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MiR-769-5p functions as an oncogene by down-regulating RYBP expression in gastric cancer

P.-B. LUAN[1], X.-Z. JIA[2], J. YAO[2]

[1]Department of General Surgery, Yantaishan Hospital, Yantai, China
[2]Central Ward Operating Room, Yantai Yuhuangding Hospital, Yantai, China

Pengbo Luan and Xizhen Jia contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to detect the relative expression level of micro-ribonucleic acid (miR)-769-5p in gastric cancer (GC) tissues and cells, and to investigate the clinical significance, biological function, and mechanism of miR-769-5p in GC.

PATIENTS AND METHODS: The relative expression level in 62 cases of GC tissues and paracancerous tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between miR-769-5p expression and clinicopathological characteristics of GC patients was analyzed by Chi-square test. Besides, the relative expression level of miR-769-5p in GC cells and the interference efficiency of si-miR-769-5p were detected by qRT-PCR, and the biological function of miR-769-5p was studied by *in vitro* experiments [Thiazolyl Blue Tetrazolium Bromide (MTT), flow cytometry, 5-ethynyl-2'-deoxyuridine (EdU)]. Next, the effect of miR-769-5p on the tumorigenicity of GC cells *in vivo* was investigated by nude mouse tumorigenicity assay. Moreover, the downstream target genes of miR-769-5p were predicted by bioinformatics. Finally, qRT-PCR and Western blotting were used to screen the downstream target genes.

RESULTS: In the 62 cases of GC tissues, the expression of miR-769-5p was upregulated in 48 cases. MiR-769-5p was divided into high-expression group and low-expression group. Chi-square analysis showed that the high expression of miR-769-5p was positively correlated with tumor-node-metastasis (TNM) stage (p=0.005), lymph node metastasis (p=0.010), and infiltration depth (p=0.011) in patients with GC. The results of qRT-PCR indicated that the expression of miR-769-5p was upregulated in GC cells. *In vitro* experiments (MTT, flow cytometry, EdU) results showed that after interfering in the expression of miR-769-5p, the proliferation ability of GC cells was decreased, and apoptosis was increased. Furthermore, the results of *in vivo* experiments manifested that the tumorigenic ability of GC cells declined after interference in the expression of miR-769-5p. Finally, the results of qRT-PCR and Western blotting revealed that the expression of RING1 and YY1-binding protein (RYBP) was regulated by miR-769-5p.

CONCLUSIONS: The expression of miR-769-5p is upregulated in GC and positively correlated with TNM stage in GC patients. By regulating the expression of RYBP, the proliferation of GC cells was promoted, and the apoptosis was inhibited.

Key Words:
MiR-769-5p, Gastric cancer, Proliferation, Apoptosis, RYBP.

Introduction

Gastric cancer (GC) is one of the most common cancers, mainly in Asian countries, such as South Korea, Japan, and China[1]. The incidence of GC is affected by many factors, including high salt food, nitrated food, and excessive alcohol intake, low intake of fruits and vegetables, and EB virus and Helicobacter pylori infection[2]. Genetic and epigenetic factors are involved in the pathogenesis of GC, as other malignant tumors[3]. If GC is diagnosed in the early stage, its mortality will be greatly reduced[4]. Therefore, it is of importance to investigate the pathogenesis of GC and to screen early specific tumor markers.

Micro-ribonucleic acids (miRNAs) are a kind of non-coding RNAs with a fragment size of 21-25 bp. They can inhibit the translation of messenger RNA (mRNA) molecules by binding to the 3'-untranslated region (UTR) of mRNA, thus silencing their homologous target genes, exerting an important role in different cell processes and maintaining normal physiological environment[5-7]. In cancer, special miRNAs can serve as a tumor suppressor or carcinogen, so it is considered as a biomarker for early diagnosis and judgment of prognosis of cancer[8], which can control many functions of tumor, including cell proliferation, metastasis, differentiation, apoptosis, and angiogenesis[9].

Corresponding Author: Jie Yao, BM; e-mail: yt6709936@163.com

MiR-769-5p is located on chromosome 19q13.32. In 2015, foreign researchers found that miR-769-5p expression can distinguish EGFR, K-Ras gene mutation status, and ALK gene rearrangement in non-small cell lung cancer (NSCLC) tissues[10]. Recently, it has been reported that miR-769-5p is dysregulated in many kinds of tumors, such as hepatocellular carcinoma, and can promote the proliferation, invasion, and metastasis of tumor cells[11]. However, its expression, clinical significance, and biological function in GC have not been reported. Our research group is the first to investigate and find out that the high expression of miR-769-5p in GC can promote the proliferation and inhibit the apoptosis of GC cells, which is correlated with the clinical stage of GC patients.

Patients and Methods

Tissue Samples

After obtaining the informed consent of patients, the GC tissue samples and normal paracancerous tissues (more than 3 cm away from the cancer tissues) which were surgically removed from January 2015 to December 2017 in Yantaishan Hospital were collected and stored in a refrigerator at -80°C. There were 62 patients, male in 23 cases and female in 13 cases, aged 40-79 years old. The clinical staging of GC tissues was performed according to the tumor-node-metastasis (TNM) staging standard of UICC/AJCC in 2010. This study was approved by the Ethics Committee of Yantaishan Hospital.

Cell Culture

Normal gastric cell line HFE-145 and GC cell lines BGC-823, SGC7901, BGC803, MKN45, MKN28 were preserved by our research group. All the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS) or Dulbecco's Modified Eagle's Medium (DMEM, HyClone, South Logan, UT, USA) and placed in an incubator at 37°C with 5% CO_2. The cells in good growth condition were selected for the experiment.

Cell Transfection

The GC cells in the logarithmic growth phase were transfected with miR-769-5p inhibitor according to the Lipofectamine 2000 transfection instructions (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, total RNA was extracted for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiR-769-5p inhibitor: 5'-AGAGGUCGACCGUGUAAUGUG-3'.

RNA extraction

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added into GC tissues and cells. After 0.5 h, TRIzol was used to extract the total RNA according to the experimental steps in the instructions. After that, the total RNA integrity was detected by 1% agarose gel electrophoresis, and the purity of RNA was determined to be qualified by A_{260}/A_{280} ratio. Then, RNA products were stored at -80°C for reservation.

Reverse Transcription and qRT-PCR

The complementary deoxyribose nucleic acid (cDNA) (TaqMan Reverse Transcription Kit, Thermo Fisher Scientific, Waltham, MA, USA) was synthesized *via* reverse transcription, and stored at -80°C for reservation. TaqMan RT-PCR kit (Thermo Fisher, Waltham, MA, USA) was applied for PCR amplification to detect miR-769-5p. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the internal reference genes to detect the expression level of RYBP mRNA. The reaction was carried out on ABIQ6 fluorescent qRT-PCR instrument (Applied Biosystems, Foster City, CA, USA). Finally, the Ct value of each sample was measured, and the relative content was detected through $2^{-\Delta\Delta Ct}$ method. U6 F: 5'-CACAUUACACGGUCGA CCUCU-3', R: 5'-AGGUCGACCGUGUAAUGUGUU-3', miR-769-5p F: 5'-GGCTGAGACCTC TGGGITC-3', R: 5'-CAGTGCGTGTCGTGGAGT-3'. RYBP F: 5'-AGGCCCCGGCTGAAAAACGTG-3', R: 5'-CATGTCGCCCTGGCGTGGA-3'.

Thiazolyl Blue Tetrazolium Bromide (MTT)

The cells in good condition were digested and counted accurately, followed by inoculation into a 96-well plate (3000 cells/well). The cells were taken out on the first to the fifth day of culture, added with 5 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA), and incubated in the incubator for 4 h. Then, dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added to each well for incubation at room temperature for 10 min, and slight vibration for 15 min. The absorbance $A_{520\ nm}$ was read on a microplate reader. In each group, 3 repeat wells were set up, and the average value was taken. The *in-vitro* growth curve of the cells was drawn.

Flow Cytometry

The GC cells were inoculated into a 6-well plate. After adhering to the wall, the cells were transfected for 48 h, and then, the cells were collected. The binding buffer, Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining liquid were added in sequence according to the instructions of Annexin V-FITC/PI flow cytometry. Finally, the cell apoptosis was analyzed on a flow cytometer.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Test

EdU proliferation test was conducted according to the EdU reagent instructions (Sigma-Aldrich, St. Louis, MO, USA). GC cells were inoculated into a 6 cm culture dish. After 48 h of transfection, the cells were incubated with 50 μm of EdU for 5 h, fixed with 4% paraformaldehyde at room temperature for 30 min, and then, washed twice by phosphate-buffered saline (PBS). Next, the cells were cultured by 0.5% Triton X-100 for 10 min, and then stained in the staining liquid for 30 min. All images were taken by a fluorescence microscope.

Tumorigenicity Assay In Vivo

GC cells were digested with trypsin into single cell suspension. The cells were counted after three times of washing in serum-free medium. Five 4-week-old female BALB/c nude mice were selected. The cells (2×10^6) in the miR-769-5p inhibitor group and inhibitor normal control (NC) group were injected into the left back subcutaneous and right back subcutaneous tissues of the nude mice, respectively. The tumor diameter was measured with a vernier caliper every 3-5 d, and continuously observed for 20 d. This study was also approved by the Ethics Committee of the Animal Center of the Yantaishan Hospital.

Western Blotting

The cells in the experimental group and control group were collected and lysed at 4°C, and the total protein was obtained. Then, the total protein concentration was determined by bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA), and 30 g of samples were loaded for sodium dodecyl benzene sulfonate gel electrophoresis for 1-2 h, followed by transfer onto a polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) for 30-50 min. After that, the membrane was incubated by 2% of bovine serum albumin (BSA) at room temperature for 1 h, by primary antibody solution (GAPDH, RYBP) at 4°C overnight, and by the second antibody at room temperature on the next day. Finally, the protein bands were exposed in the gel imaging system according to the instructions of the chemiluminescent immunoassay kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The measurement data were expressed by mean ± standard deviation. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Upregulation of MiR-769-5p Expression in GC

The results of qRT-PCR indicted that in the 62 cases of GC tissues, the expression level of miR-769-5p was upregulated in 48 cases, compared with that of paracancerous tissues (Figure 1A). The average relative expression level of miR-769-5p (2.69) was taken as the cut-off point, and the high-expression group (n=31) and low-expression group (n=31) (Figure 1B) were set up. Then, the clinicopathological data of patients were collected. The results of the Chi-square test showed that the high expression of miR-769-5p was positively correlated with TNM stage, lymph node metastasis, and infiltration depth in patients with GC (Table I). Besides, the results of qRT-PCR manifested that the expression level of miR-769-5p was upregulated in GC cells (Figure 1C). Finally, the miR-769-5p interference sequence was designed and transiently transfected into the GC cells, and after 48 h, the interference efficiency was detected by qRT-PCR (Figure 1D).

Inhibition of Proliferation of GC Cells After Interference in the Expression of MiR-769-5p

The results of MTT assay showed that after interference in the expression of miR-769-5p in GC cells, the proliferation ability of GC cells was inhibited (Figure 2A and 2B). The results of EdU assay revealed that the proliferation of cells was inhibited after interference in the expression of

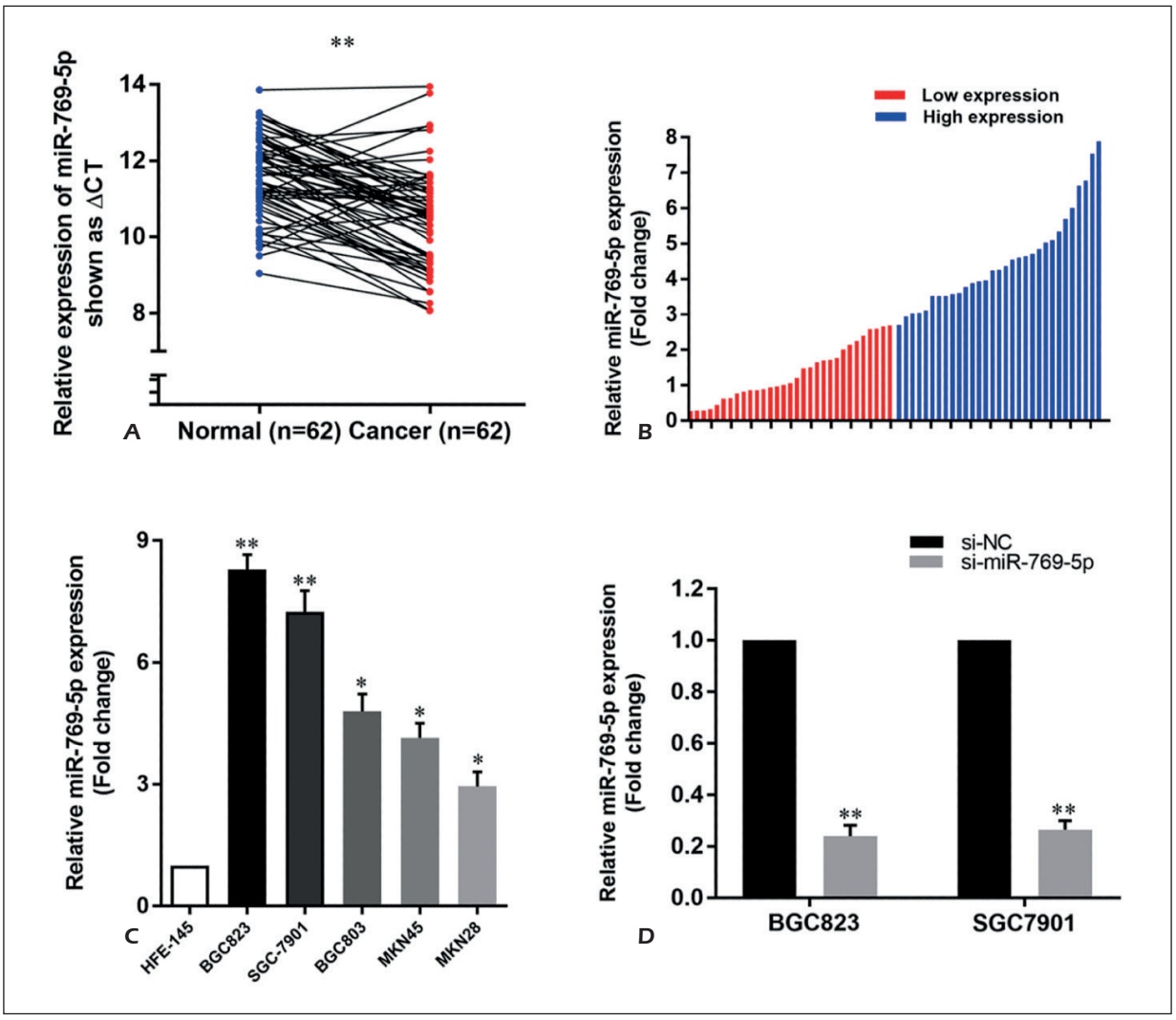

Figure 1. Upregulation of miR-769-5p expression in GC. **A**, The results of qRT-PCR indicate that in the 62 cases of GC tissues, the expression level of miR-769-5p is upregulated in 48 cases. **B**, The average relative expression level of miR-769-5p is taken as the cut-off point, and the high-expression group and low-expression group are set up. **C**, The results of qRT-PCR manifest that the expression level of miR-769-5p is upregulated in GC cells. **D**, The miR-769-5p inhibitor is transiently transfected into the GC cells, and the interference efficiency is detected by qRT-PCR.

miR-769-5p (Figure 2C and 2D). Then, the effect of miR-769-5pd on the apoptosis of GC cells was detected by flow cytometry, which suggested that compared with that in the control group, the apoptosis rate of GC cells in the experimental group was increased (Figure 2E and 2F).

Effect of MiR-769-5p on the Tumorigenic Ability of GC Cells In Vivo

The 4-week-old nude mice were selected as the objects of study. The cells of the experimental group and the control group were separately injected into the subcutaneous tissues of the nude mice, and the nude mice were observed and weighed every 3 days. After 20 days, all the nude mice were killed, and the transplanted tumor was taken out, photographed (Figure 3A), and weighed (Figure 3B). The results manifested that the tumorigenic ability of the GC cells was inhibited after interfering in the expression of miR-769-5p. Next, the relative expression level of miR-769-5p in the transplanted tumor was detected by qRT-PCR (Figure 3C). The downstream target genes of miR-769-5p were predicted online by TargetScan and verified by qRT-PCR (Figure 3D). The results of Western blotting revealed that the expression of RYBP was downregulated at the protein level after interference in the expression of miR-769-5p (Figure 3E).

Table I. Correlation between miR-769-5p expression and clinicopathological characteristics of gastric cancer patients (n = 62).

Characteristics	MiR-769-5p Low No. case (%)	MiR-769-5p High No. case (%)	*p*-value Chi-squared test
Age (years)			
>60	12	17	0.309
≤60	19	14	
Sex			
male	14	22	0.071
female	17	9	
Tumour size (cm)			
>4	18	14	0.446
≤4	13	17	
Differentiation			
well	3	2	0.326
moderate	11	5	
poor	12	21	
undifferentiated	5	3	
TNM staging			
I+II	20	8	0.005*
III+IV	11	23	
Lymph lode metastasis			
No	21	10	0.010*
Yes	10	21	
Tumor location			
gastric antrum	12	9	0.499
corpora ventriculi	12	16	
preventriculus	7	6	
Invasion degree			
T1	10	4	0.011*
T2	11	6	
T3	4	11	
T4	6	10	

*Overall p<0.05.

Discussion

GC is one of the common digestive system tumors, and according to the statistical data of the International Agency for Research on Cancer (IARC), the incidence and mortality of GC ranked the fifth and the third place, respectively, in the world in 2018[12]. In 2015, the incidence and mortality of GC in China ranked the second and third, respectively, among malignant tumors[13]. Recently, with the screening for GC and the control for related risk factors, the incidence and mortality of GC in China have declined, but the 5-year survival rate is still low[14]. The malignant proliferation of tumor is the main cause leading to the poor prognosis of GC. Therefore, it is of significance to elucidate the molecular mechanism of malignant proliferation of GC cells and improve the sensitivity of GC to radiotherapy and chemotherapy, thus ameliorating the prognosis and improving the survival rate.

MiRNAs play important roles in the occurrence, invasion, and metastasis of tumors *via* targeted regulation of specific oncogenes or anti-oncogenes. Of note, miRNA-137, regulated by transcription factor HMGA1, inhibits the invasion and metastasis of colorectal cancer through target gene FMNL2[15]. MiRNA-192/215 promotes the proliferation and invasion of GC cells by regulating multiple downstream target genes[16,17]. In prostate cancer, miRNA-223 inhibits cell invasion and migration *via* targeting on the proto oncogene integrin subunit alpha 3 (ITGA3)/ITGB1[18]. Our research group is the first to find that the expression of miR-769-5p in GC is upregulated and it functions as an oncogene.

RYBP, as an important member of PcG protein family, is highly correlated with biological processes, such as occurrence of malignant tumor and apoptosis of tumor cells[19]. Voruganti et al[20] reported the expression of RYBP in human NSCLC and its relationship with the survival of NSCLC patients and confirmed the molecular basis of antitumor effect of RYBP in NSCLC through *in vitro* and *in vivo* studies.

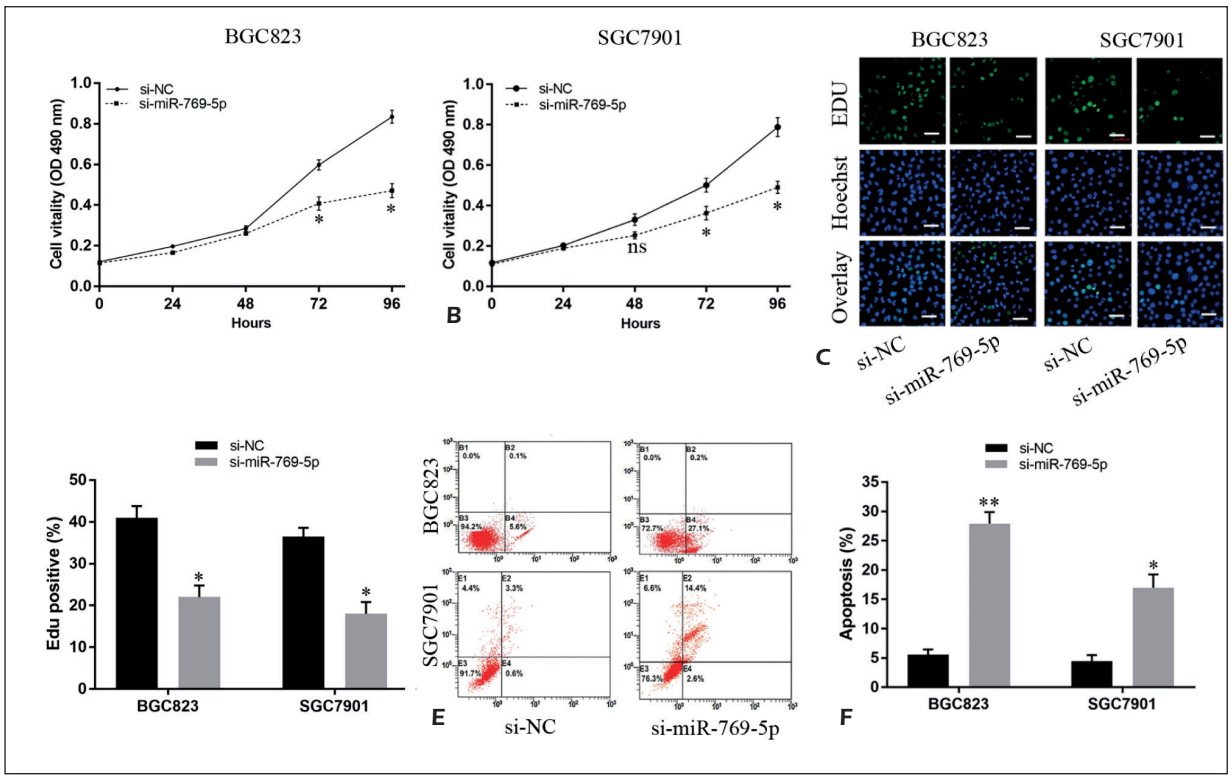

Figure 2. Inhibition of proliferation of GC cells after interference in the expression of miR-769-5p. **A**, and **B**, The results of MTT assay show that after interference in the expression of miR-769-5p, the proliferation ability of cells is decreased. **C**, and **D**, The results of EdU assay reveal that compared with that in control group, the proliferation ability of cells is inhibited in the miR-769-5p inhibitor group (magnification: 400×). **E**, and **F**, The results of flow cytometry indicate that the apoptosis rate of GC cells is increased after interference in the expression of miR-769-5p.

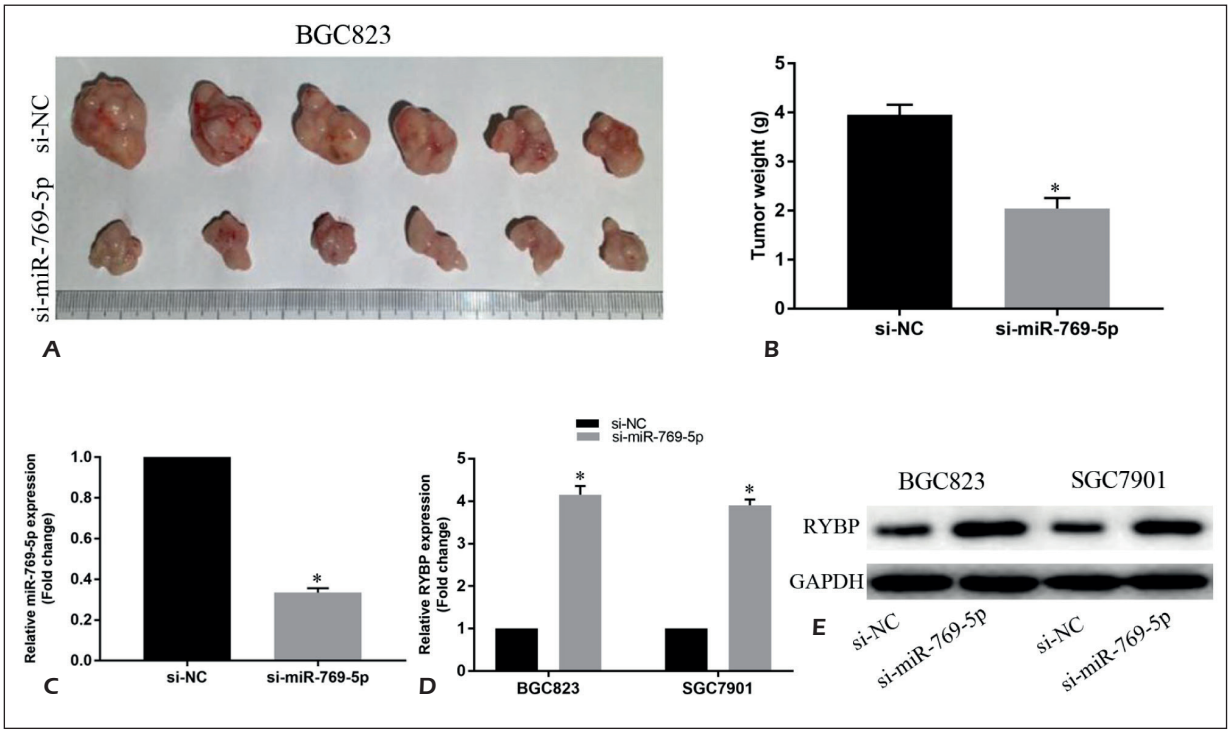

Figure 3. Effect of miR-769-5p on the tumorigenic ability of GC cells *in vivo*. **A**, Transplanted tumors of nude mice. **B**, Transplanted tumors of the experimental group and the control group are weighed. **C**, Relative expression level of miR-769-5p in the transplanted tumors determined via qRT-PCR. **D**, After interference in the expression of miR-769-5p, the changes in RYBP expression at the mRNA level determined via qRT-PCR. **E**, After interference in the expression of miR-769-5p, the changes in RYBP expression at the protein level detected via qRT-PCR.

Lando et al[21,22] have found that the survival rate of cervical cancer patients after radiotherapy is reduced, which is significantly related to the decrease of RYBP expression level, and the transcription and translation of RYBP are associated with the radiotherapy inhibition of cervical cancer patients. In this study, it was shown by *in vitro* experiments that miR-769-5p could promote the proliferation and inhibit the apoptosis of GC cells by targeted regulation of the expression of RYBP.

Conclusions

The results above showed that miR-769-5p is upregulated in GC and functions as an oncogene. Targeting for miR-769-5p/RYBP can provide a direction for clinical development of targeted drugs for the treatment of GC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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