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MiR-1-3p suppresses cell proliferation and invasion and targets STC2 in gastric cancer

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Abstract. – OBJECTIVE: MiR-1 has been reported to act as an inhibitory microRNA in gastric cancer (GC). This study aimed to investigate the regulatory mechanism by which miR-1-3p blocks the progression of GC by targeting stanniocalcin 2 (STC2).

PATIENTS AND METHODS: The expression level of miR-1-3p in GC was assessed via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Expressions of STC2 were measured by qRT-PCR and Western blot analysis. Proliferation and invasion assays were detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays, respectively. Moreover, the dual-luciferase reporter assay was used to confirm the binding sites between miR-1-3p and STC2.

RESULTS: MiR-1-3p was significantly down-regulated in GC. Moreover, abnormal expression of miR-1-3p was correlated with GC tumor size. Functionally, overexpression of miR-1-3p inhibited proliferation and invasion in GC by inhibiting stanniocalcin 2 (STC2) expressions. In contrast, STC2 was significantly up-regulated in GC. Furthermore, miR-1-3p negatively regulated STC2 expression in GC. The upregulation of STC2 weakened the inhibitory effect of miR-1-3p in GC.

CONCLUSIONS: MiR-1-3p suppressed cell proliferation and invasion by targeting STC2 in GC, providing a novel therapeutic target for GC.

Key Words:
Gastric cancer, MiR-1-3p, Invasion, Proliferation, STC2.

Introduction

Gastric cancer (GC) is a malignant tumor derived from the epithelium of the gastric mucosa. The prevalence of GC is around 50 years old, and the ratio of male to female is 2:1[1]. GC shows a trend toward younger due to dietary changes, increased work stress and infection of *H. pylori*[2]. GC can occur anywhere in the stomach, and most GC tumors are adenocarcinomas. In addition, there are no symptoms or epigastric discomfort and other non-specific symptoms in the early stages of GC[3]. GC is easily overlooked because its symptoms are usually similar to gastritis, gastric ulcer, and chronic gastric disease. Therefore, the early diagnostic rate of GC is still very low.

MicroRNAs (miRNAs) are evolutionarily conserved non-coding small molecule RNAs that function to regulate gene expression at the translation level. Although the first miRNA was discovered as early as 1993, its diversity and universality are revealed in recent years[4]. MiRNAs block gene expression by inhibiting translation or degradation of mRNA. Therefore, the aberrant expression of miRNAs can affect many biological activities, including cell differentiation, proliferation, and metastasis in human cancers[5-7]. Moreover, a number of miRNAs have been identified to regulate the development of GC, such as miR-324[8], miR-329[9], miR-599[10], and miR-937[11]. In particular, miR-1, which was an inhibitory miRNA, was downregulated in various human cancers including oral squamous cell carcinomas[11], ovarian cancer[12], bladder cancer[13], esophageal carcinoma[14], and colorectal cancer[15]. However, miR-1 was reported to be up-regulated in GC[16], while Xie et al[17] indicated a decrease in miR-1 expression in GC. These conflicting findings suggest that the specific role of miR-1 in GC remains to be further investigated.

As a member of the STC family, stanniocalcin 2 (STC2) affects the homeostasis of calcium and phosphate[18]. Additionally, STC2 was shown to be involved in cell proliferation, migration, and

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invasion of glioblastoma[19] and hepatocellular carcinoma[20]. Furthermore, the clinical significance of STC2 expression has been shown to be a predictor of tumor progression in GC[21]. Therefore, we speculated that STC2 might be involved in biological processes associated with GC development.

Here, we explored the regulatory mechanism of miR-1-3p on GC cell proliferation and invasion. In particular, STC2 and its relationship to miR-1-3p in GC were investigated as well. This study will provide a novel therapeutic target for GC.

Patients and Methods

Clinical Tissues

Sixty-two surgical tumor specimens and adjacent tissues were obtained from the Xiyuan Hospital, China Academy of Chinese Medical Sciences. All of GC patients provided written informed consents. All patients did not receive any treatment before surgery. Human tissues were frozen in liquid nitrogen and stored in an −80°C refrigerator for further experiments. This study was approved by the Institutional Ethics Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences.

Cell Cultures and Cell Transfection

Human normal gastric epithelial cell line GES-1 and AGS, MGC803 GC cell lines were used for this experiment. All cell lines were bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were seeded in Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and cultured in an incubator at 37°C with 5% CO_2.

MiR-1-3p mimics and inhibitor, STC2 siRNA (si-STC2) were purchased from GenePharma (Shanghai, China). They were transferred to MGC803 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA containing miRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to quantify the expression of miR-1-3p in GC tissues and cell lines. We performed qRT-PCR on ABI StepOne Plus system using SYBR Green Master Mix (Roche, Basel, Switzerland). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-1-3p and STC2. And their expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The primers used in our work were as follows: miR-1-3p, forward primer: 5'-CAG TGC GTG TCG TGG AGT-3', reverse primer: 5'-GGCCTGGAATGTAAAGAAGT-3'; U6, forward primer: 5'-CTCGCTTCGGCAGCACA-3', reverse primer: 5'-AACGCTTCACGAATTTGCGT-3'; STC2 forward primer: 5'-ATGCTACCTCAAGCACGACC-3', reverse primer: 5'-TCTGCTCACACTGAACCTGC-3'; GAPDH forward, 5'-ACATCGCTCAGACACCATG-3', reverse, 5'-TGTAGTTGAGGTCAATGAAGGG-3'.

Luciferase Activity Assay

The 3'-UTR of wild or mutant STC2 was inserted into the pGL3 promoter vector (Invitrogen, Carlsbad, CA, USA) for luciferase reporter experiments. Next, the above vector and miR-1-3p mimics were transfected into MGC803 cells. Finally, luciferase assay was performed using the dual luciferase reporter assay (Promega, Madison, WI, USA).

MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) Assay For Cell Proliferation

Cell proliferation was measured using the MTT assay. The transfected cells (1×10^3/well) were seeded into 96-well plates. MGC803 cells containing miR-1-3p mimics or inhibitor were incubated for 24-96 h. After the incubation, the cells were incubated with MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C. The absorbance at 490 nm (OD=490 nm) was measured with a spectrophotometer.

Cell Invasion Assay

Cell invasion was assessed by performing the transwell assay. The upper chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to detect cell invasion. 5×10^5 cells were seeded into the upper chamber (8 μm pore size; Corning Incorporated, Corning, NY, USA), and the medium with 20% FBS was placed in the lower chamber. The cells were then incubated for 18 h at 37°C with 5% CO_2. The invading cells on the lower surface were fixed with 4%of paraformaldehyde (PFA) and stained with 0.1% of crystal violet. Cells were counted by a light microscope.

Western Blot Analysis

Protein samples were obtained using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein was separated by 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocked with 5% non-fat milk, the protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at room temperature. Next, we incubated the membranes with anti-STC2, anti-GAPDH overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Finally, protein expression levels were measured by the enhanced chemiluminescence (ECL) assay.

Statistical Analysis

Data were shown as mean ± SD (Standard Deviation). Statistical analysis was analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA). Differences between groups were calculated using Student *p*-test and One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). *p*-value <0.05 was considered to indicate a statistically significant.

Results

MiR-1-3p was Downregulated in GC Tissues and Cells

Primarily, the expression level of miR-1-3p was identified in the collected GC tissues. Moreover, the low expression of miR-1-3p was detected in GC tumor tissues compared to normal tissues (Figure 1A). Not surprisingly, the same results were found in MGC803 and AGC cell lines, as shown in Figure 1B. In addition, MGC803 cells were selected for the next experiment, because the difference in miR-1-3p expression was more significant in MGC803 cells than in AGC cells. Besides that, the correlation between miR-1-3p expression and clinicopathological features of GC patients was also analyzed. Surprisingly, there was almost no significant difference between miR-1-3p and clinic features except for tumor size (Table I).

MiR-1-3p Inhibited GC Cell Proliferation and Invasion

MiR-1-3p mimics or inhibitor was transfected into MGC803 cells to explore its function in GC. Transfection efficiency was assessed by qRT-PCR (Figure 2A). Functionally, proliferation and invasion in transfected MGC803 cells were measured by MTT and transwell assays. We found that the proliferation of miR-1-3p mimics group was decreased compared to the control group, whereas miR-1-3p inhibitor enhanced MGC803 cell proliferation (Figure 2B). As we expected, cell invasion in GC also found the same result. As shown in Figure 2C, the overexpression of miR-1-3p inhibited cell invasion in GC. Knockdown of miR-1-3p was found to promote cell invasion in MGC803 cells. Therefore, we considered that miR-1-3p had an inhibitory effect on the proliferation and invasion of GC cells.

STC2 is a Direct Target of MiR-1-3p

Based on the prediction of Targetscan (http://www.targetscan.org/), STC2 was selected as a po-

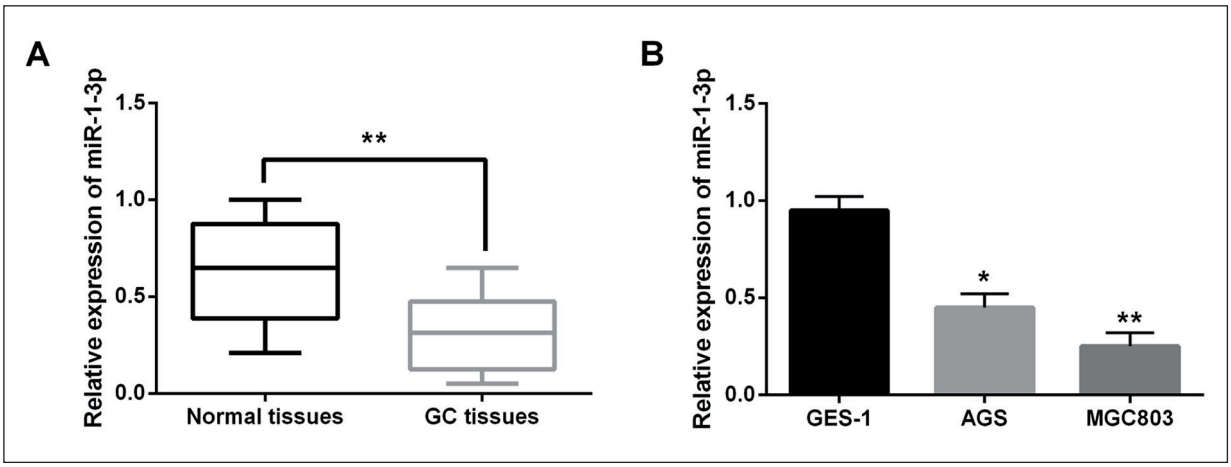

Figure 1. MiR-1-3p was downregulated in GC tissues and cells. **A,** Expressions of miR-1-3p in GC tissues and normal tissues were detected *via* qRT-PCR. **B,** MiR-1-3p expression in MGC803, AGS and GES-1 cells (control). **p*<0.05, ***p*<0.01.

Table I. Relationship between miR-1-3p expression and their clinicopathological characteristics in patients with gastric cancer.

Characteristics	miR-1-3p		*p*-value
	High	Low	
Age (years)			0.458
≥ 60	16	27	
< 60	5	14	
Gender			0.257
Male	12	28	
Female	9	13	
Tumor size (cm)			0.016*
≥ 3	14	29	
< 3	7	12	
TNM stage			0.124
I+ II	13	26	
III+IV	8	15	
Tumor Stage			0.486
I + II	4	13	
III+IV	17	28	
Lymph node metastasis			0.124
None	15	28	
Yes	6	13	

*$p < 0.05$ was considered significant.

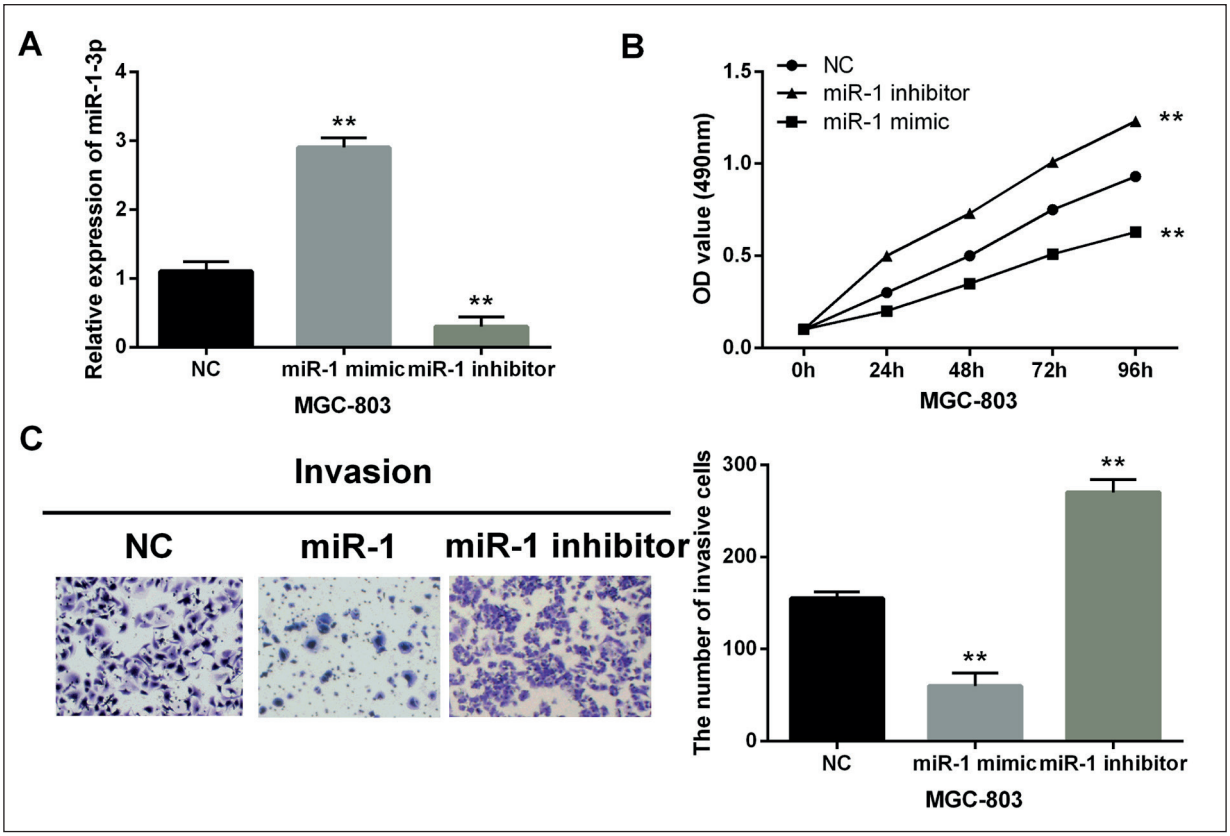

Figure 2. MiR-1-3p inhibited GC cell proliferation and invasion. **A,** MiR-1-3p expressions were examined in MGC803 cells with miR-1-3p mimics or inhibitor *via* qRT-PCR. **B,** Cell proliferation was measured in cells with miR-1-3p mimics or inhibitor *via* MTT. **C,** Cell invasion was measured in cells with miR-1-3p mimics or inhibitor *via* transwell assay (magnification: 40×) (**p<0.01).

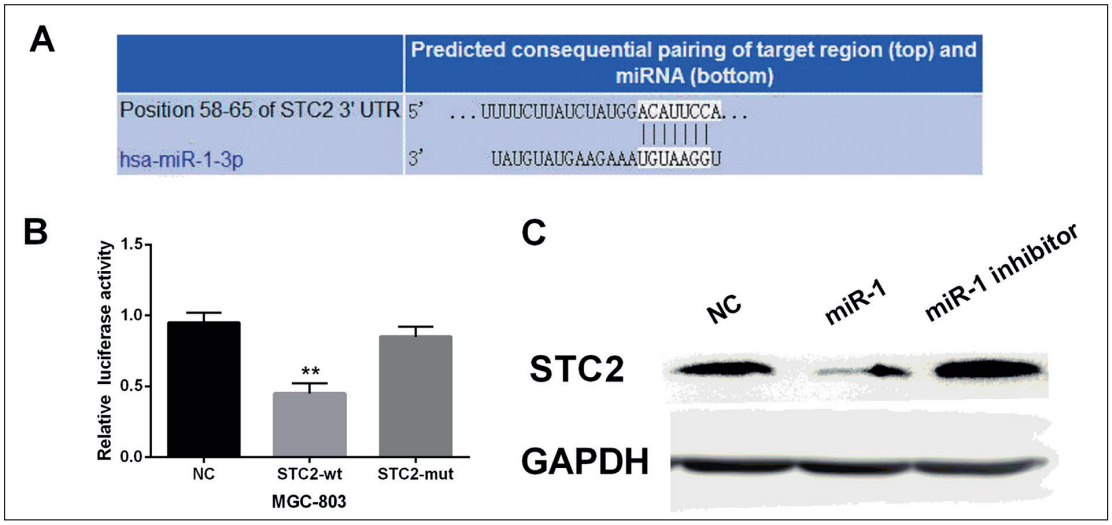

Figure 3. MiR-1-3p directly targeted STC2 in GC. **A,** MiR-1-3p has binding sites with the 3'UTR of STC2. **B,** Luciferase reporter assay. **C,** Protein expressions of STC2 were analyzed in MGC803 cells with miR-1-3p mimics or inhibitor (**p<0.01).

tential target of miR-1-3p (Figure 3A). Moreover, dual-luciferase reporter assay was performed to further verify the above prediction. As expected, the luciferase activity was significantly decreased in MGC803 cells with miR-1-3p mimics and STC2-wt vector. However, there was almost no change in MGC803 cells with miR-1-3p mimics and STC2-mut vector (Figure 3B). We also found that the protein expression of STC2 was reduced by miR-1-3p mimics and enhanced by the miR-1-3p inhibitor (Figure 3C). Therefore, miR-1-3p was considered to directly target STC2 and negatively regulated its expression in GC.

STC2 Was Upregulated and Promoted Proliferation and Invasion of GC Cells

To investigate the biological function of STC2 in GC, si-STC2 was transfected into MGC803 cells (Figure 4A). And upregulation of STC2 was identified in MGC803 and AGC

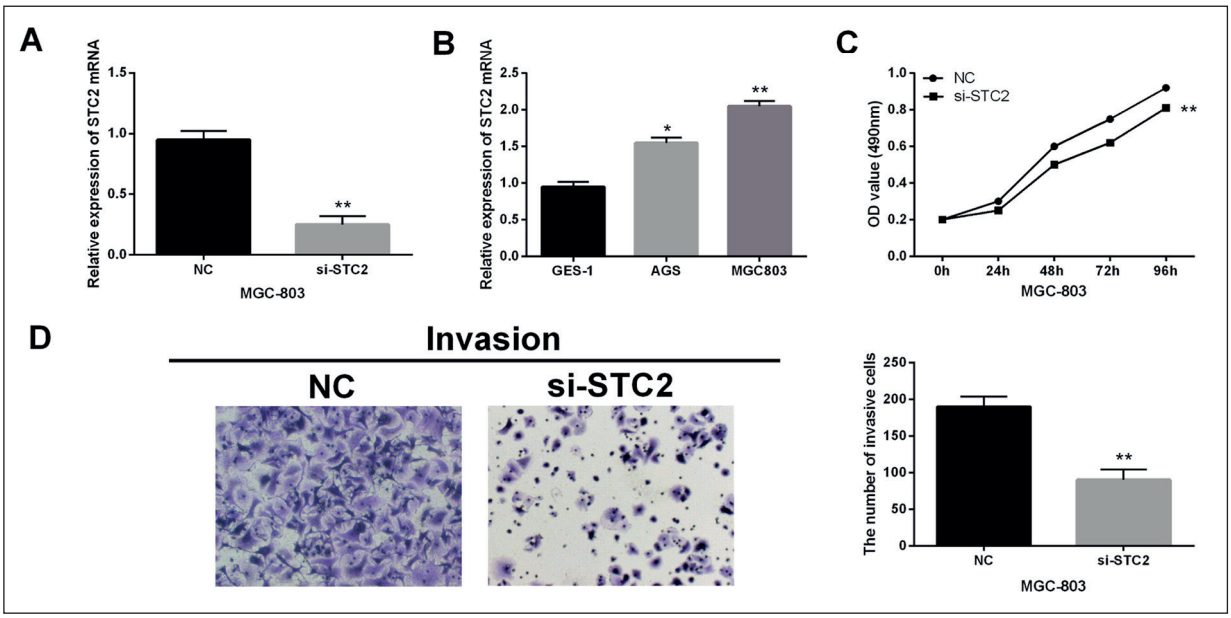

Figure 4. STC2 promoted the proliferation and invasion of GC cells. **A,** The mRNA expression of STC2 was measured in cells with STC2 siRNA. **B,** STC2 expression in MGC803, AGS and GES-1 cells (control). **C,** Cell proliferation in cells containing si-STC2. **D,** Cell invasion analysis of MGC803 cells with si-STC2 (magnification: 40×) (**p<0.01).

cell lines compared to GES-1 cells (Figure 4B). What's more, cell proliferation of si-STC2 group was significantly decreased compared to the control group in MGC803 cells (Figure 4C). At the same time, si-STC2 also suppressed cell invasion in MGC803 cells (Figure 4D). These results indicated that STC2 acted as an oncogene in GC.

MiR-1-3p Inhibited GC Proliferation and Invasion Through Downregulation of STC2

STC2 vector was transfected into MGC803 cells with miR-1-3p to verify their interaction in GC. Furthermore, we found that STC2 vector restored the decrease in STC2 mRNA and protein expressions induced by miR-1-3p mimics in MGC803 cells (Figure 5A and 5B). More importantly, the inhibitory effect of miR-1-3p mimics on the proliferation of GC cells was impaired by STC2 vector (Figure 5C). Furthermore, the inhibitory effect of miR-1-3p on cell invasion almost disappeared in the STC2 over-expression group (Figure 5D). All of these findings indicated that miR-1-3p inhibited cell proliferation and invasion by regulating STC2 expression in GC.

Discussion

Many previous studies have shown that miRNAs regulate the expression of different genes by inducing degradation of mRNA or inhibiting translation. Furthermore, there is growing evidence that miRNAs play important roles in the development of GC. In the current study, the expression of miR-1-3p was decreased in GC. Moreover, the overexpression of miR-1-3p suppressed cell proliferation and invasion in GC. Furthermore, miR-1-3p inhibited the progression of GC by targeting STC2.

Recently, several studies have shown that miR-1 was downregulated in various cancers. The dysregulation of miR-1 induced changes in biological activities. For example, overexpression of miR-1 showed significant inhibition of proliferation, migration, and invasion in prostate cancer[22]. Similarly, miR-1 inhibited cell proliferation, invasion, and migration in head and neck squamous cell carcinoma[23]. Besides that, downregulation of miR-1 was correlated with a low survival rate of clear cell renal cell carcinoma patients and obstructed cell cycle and metastasis[24]. More importantly, miR-1 was found to inhibit GC cell proliferation and migration by targeting MET[25]. Consistent with the above studies, it was also

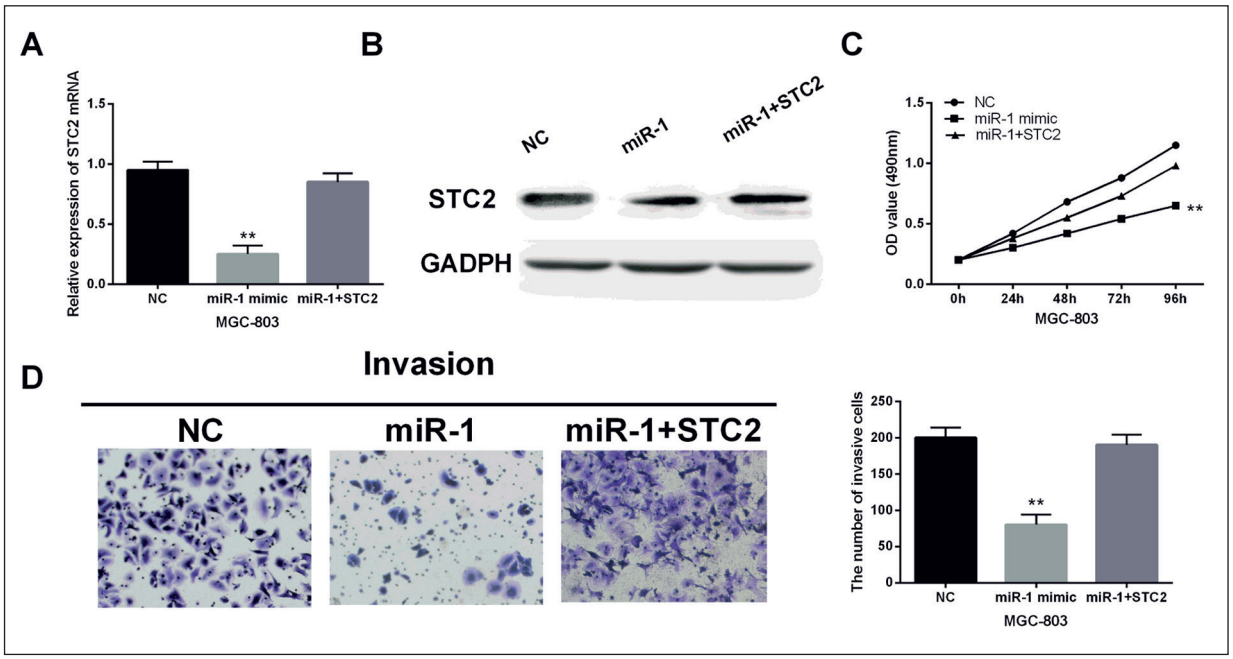

Figure 5. MiR-1-3p regulated the proliferation and invasion of GC cells through suppressing STC2. **A, B,** The mRNA and protein expressions of STC2 were measured in cells containing STC2 vector and miR-1-3p. **C,** Cell proliferation in cells containing STC2 vector and miR-1-3p. **D,** Cell invasion in cells containing STC2 vector and miR-1-3p (magnification: 40×) (**p<0.01).

found that miR-1-3p had an inhibitory effect on proliferation and invasion in GC. In addition, STC2 was detected as a direct target gene of miR-1-3p.

The STC2 gene is normally found in the human heart, spleen, kidney, and pancreas by encoding a protein with 302-amino acid-long[26]. It had also been found that altered expression of STC2 affected cellular biological activities in several cancers. In particular, STC2 was upregulated in hepatocellular carcinoma and promoted cell proliferation and migration *in vitro*[27]. In contrast, STC2 was reported to inhibit cell proliferation in breast cancer[28]. It had also been demonstrated that the clinical significance of STC2 can serve as a prognostic marker in GC[29]. The upregulation of STC2 was identified in GC, which resulted in the suppression of GC cell proliferation. Furthermore, knockdown of STC2 was found to suppress cell proliferation and invasion in GC. Shen et al[30] have also found that STC2 silencing can inhibit the invasion of cervical carcinoma cells. These findings implied that STC2 can act as an oncogene in GC.

Conclusions

In this study, downregulation of miR-1-3p and upregulation of STC2 were identified in GC. Moreover, miR-1-3p was shown to directly target STC2. STC2 expression was negatively associated with miR-1-3p in GC. Functionally, miR-1-3p inhibited GC cell proliferation and invasion by down-regulating STC2. This is the first time we verify the relation between miR-1-3p and STC2 in GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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