ORIGINAL RESEARCH

miR-22-3p Suppresses Cell Proliferation and Migration of Gastric Cancer by Targeting ENO1

Hui Qiao, MD; Na Wang, MM; Quan-Lin Guan, MD; Peng Xie, MM; Xiang-Kai Li, PhD

ABSTRACT

Background and Objective • miR-22-3p functions as a tumor suppressor by targeting a variety of downstream genes, while its role and downstream targets in gastric cancer (GC) remain to be determined. We aimed to explore the role of miR-22-3p in gastric cancer and the potential mechanism.

Methods • miR-22-3p mimic and inhibitor were used to overexpress or knockdown the expression of miR-22-3p separately. Quantitative real-time PCR (RT-qPCR) and Western blot were used to analyse the abundance of mRNA or protein level respectively. CCK-8 assay, cell colony formation assay, and flow cytometry were implemented to investigate the effect of miR-22-3p on gastric cancer cell proliferation and apoptosis. Luciferase assay was used to evaluate the role of miR-22-3p on the expression of glycolytic enzyme enolase 1 (ENO1).

Results • In this study, we found that miR-22-3p was downregulated in GC cells. By transfecting the cells with miR-22-3p inhibitors or mimics, we showed that miR-22-3p suppressed GC cell proliferation and migration, as well as triggered cell death. In addition, we discovered that miR-22-3p was engaged in glycolysis by controlling the generation of lactate as well as the consumption of glucose. TargetScan database suggested that the ENO1 may be a target of the miR-22-3p, and the luciferase experiment verified this hypothesis. Recovery assays showed that the proliferation and migration of GC cells suppressed by miR-22-3p could be rescued by overexpression of ENO1.

Conclusion • Collectively, we identified a new axis of miR-22-3p/ENO1 for GC development, which could be investigated as a therapeutic target for GC. (*Altern Ther Health Med.* 2023;29(5):278-283).

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INTRODUCTION

Gastric cancer (GC) is the fifth most common type of cancer and the third most common cause of cancer-related death globally.[1] Furthermore, GC remains highly lethal with a median survival of less than 1 year for metastatic disease.[2] On the other hand, the prognosis for gastric cancer is often dismal since the disease is frequently detected at a more advanced stage.[3] As a result, it is essential to investigate potential novel therapeutic targets or indicators for diagnosis and prognosis in GC.

The microRNAs, also known as miRNAs, are a family of single-stranded and highly conserved short noncoding RNAs. They have a role in the early stages of cancer as well as in its progressive stages, and they may either stimulate or inhibit tumor growth.[4] Numerous studies have been conducted to elucidate the role of microRNAs in the onset, development, progression, invasion, and dissemination of GC, as well as their use as diagnostic biomarkers and therapeutic targets.[5,6] miR-22-3p were found to be significantly higher in pancreatic cancer (PC) patients and may prove to have clinical utility in diagnosis of PC.[7] Dong et al. showed that miR-22-3p is less expressed in lung adenocarcinoma tissues and the low expression of miR-22-3p is closely associated with clinicopathological characteristics and the prognosis of the disease.[8] miR223p promotes chemoresistance in bladder cancer (BCa) by targeting neuroepithelial cell transforming 1 (NET1) and may serve as a new prognostic biomarker for BCa patients.[9] Long noncoding (Lnc) RNA RGMB antisense RNA 1 (RGMB-AS1) accelerated the progression of GC by targeting miR-22-3p/NFIB axis.[10] LncRNA NCK1-AS1 exerts oncogenic effects in gastric cancer by targeting the miR-22-3p/BCL9 axis to activate the Wnt/beta-catenin signaling.[11] LncRNA H19 regulates cell growth and metastasis via the miR223p/Snail1

axis in gastric cancer.[12] In this study, we discovered that miR-22-3p targets the glycolytic enzyme enolase 1 (ENO1), and that miR-22-3p controls GC cell development by inhibiting glycolysis via ENO1 targeting.

MATERIALS AND METHODS

Cell culture and transfection

The Cell Resource Center at the Shanghai Academy of Biomedical Sciences provided gastric cancer cell lines HGC27 cells, MKN45, and AGS, as well as the normal gastric mucosa epithelial cell line GES1, which were grown in Dulbecco's modified Eagle's medium that was augmented with 10% fetal bovine serum (FBS) (Gibco). The cells were kept in an incubator at a temperature of 37°C and a CO_2 concentration of five percent. The miR-22-3p mimic (5'-AAGCUGCCAGUUGAAGAACUGU-3') and inhibitor (5'-ACAGUUCUUCAACUGGCAGCUU-3'), and corresponding controls (mimics-NC,5'-UUUGUACUACACAAAAGUACUG-3'; Inhibitor-NC, 5'-CAGUACUUUUGUGUAGUACAAA-3') were synthesized by Hippobio (Huzhou, China). For overexpression, the cDNA sequence of ENO1 (NM 001428) was cloned into the pCDNA3.1 vector. Cell transfection was performed with Lipofectamine 2000 (Life Technologies, Darmstadt, Germany). Cells were collected 48 hours after transfection for further analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Trizol reagent was used to extract total RNA from gastric cell lines (Invitrogen, Carlsbad, CA). The RT-primers and qPCR primers of miR-22-3p and U6 were purchased from Ruibo (Guangzhou, China), and the reverse transcription and qPCR were performed according the manufacturer's instructions. For mRNA cDNA synthesis, a commercial cDNA synthesis kit (Yeasen, China) was employed. On the ABI StepOne RT-PCR System, RT-qPCR was performed using SYBR Green Master Mix (Life Technology Inc.) (Applied Biosystems). For mRNAs, loading controls used were GAPDH. Tianyi Huiyuan (Beijing, China) developed and synthesized the primer sequences utilized in this investigation (Supplementary Table S1). The 2-ΔΔCt method was used for calculating the gene expression levels.[13]

CCK-8 assay

Cell viability was measured using CCK-8 (Beyotime, China). MKN45 and HCG27 cells were transfected with miR-22-3p mimics or inhibitors for 24, 48, 72, and 96 hours. At each time point, 10 uL CCK-8 solution was added to the cells, and absorbance at 450 nm was measured (HBS-1096, DeTie, Nanjing, China).

Cell colony formation assay

A total of 800 cells were seeded into each well of 6-well plates, and the plates were kept in an incubator for seven to ten days. Then the cell colonies were rinsed in cold PBS, fixed in methanol at a concentration of 20%, and stained with crystal violet (0.5% w/v). After that, the colony-forming units (CFUs) that contained more than 50 cells were counted, and all of the tests were carried out in two separate sets.

Transwell assay

We used a 24-well Transwell chamber coated with Matrigel (BD Pharmingen, San Diego, CA, USA) to determine the invasive ability of cells in this study. Briefly, 5×10^4 transfected cells were kept in the upper chamber in serum-free medium, and 500 µL DMEM medium supplemented with 10% FBS was put in the lower chamber. After incubation at 37°C for 24 hours, the cells remaining in the upper chamber were removed using cotton swabs, while the cells which migrated through the membrane were fixed using 4% paraformaldehyde, and then stained the cells with 0.1% crystal violet (Beyotime Biotechnology). Therefore, the cells that were fixed were identified as invasive cells which were further observed and captured using a microscope (Leica Microsystems, Wetzlar, Germany).

Detection of cell apoptosis

Cell apoptosis was examined by flow cytometry using annexin V-FITC/propidium iodide (Yeasen, China). Indicated cells were collected, rinsed, resuspended, and stained with annexin V-FITC and propidium iodide in the dark for 15 minutes. Then the cells that had been stained were examined using a flow cytometer (Beckman Coulter Epic XL).

Measurement of glucose consumption and lactate production

A total of 5×10^4 cells per well were seeded in 24-well plates, and transfected cells were trypsinized and counted, while the supernatants of cell culture medium were collected. The media were assayed immediately for glucose and lactate levels by using glucose assay kit (Biovision, Mountain View, California) and lactate assay kit (Sigma-Aldrich, Cat. No. GAHK20-1KT) according to the manufacturer's instruction.[14]

Luciferase assay

The mutant sequence of the ENO1 3' UTR was produced after the mutation was introduced into 7 nucleotides (CCGUGCA) of the seed region for miR-22-3p. Thereafter, this mutant construct was cloned into a firefly luciferase reporter vector called pGL3-control (Promega), and the resulting vector was given the designation pGL3-luc-ENO1 3'UTR. Co-transfection of the produced reporter plasmids into MKN 45 cells was accomplished with the help of Lipofectamine® 2000. In addition, miR-22-3p mimics or miR-22-3p-NC was added to the mixture that was used for transfection (Invitrogen; Thermo Fisher Scientific, USA). After a duration of forty-eight hours, the luciferase activities were analyzed using a Dual-Luciferase® Reporter Assay kit (Promega, Madison, WI, USA). Relative luciferase activity was calculated by the ratio of firefly luciferase to Renilla luciferase.[9]

Western blot

Proteins were extracted with RIPA lysis buffer, which was manufactured by Beyotime in Jiangsu, China. The

protein concentration was determined using a BCA kit that was manufactured by Beyotime in Jiangsu, China. In order to separate the proteins, a sodium dodecyl sulfate-polyacrylamide gel with a concentration of 10% was utilized. The proteins on the gel were subsequently transferred to a PVDF membrane (Millipore). After blocking the membranes with 5% non-fat milk for 1 hour, the membranes were placed in a refrigerator at 4°C and incubated overnight with primary antibodies (anti-ENO1, proteintech, 11204-1-AP, 1:1000; anti-Vinculin, ABclonal, A2752, 1:1000). Following this step, the membranes were rinsed with PBS three times before being subjected to an incubation with secondary antibodies (Rabbit IgG Secondary Antibody, PA1-86329, Thermo Fisher) at room temperature for one hour. The enhanced chemiluminescence reaction (Pierce, Rockford, Illinois, USA) was used to reveal the protein bands, and an Odyssey Infrared scanning system was employed for the analysis (Li-Cor, Lincoln, NE, USA).

Statistical analysis

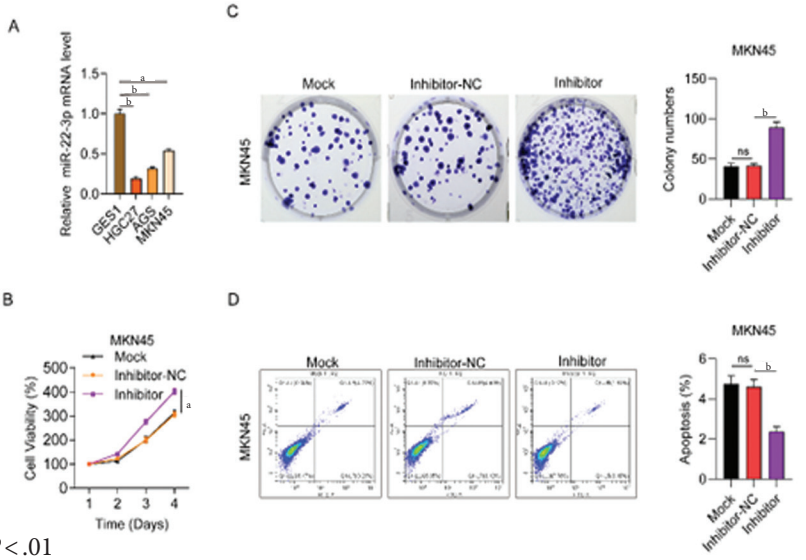
Each experiment was performed for three independent times. The data have been analyzed using GraphPad Prism version 6.0, and the results are shown as the mean along with the standard deviation (GraphPad Software, Inc., La Jolla, CA, USA). Differences among groups were determined by the use of student's t-test or one-way ANOVA analysis. $P < .05$ was considered significant and $P > .05$ was considered non-significant (NS).

RESULTS

miR-22-3p knockdown promotes cell proliferation and reduces apoptosis

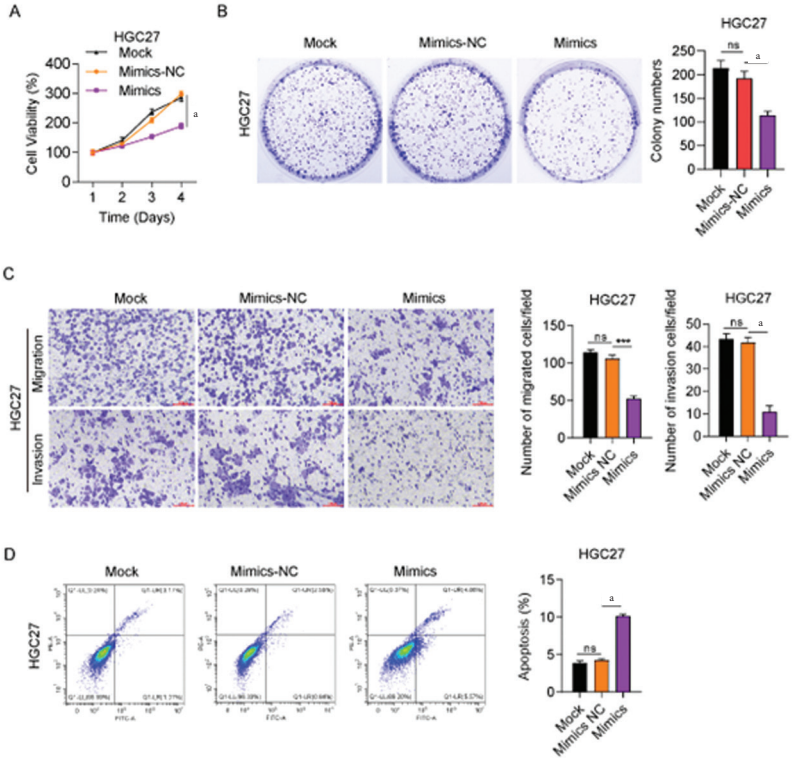
In order to examine the level of miR-22-3p in three distinct cell lines derived from patients with gastric cancer, RT-qPCR was used. According to the results, miR-22-3p expression was noticeably lower in the HGC27, MKN45, and AGS cells compared with the normal gastric mucosa epithelial cell line GES1 (Figure 1A). In order to get a better understanding of miR-22-3p's role in gastric cancer cells, we transfected MKN45 cells with inhibitors NC or miR-22-3p for a period of three days. Both the cell colony formation test and the CCK-8 assay were used to

Figure 1. miR-22-3p Downregulation Promotes Cell Proliferation and Reduces Cell Apoptosis. (A) miR-22-3p Levels were Shown to be Lower in all Three GC Cell Lines. (B) miR-22-3p Inhibitor Enhanced MKN45 Cells Viability. (C) miR-22-3p Inhibitor Enhanced MKN45 Cells Colony Formation Ability. (D) miR-22-3p Inhibitor Reduced MKN45 Cells Apoptosis Rate.



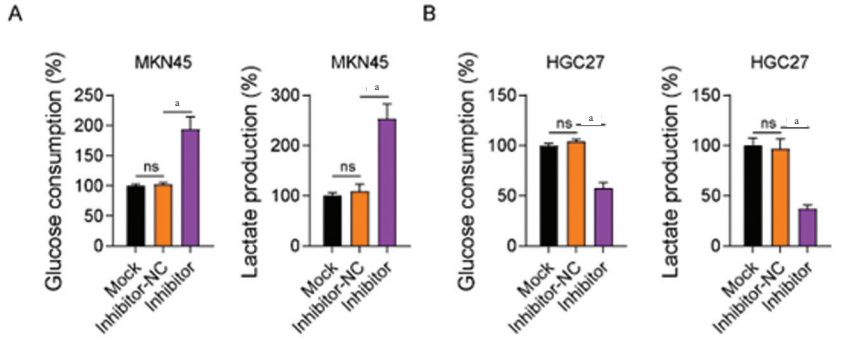
[a]$P < .01$
[b]$P < .001$

Figure 2. Overexpression of miR-22-3p Limits Cell Proliferation and Migration and Induces Cell Apoptosis. (A) miR-22-3p Mimics Decreased HGC27 Cells Viability. (B) MiR-22-3p Mimics Hindered HGC27 Cells Colony Formation. (C) MiR-22-3p Mimics in HGC27 Cells Repressed Cell Migration and Invasion. (D) MiR-22-3p Overexpression Induced HGC27 Cells Apoptosis.



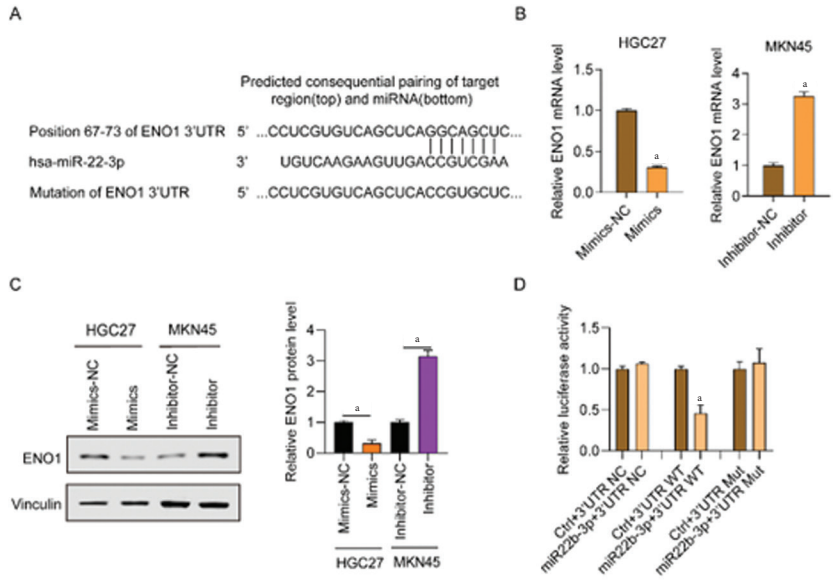
[a]$P < .001$

Figure 3. The miR-22-3p Controls Glucose Intake and Lactate Production in Gastric Cells. (A) In MKN45 Cells, the Depletion of miR-22-3p led to an Increase in Glucose Consumption and Lactate Production. (B) Overexpression of miR-22-3p led to a Decrease in Glucose Consumption and Lactate Production in HCG27 cells.



[a]$P < .001$

Figure 4. ENO1 Expression is Negatively Regulated by miR-22-3p. (A) A miR-22-3p Binding Site with ENO1 Gene is Predicted using TargetScan. (B) ENO1 mRNA Expression was Controlled by miR-22-3p in both HGC27 and MKN45 Cells. (C) ENO1 Protein Expression was Controlled by miR-22-3p in both HGC27 and MKN45 Cells. (D) The Interaction of ENO1 Gene 3'UTR and miR-22-3p was Detected Using Dual Luciferase Reporter Assay.



[a]$P < .001$

quantify cell proliferation. Results showed that after treatment with the miR-22-3p inhibitor, both the number of cell colonies and the percentage of viable cells had significantly increased in MKN45 cells (Figure 1B, C). After conducting additional research into the role of miR-22-3p on the apoptosis of gastric cancer cells, we discovered that when miR-22-3p was knocked down in MKN45 cells, the apoptosis rate was significantly decreased (Figure 1D). On the basis of these observations, we were able to arrive at the conclusion that miR-22-3p performs a function similar to that of a tumor suppressor in gastric cancer.

miR-22-3p overexpression suppresses cell proliferation and induces apoptosis

The usage of a synthetic miR-22-3p mimic was required in order to obtain the desired result of overexpression of miR-22-3p. Overexpression of miR-22-3p in HGC27 cells, which resulted in a considerable reduction in cell proliferation, was one of the key observations (Figure 2A, B). In keeping with the findings that were provided earlier, transwell assays showed that miR-22-3p overexpression inhibited migration and invasion of HGC27cells (Figure 2C). The results of flow cytometry showed that the apoptosis rate increased markedly after miR-22-3p overexpression (Figure 2D).

miR-22-3p inhibits the glycolysis in gastric cancer cells

It has been suggested that miRNAs have a role in the glycometabolism in gastric cancer, and both the consumption of glucose and the creation of lactate are essential glycolysis processes.[15] In order to determine whether or not miR-22-3p might influence glycolysis in gastric cancer cells, we measured the amount of glucose that MKN45 and HCG27 cells consumed as well as the lactate that they produced. As shown in Figure 3, miR-22-3p inhibitor increased glucose consumption and lactate production compared with the inhibitor control (inhibitor-NC), whereas miR-22-3p mimics decreased glucose consumption and lactate production about fifty percent and sixty percent, respectively. These findings provide further evidence that miR-22-3p is a critical regulator of glycolysis in gastric cancer cells.

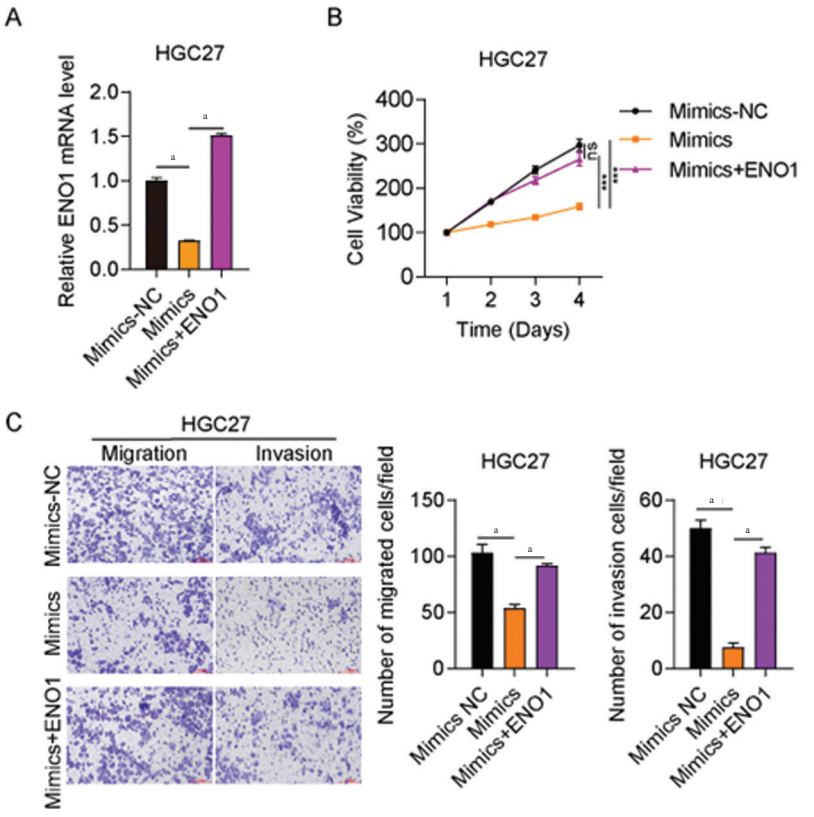
miR-22-3p suppresses ENO1 expression

We further investigated the underlying mechanism of ENO1 in GC cells. Through searching TargetScan (https://www.targetscan.org/vert_80/), miR-22-3p was found to have a binding site for ENO1 (Figure 4A). In order to demonstrate the connection between miR-22-3p and ENO1, we checked whether the expression of ENO1 was present in HGC27 and MKN45 cells after being transfected with miR-22-3p mimics or with an inhibitor of the miR-22-3p. Additionally, RT-qPCR results showed that the overexpression of miR-22-3p evidently decreased the expression of ENO1 in HGC27 cells, and inhibition of miR-22-3p evidently increased the expression of ENO1 in MKN45 cells (Figure 4B, C). Dual-luciferase reporter assay demonstrated that ENO1 was the direct target of the miR-22-3p (Figure 4D).

miR-22-3p impedes gastric cancer cell proliferation and migration through inhibition of ENO1

In order to further study whether miR-22-3p modulates the biological activity of GC cells through ENO1, we carried out rescue assay. HGC27 cells were used in these experiments throughout. After transfecting the cells with miR-22-3p mimics and ENO1 plasmids, RT-qPCR was used to evaluate the amount of ENO1 (Figure 5A). The proliferation ability of HGC27 cells, which was inhibited by miR-22-3p mimics, was significantly improved by ENO1 (Figure 5B). In addition, we observed that an increase in miR-22-3p inhibited migration and invasion of HGC27 cells. The ability of migration and invasion of the HGC27 cells were rescued by ENO1 overexpression (Figure 5C). As a consequence, we were able to show that miR-22-3p specifically targets ENO1 and suppresses the proliferation and migration of GC cells.

Figure 5. ENO1 Overexpression Rescued GC Cell Proliferation and Migration. (A) The Expression of ENO1 in HGC27 Cells Transfected with miR-22-3p Mimics or Mimics + ENO1. (B) The CCK-8 Assay was Used to Determine Cell Viability. (C) Transwell Assay was Used to Determine Cell Migration and Invasion.



[a]$P < .001$

DISCUSSION

Hundreds of microRNAs, which are fewer than 22 nucleotides in length, have been found in humans and are associated with the controlling of gene expression after transcription.[16] In 2002, researchers made the initial discovery on the link between cancer and miRNA dysregulation.[17] Kipleeva et al.[18] reported that hundreds of miRNAs participated in the development, metastasis, chemoresistance, prognosis, and diagnosis of gastric cancer. They found that microRNAs are associated with GC metastasis through the interaction with the Wnt/β-catenin pathway and microRNAs affecting chemoresistance and apoptosis were associated with the PI3K/AKT/mTOR pathway. In this study, we showed that the expression of miR-22-3p was reduced in three different cell lines derived from patients with gastric cancer. Additionally, miR-22-3p prevented cell proliferation and migration and induced apoptosis in GC cells. These findings are in line with what has been reported in the previous studies.[12,19]

One of the so-called "hallmarks of cancer" is an improperly regulated or changed energy metabolism, and the connection between aerobic glycolysis and carcinogenesis has been called the "Warburg effect" for several decades.[20] It has been suggested that inhibiting the Warburg effect might be a useful method to treat cancer.[21] New results revealed that abnormal miRNAs have a role in the altered glycometabolism in gastric cancer.[22,23] Using a high-throughput lactate-production screening platform in HeLa cells, Guo et al. discovered 100 glycometabolism-regulating miRNAs from a library of 854 miRNAs; however, miR-22-3p was not one of the miRNAs that made to the list.[24] Within the scope of this paper, we investigated whether or not miR-22-3p had a role in glycolysis. In order to develop the necessary biomass for their growth, tumor cells raise their glucose intake.[25] Lactate, the ultimate product of aerobic glycolysis, is frequently produced in excess in a variety of tumors. This excess production of lactate in many types of cancer is utilized in assessing the specific metabolomic characteristics and acidic microenvironment of tumor cells.[26] In MKN45 and HGC27 cells, we discovered that miR-22-3p was engaged in glycometabolism by measuring the amount of glucose consumed and the amount of lactate produced. For the sake of glycometabolism, miR-22-3p has been proven to be able to control the amounts of glucose intake and lactate formation.

During glycolysis, alpha-enolase (ENO1), which is also known as Hydrolases, convert 2-phosphoglyceric acid to phosphoenolpyruvic acids.[27] Overexpression or activation of ENO1 has been shown to be a feature of cancer cells. Its overexpression leads to the growth of tumors and has the potential to serve as a diagnostic and prognostic marker in a wide variety of cancer types.[28] It is possible that ENO1 might serve as a therapeutic target for cancer.[29] ENO1 overexpression in gastric cancer, as described by Qiao et al., has been shown

to enhance the development of GC cells and to predict poor survival rate of patient, and silencing ENO1 limits the proliferation of GC cells.[30,31] In addition, research conducted by Qian et al. and Yang et al. demonstrated that ENO1 either promoted chemoresistance or controls stem cell-like feature by stimulating glycolysis in gastric cancer cells.[32,33] In this study, we used TargetScan database to analyze the genes that were potentially targeted by miR-22-3p. We found that ENO1 was a potential downstream gene. After that, we examined the levels of mRNA and protein produced by ENO1 in gastric cancer cell lines that had either miR-22-3p overexpressed or knocked down. Following treatment with miR-22-3p mimics, the results indicated that the expression of ENO1 was dramatically lowered, while following treatment with miR-22-3p inhibitor, the expression of ENO1 was significantly elevated. Based on these findings, it was determined that miR-22-3p controlled the expression of ENO1. In addition, dual luciferase reporter assay demonstrated that miR-22-3p was capable of interacting with the 3' UTR of the ENO1 gene. As a result, we can only speculate as to whether or not miR-22-3p influences the formation of GC through ENO1. According to the results of the rescue assay, ENO1 production largely reversed the inhibitory effects of miR-22-3p mimics on GC proliferation and migration. However, the limitation of this study was that whether and how ENO1 deletion affects the gastric cancer cell proliferation and migration *in vivo* were not investigated.

CONCLUSION

In conclusion, we showed that ENO1 was a target gene of miR-22-3p in gastric cancer cells. Additionally, miR22-3p's ability to target ENO1 in order to stimulate glycolysis might govern the proliferation and migration of GC cells.

AUTHOR DISCLOSURE STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this article.

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Supplementary Table S1. Nucleotide Sequence of the Oligonucleotide Primers Used in this Study

GENE	Forward primer	Reverse primer
miR-22-3p	5'-AAGCTGCCAGTTGAAGAACTGT-3'	
U6	5'-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3'	
ENO1	5'-TGGTGTCTATCGAAGATCCCTT-3'	5'-CCTTGGCGATCCTCTTTGG-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'