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The role of miR-144/GSPT1 axis in gastric cancer

Q.-G. TIAN[1], R.-C. TIAN[2], Y. LIU[1], A.-Y. NIU[3], J. ZHANG[1], W.-F. GAO[1]

[1]Department of General Surgery, The Fourth Affiliated Hospital of Baotou Medical College, Baotou, China
[2]The Clinical Medicine School of Dali University, Dali, China
[3]Department of Gastroenterology, The Fourth Affiliated Hospital of Baotou Medical College, Baotou, China

Abstract. – OBJECTIVE: To investigate the potential effects of miR-144/GSPT1 axis on the development of gastric cancer.

PATIENTS AND METHODS: The expressions of GSPT1 (G1 to S Phase Transition 1) and miR-144 were detected in gastric cancer tissues and the adjacent normal tissues. We also explored the levels of GSPT1 and miR-144 in both normal gastric cell line (GES-1) and gastric cells (SGC7901). Luciferase assay was conducted to evaluate the interaction between miR-144 and GSPT1. The effects of the miR-144/GSPT1 axis on SGC7901 cells were determined via investigating cell proliferation, invasion and metastasis.

RESULTS: miR-144 was found to be down-regulated in gastric cancer tissues while GSPT1 expression level was markedly increased. Bioinformatics analysis showed that GSPT1 was a direct target of miR-144. Luciferase assays confirmed our hypothesis. The subsequent experiments showed that miR-144 could promote cell proliferation, invasion and migration in gastric cancer cells via inhibiting GSPT1.

CONCLUSIONS: We showed that miR-144/GSPT1 axis could be a potential therapeutic target in treatment of gastric cancer.

Key Words:
miR-144, Eukaryotic release factor, GSPT1, Gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors with the 4th incidence rate and the 2nd mortality rate worldwide[1]. It is estimated that about 700,000 people die of GC each year worldwide, and there are about 900,000 new cases annually[2]. In East Asia, the incidence and mortality rates of GC are higher than those in other regions[3]. In China, the incidence rate of GC ranks 2nd. Nearly 300,000 people die of GC and 400,000 new cases are diagnosed per year [4]. In recent years, despite the improvement in the diagnosis and treatment techniques, the early diagnosis rate of GC is still not high. Most patients have been in the advanced stage with local infiltration and lymph node metastasis when diagnosed[5,6]. The overall prognosis is still poor and the 5-year survival rate after operation is about 60%. The 5-year survival rate of patients with metastasis is only 2% when diagnosed[7]. Therefore, elucidating the mechanism of progression and metastasis in GC is very important in improving the prognosis of GC.

During tumorigenesis, the overexpression of some specific proteins involved in cell cycle and proliferation, is often related to the changes in many translation factors[8]. Although accumulating evidence has identified the roles of eukaryotic translation factors such as translation initiation and elongation factors, in the occurrence and development of tumors[9], little is known about the releasing factors. Eukaryotic peptide chain releasing factors (eRF) is a group of important proteins released by nascent peptide chain in eukaryotes involved in intracellular protein synthesis, including two types (eRF1 and eRF3). Researches have shown that the eRF3 subtype (eRF3a) is a major factor in the termination process of protein translation in mammals, whose expression level affects the stability of eRF1 protein[10]. ERF3a is mainly encoded by G1 to S phase transition 1 (GSPT1). Various studies[11] have also identified that GSPT1 in patients with GC is significantly increased. Given this, GSPT1 protein has been demonstrated to be a potential therapeutic target for GC.

MicroRNA (miRNA) is a newly-discovered short-chain non-coding regulatory RNA with an average length of 18-25 nucleotides. MiRNA

Corresponding Author: Ruochen Tian, MD; e-mail: 2311030163@qq.com

plays a crucial regulatory role in various physiological and pathological processes, including embryonic development[12], organ formation[13] and tumorigenesis[14]. The abnormal expression of miRNA in tumors is closely related to the occurrence and development of tumors and prognosis[15-17]. Up to date, no study has been published on the role of miR-144/GSPT1 axis in GC development and treatment.

In this study, we aimed at investigating the molecular mechanism of highly expressed GSPT1 in GC. MiR-144 induced GSPT1 downregulation was first screened using bioinformatics in the present work. The purpose of our current study was to detect the functions of miR-144/GSPT1 axis in the occurrence and development of GC, so as to provide new basis for the clinical treatment and prevention of GC.

Patients and Methods

Gastric Cancer Cases and Cells

Data of 100 gastric cancer (GC) patients were collected. These patients were confirmed as GC cases *via* pathology and underwent a surgical procedure in our hospital. Preoperative chemotherapy or radiotherapy treatments were forbidden. Liquid nitrogen was used to freeze GC tissues. The corresponding adjacent normal tissues were kept in -70ºC refrigerator. This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Baotou Medical College. Signed written informed consents were obtained from all participants before the study. The human gastric cell line (SGC7901) and normal gastric cell line (GES-1) were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C with 5% CO_2.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted using TRIzol Reagent in accordance with the manufacturer's protocol. SYBR green qPCR assay was performed to measure the level of GSPT1 expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as internal control. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to determine the level of miR-144 expression. U6 was served as the internal control.

Cell Transfection

MiR-144 mimics and inhibitors were synthesized and transfected to cells, which were divided into three different groups: NC group (negative control), miR-144 mimics (SGC7901 cell transfected with miR-144 mimics) and mimics+GSPT1 (SGC7901 cell transfected with miR-144 mimics and siGSPT1). All the reagents were purchased from RiboBio (Guangzhou, China). Cells were transfected using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Luciferase Reporter Assays

SGC7901 cells were co-transfected with pMIR-3'UTR-GSPT1 or pMIR-3'UTR-Mut-GSPT1, miR-144 mimic or negative control (NC), and the pMIR-Renilla plasmid (Promega, Madison, WI, USA). Next, the cells were seeded into a 24-well plate. Luciferase activity was assessed using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

Western Blotting Analysis

Protein concentration was measured using bicinchoninic acid (BCA) reagent kit (Merck, Billerica, MA, USA). SGC7901 Cell lysates were separated on polyacrylamide gels and blotted onto nitrocellulose membranes. The membrane was then blocked with a blocking buffer TBST-20 (Tris-buffered saline with 0.05% Tween 20, pH 7.6 with 5% skimmed milk). After that, the blotted proteins were washed and incubated with anti-GSPT1 antibody (1:1000; Proteintech, Rosemont, IL, USA) or anti-GAPDH (1:1000; Cell Signaling Technology, Danvers, MA, USA) antibody at 4°C overnight.

Cell Proliferation

SGC7901 cells were harvested and inoculated into 96-well plates at a density of 2´10^{3} cells for 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL, MultiSciences, Hangzhou, China) was added to each well after incubation. After 30 min, the absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

Cell Invasion Assay

Cell invasion assay was performed using transwell plates (Corning, Corning, NY, USA) with 8-μm-pore size membranes with Matrigel. Briefly, $2´10^4$ cells were plated into the upper chambers with serum free medium. On the other hand, the lower chamber was added with medium containing 10% FBS as a chemoattractant. At 2 days after incubation, the cells on the top of membrane were wiped by a brush. Subsequently, the membrane was stained by 0.2% crystal violet and then drenched by 95% ethanol. The invaded cells were observed by an inverted microscope.

Cell Migration Assay

Cell migration was performed by scratch-wound assay. SGC7901 cells were seeded in 6-well plates. After transfection, each well was scraped with a 10 μL pipette tip to create a linear region devoid of cells. Subsequently, the cells in each well were cultured with RPMI-1640 medium containing 2% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). We monitored wound healing at 0, 12, 24, and 48 h after scraping.

Statistical Analysis

Statistical analysis was performed using a Student's *t*-test or F-test. All *p*-values were two-sided and analyzed by Prism 6 software (San Diego, CA, USA). $p < 0.05$ represented that the difference was statistically significant.

Results

Expression of GSPT1 and miR-144 in Both Tissues and Cells of GC

The levels of GSPT1 expression in GC tissues and the adjacent normal tissues were detected to determine the role of GSPT1 in GC development. It was found that the level of GSPT1 expression in GC tissues was much higher than that in the adjacent normal tissues (Figure 1A). Previous reports[18-22] have demonstrated that miR-144 was dysregulated in various cancers. MiR-144 has been found to be downregulated in GC according to a previous investigation[23], but its molecular mechanism remains unclear. Another work has shown that miR-144 regulates GSPT1 in colorec-

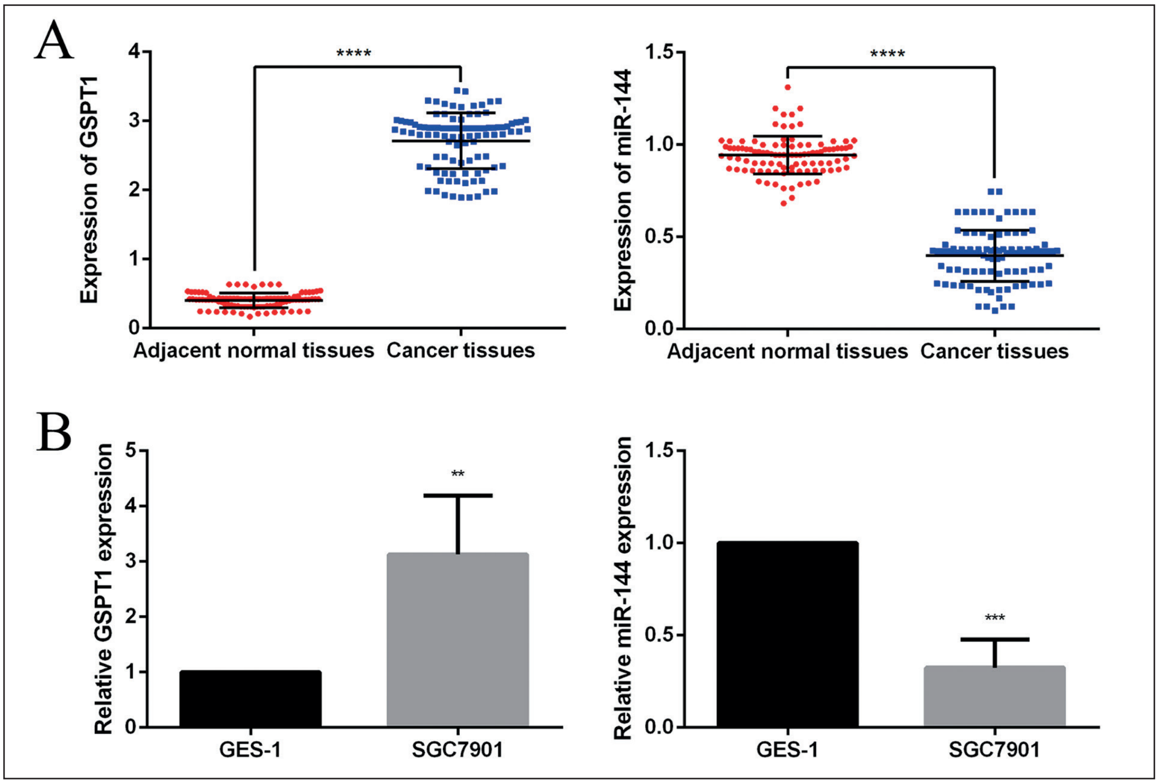

Figure 1. The expressions of GSPT1 and miR-144 in gastric tissue samples and cells. ***A.*** Difference in the expression of GSPT1 and miR-144 between GC tissues and corresponding adjacent normal gastric tissues. (****compared with adjacent normal gastric tissues, $p < 0.0001$). ***B.*** The expression of GSPT1 and miR-144 in GC cell line (SGC7901) and normal gastric epithelial cells (GES-1) (****compared with GES-1, $p < 0.0001$).

tal cancer[24], so we measured the expression of miR-144 in the GC tissue. As we expected, the result revealed that the level of miR-144 expression was significantly decreased in GC tissues compared with that in the normal tissues (Figure 1A). Furthermore, we found the same results in GC cell line (SGC7901) in comparison with the normal gastric cell line (GES-1) (Figure 1B).

GSPT1 is a Direct Target of miR-144 in GC Cells

In order to confirm whether miR-144 has a regulatory effect on GSPT1, we made a search based on the databases of TargetScan, miRDB and PicTar. The results showed that the GSPT1 was a potential target of miR-144 (Figure 2A). We established luciferase reporter vectors containing the wild or mutant-type miR-144 seed sequences of the GSPT1 3'UTR. The increased expression of miR-144 with mimics resulted in the decrease of the luciferase activity of the wide-type GSPT1 3'UTR reporter gene, but it had no effect on mutant-type (Figure 2B). Moreover, the expression of GSPT1 was negatively correlated with the expression of miR-144 in GC tissues (Figure 2C). These results indicated that the expression of GSPT1 can be regulated by miR-144.

MiR-144 Decreases the Expression Level of GSPT1

We set up three groups to conduct similar experiments (miR-NC group, miR-144 mimics group and the mimics + GSPT1 group) for SGC7901 cells. We found that the protein expression of GSPT1 was decreased by up-regulating miR-144 in SGC7901 cells (Figure 2C, D). These data indicated that GSPT1 could be negatively regulated by miR-144.

MiR-144 Suppressed Proliferation of GC Cell

To examine the function of miR-144 on proliferation of GC cells, MTT assay was performed. Results showed that the cell proliferation rate of SGC7901 cells was decreased after miR-144 mimics transfection. In contrast, inhibition of miR-144 increased the growth of GC cells (Figure 3).

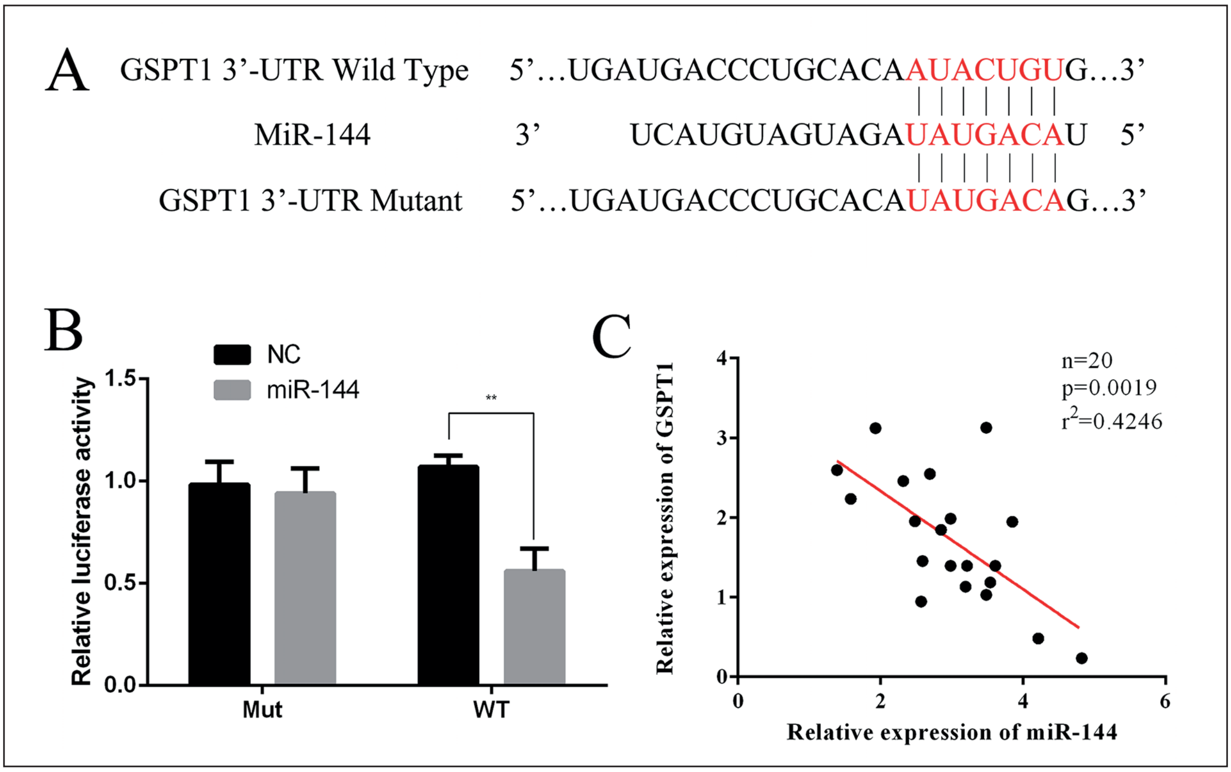

Figure 2. GSPT1 is a direct and functional target of miR-144. SGC7901 cell is transfected with miR-144 mimics and inhibitor. ***A.*** Diagram of putative miR-144 binding sites of GSPT1. ***B.*** Relative activities of luciferase reporters (**$p < 0.01$). ***C.*** The expression of GSPT1 is negatively correlated with the expression of miR-144 in GC tissues.

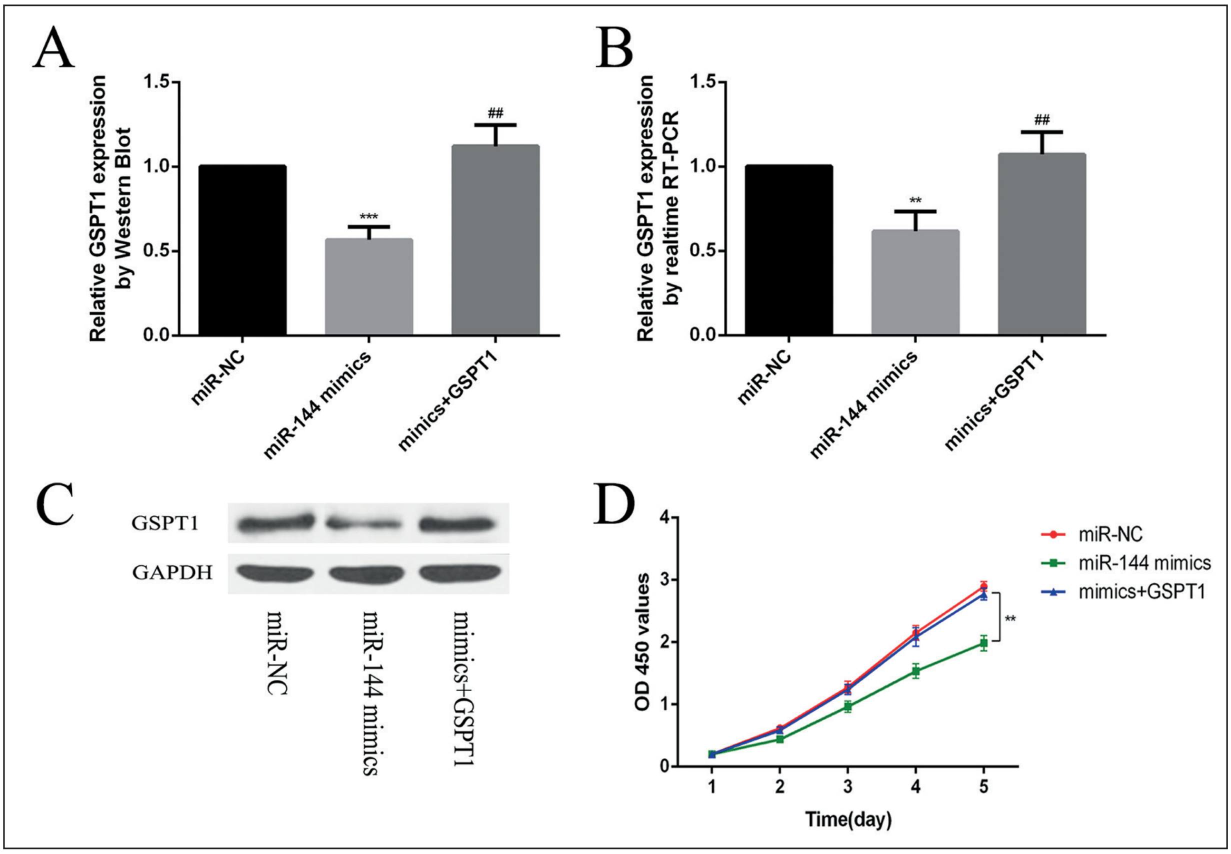

Figure 3. MiR-144 decreases the expression level of GSPT1. ***A.*** The content of GSPT1 by Western blot experiment. ***B.*** Expression level of GSPT1 by Real-time PCR analysis (**$p < 0.01$, ***$p < 0.001$ *vs.* NC group; ##$p < 0.01$ *vs.* Mimics group). ***C.*** Protein content of GSPT1 by Western blot. ***D.*** MiR-144/GSPT1 axis inhibits the proliferation of GC cell (**$p < 0.01$).

MiR-144 Inhibited Invasion and Migration of GC cell

Migration and invasion are key factors in cancer cell proliferation. In the transwell experiments, we tested the invasive ability of SGC7901 cells. Result revealed that the up-regulation of miR-144 could inhibit the invasive ability of SGC7901 cells (Figure 4A). Meanwhile, the results of scratch-wound assay demonstrated that the migration ability of SGC7901 cells was attenuated by miR-144 (Figure 4B).

Discussion

In recent years, studies on miRNA have suggested that miRNA plays an extremely important regulatory role in the occurrence and development of GC. Scholars[25-28] have also confirmed that miRNA is involved in important physiological processes. A number of large-sample sequencing data assays showed that the difference in miRNA expression in GC tissues or serum was closely related to the malignant clinical phenotypes (metastasis, recurrence, chemotherapy resistance and survival prognosis) of GC[29-31]. The above findings revealed that miRNA may not only provide novel clinical markers for the early diagnosis and detection of GC, but also serve as a tool to assess the malignant clinical phenotype and prognosis of GC. Therefore, further researches on the important role of miRNAs in the occurrence and development of GC can provide new insights for the precise treatment of GC.

Currently, the protein synthesis involved in cell proliferation and cancer progression is an important research hotspot. Changes in the expression pattern of translation factors may lead to some changes in tumor cells. Existing evidence proved that eukaryotic transcription factors play important roles in kinds of cancers[32]. However, there is still no evidence regarding translation releasing factors and cancers. The termination of translation and release of nascent peptide chain

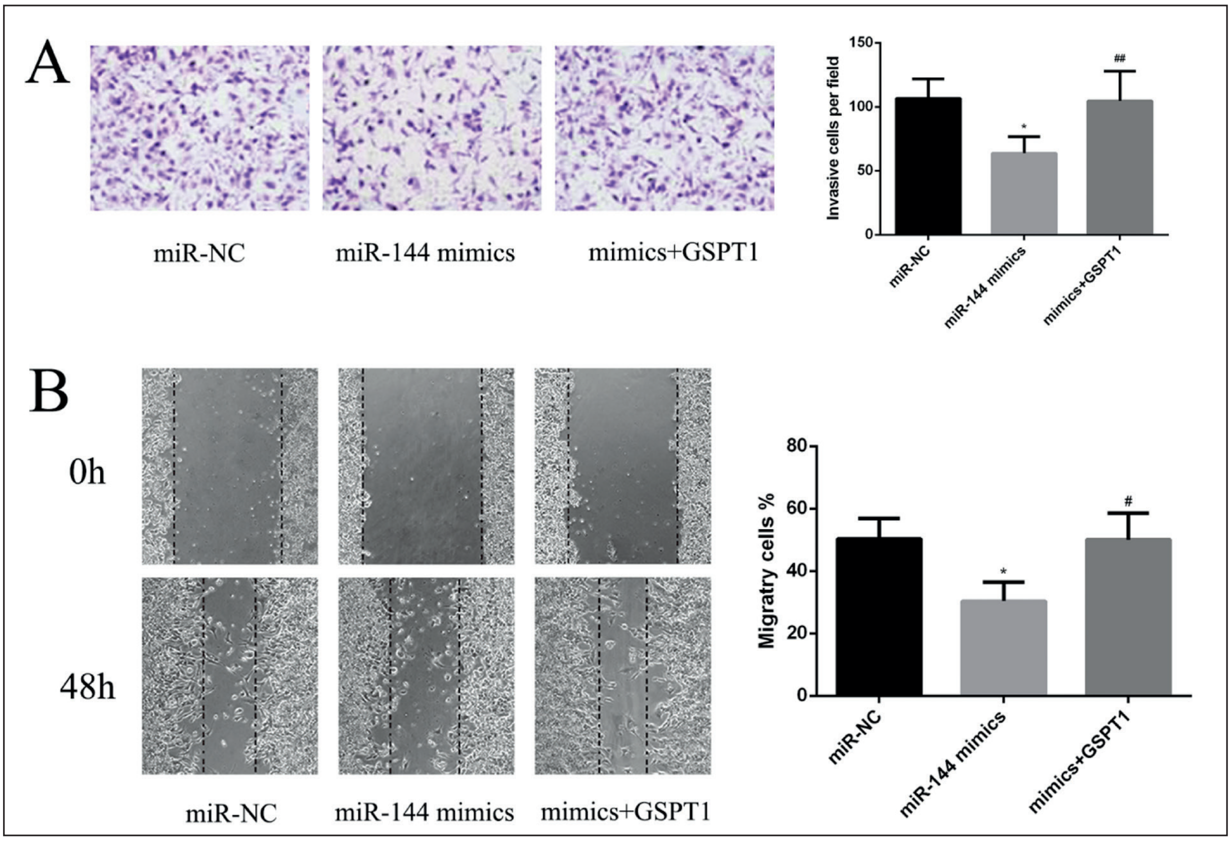

Figure 4. MiR-144/GSPT1 axis inhibits the invasion and migration of GC cell. GSPT1 overexpression attenuates the suppressive effect of miR-144 in SGC7901 cells. ***A.*** The invasion test by transwell assay. ***B.*** The migration test by scratch-wound assay (*$p < 0.05$, **$p < 0.01$ *vs.* NC group; $^{\#}p < 0.05$ *vs.* mimics group).

are the last stages in the process of protein synthesis. The transcriptional level of mRNA expression of GSPT1 is different in the cell cycle[33]. Experiments have shown that the GSPT1 mRNA level is increased in intestinal-type GC and breast cancer[34], indicating that eRF3a may be related to cell proliferation and apoptosis.

In this study, the expression of GSPT1 in GC tissues was analyzed. We also found that GSPT1 was significantly increased in GC tissues. In order to study the molecular mechanism of abnormally expressed GSPT1 in GC, miR-144 was first screened using bioinformatics. Our results showed that miR-144 inhibited the expression of GSPT1 in a targeted way. Subsequent analysis revealed that the expression level of miR-144 was significantly decreased in GC tissues. Existing studies have shown that miR-144 is abnormally expressed in various cancers, which is closely related to the proliferation, migration and invasion of tumors, indicating that miR-144 might be a tumor suppressor gene. Moreover, up-regulation of miR-144 resulted in significantly decreased proliferation, migration and invasion of GC cells. The current study therefore demonstrated that miR-144/GSPT1 regulatory axis might contribute to the occurrence and development of GC, providing a new detection for the diagnosis and treatment of GC.

Conclusions

We found that GSPT1 was found to be a direct and functional target of miR-144. MiR-144 could attenuate cell proliferation, invasion and migration in gastric cancer cells via targeting GSPT1. Thus, the restoration of miR-144/GSPT1 axis may be a potential therapeutic target for the treatment of gastric cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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