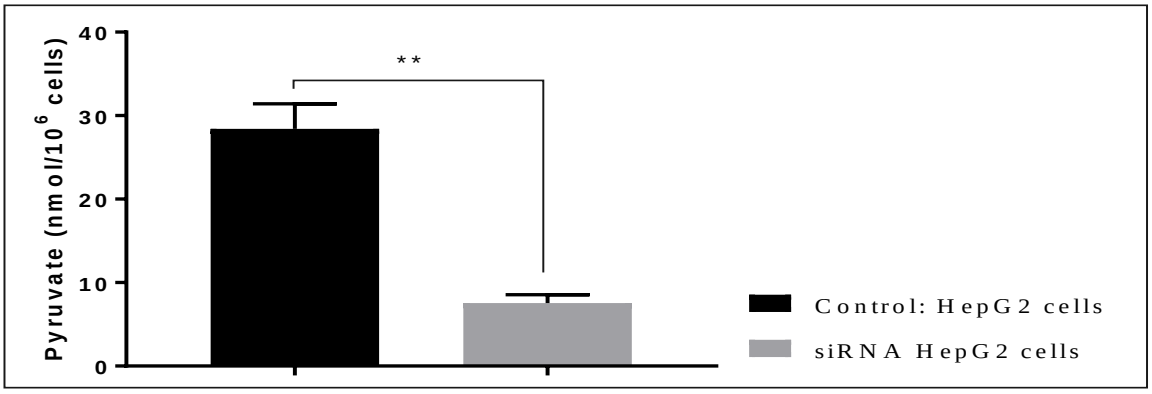


**Figure 2. Pyruvate level in siRNA-ENO1 HepG2 cells and control HepG2 cells, p<0.005.**

***Decreased proliferation of HepG2 cells after ENO1 siRNA transfection***

For the transfected hepatic cancer ENO1 siRNA/HepG2 cell line, the growth rate was significantly slower than that of the blank control (HepG2) and negative control groups, (p<0.005); as shown in Figure 3.

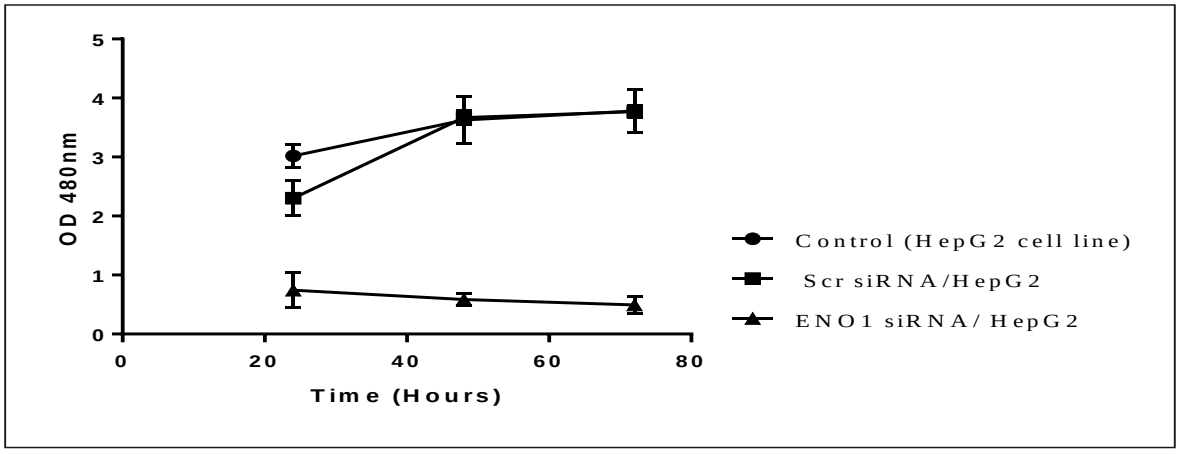


**Figure 3. The proliferation of α-enolase (ENO1) small interference RNA (siRNA)/HepG2 cells was tested by tetrazolium salt WST-8. The data are represented as the mean ± standard error of triplicate assays and are representative of 5 independent experiments. ENO1 siRNA/HepG2 cells group versus Scrambled siRNA/ HepG2 group and control HepG2 group ($p < 0.005$).**

## Discussion

Three isoenzymes of the metalloenzyme enolase catalyze, in the course of glycolysis, the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate as well as the hydration of phosphoenolpyruvate to 2-phospho-D-glycerate in the course of gluconeogenesis [14-15]. *ENO1, ENO2* and *ENO3* are the three genes which encode three isoforms of the enzyme in mammals, with expression being regulated in a tissue specific manner. α-Enolase (ENO1) is expressed in most tissues, whereas γ-enolase (ENO2) is mainly expressed in neurons and neuroendocrine tissues, while β-enolase (ENO3) is found in muscle tissues [16]. In tumors including HCC apart from its role in glycolysis, α-enolase is a multifunctional enzyme [17-18]; it has been reported to be an essential regulator of tumor cell metabolism, proliferation and survival , so this make it a perfect target for anticancer therapy. α-Enolase silencing in tumor cells causes decreasing in proliferation and also affect *in vivo* tumor growth [19-20]. Gene therapy plays an important role in the treatment of cancer and is popular topic in tumor therapy research. Our research has focused on selecting a gene therapy target. Qiao *et al*. implied that silencing α-enolase caused reduction in α-enolase mRNA expression level in the human gastric cancer MKN45 cell line. As a result of this downregulation in α-enolase
expression growth and proliferation of tumor was suppressed. In the present study, to investigate the transfection effect of cells with siRNA by Lipofectamine 3000 on α-enolase in HepG2 cells, mRNA level of α-enolase was measured. This transfection cause a significant reduction in mRNA level of α-enolase ($p<0.05$) which is consistent with the study of Qiao *et al* [21]. Several studies have reported a critical role of α-enolase in energy metabolism during growth of tumor cells that are characterized by high energy demands [22-23], so in the present study, to investigate the glycolytic function of α-enolase in HepG2 cells, pyruvate level was measured by pyruvate assay kit after knockdown of α-enolase in cells by siRNA; the results revealed that pyruvate level was significantly lowered. Furthermore, α-enolase was shown to affect proliferation, metastasis, and drug resistance in cancer cells due to Warburg effect [24]. In the present study, to evaluate the effect of glycolysis suppression by ENO1 downregulation on the proliferation of HepG2, the growth rate for these cells was measured by WST-8. The results revealed significant reduction in the growth rate for HepG2 ($p<0.005$).This study focused on the role of ENO1 in glycolytic pathway and in cellular proliferation. However, additional depth research and *in vivo* experiments are needed to further confirm these findings.

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## Conflicts of Interest

The authors have no conflict of interest to declare.

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## Ethics statements

The experiment are in vitro study so it does not require ethical approval from an ethics committee .

## Author Contribution

Shaymaa H. Alasady: contributed to data gathering, analysis, practical (follow the procedure) and written parts of the study. Basma T. Al-Sudani and Bahir A. Mshimesh final: approval and agreement for all aspects of the study, supervision, revision, and rearrengment.

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