Introduction

Gastric cancer (GC) is one of the five common types of tumors and ranks the third leading cause of cancer-related mortality worldwide. Despite new improvement to the standard of care therapy for GC patients, the prognosis of patients who were diagnosed in advanced stages remains poor, with a 5-year survival rate of less than 23%. A better understanding of the regulatory mechanisms underlying the development and progression of GC is critical for developing novel and effective therapeutic targets.

MicroRNAs (miRNAs) are noncoding RNAs of approximately 19-23 nucleotides that are transcribed by RNA polymerase II into pri-miRNAs. Growing evidence has suggested that miRNAs play an important role in many physiological and pathological processes such as cell growth and differentiation, proliferation and apoptosis. Recent studies indicated that aberrant expression of miRNAs can act as tumor suppressors or oncogenes, depending on the cellular function of their targets. For instance, Zhou et al. reported that miR-27a-3p promoted gastric cancer progression by directly targeting BTG2. Shan et al. found that miR-448 acted as a tumor suppressor regulating lung cancer cells growth and metastasis by targeting DCLK1. Bao et al. indicated that miR-194 suppressed proliferation of GC cell through targeting KDM5B. Previous reports have shown that miR-802 was involved in the carcinogenesis of some tumors. However, the expression and role of miR-802 in progression of GC has not been reported.

In the present work, we firstly detect the expression levels of miR-802 in GC tissues and cells. In addition, the biological of forced miR-802 expression in proliferation, migration, invasion and...
apoptosis of GC cells was analyzed. Furthermore, we identified RAB23 as a miR-802 target in GC.

**Patients and Methods**

**Patients**

Total 46 cases of GC tissues and the matched adjacent normal tissues used in the study were collected from 46 patients between 2014 and 2015 in our hospital. All specimens were obtained from patients through surgical resection and then histologically proven to be GC. All participants signed an informed consent for the use of their samples before recruitment. Written consent was received from all the patients and all the experiments have been approved by the Ethics Committee of our hospital.

**Cell Lines and Transfection**

Four GC cell lines (SGC-7901, MGC-803, HGC-27, BGC-823), and normal gastric epithelial cell line (GES-1) were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). GC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen (Carlsbad, CA, USA), supplemented with heat-inactivated 10% fetal bovine serum (HIFBS), Invitrogen (Carlsbad, CA, USA) in six-well plates. For transfection, miR-448 mimic and corresponding negative control (miR-NC) were synthesized by RiboBio (Guangzhou, China). Cell transfections were performed using Lipofectamine 2000 kit Invitrogen (Carlsbad, CA, USA) following the manufacturer’s instructions.

**Real-time Quantitative Reverse Transcription-PCR(qRT-PCR)**

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA from the GC cell lines and the patients’ samples according to the manufacturer’s instructions. cDNA was synthesized with the PrimeScript R Taq reagent Kit 128 (Promega, Madison, WI, USA). The expression levels of miR-802 and RAB23 mRNA were detected by qRT-PCR. All qRT-PCR were performed in triplicates on an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers for miR-802, RAB23 mRNA and the internal control GADPH gene were purchased from Ambion (Applied Biosystems, Foster City, CA, USA). The relative expression levels of RNA were quantified using $2^{-\Delta\Delta CT}$ method.

**Cell Proliferation Assay**

HGC-27cells were seeded into 96-well plates (6.0×10^4 cells per well). Cell viability was investigated by MTT assay (Beyotime Institute of Biotechnology, Shanghai, China). The absorbance at 450 nm was measured with a microplate reader. Each sample was assayed in triplicate.

**Colony Forming Assay**

Cells were seeded into 6-cm plates after the cells were transfected with miR-802 mimic or miR-NC and then the cells were digested with trypsin for preparation of single cell suspension. After visible colonies formed in a fresh six-well plate, Cell colonies were fixed with cold methanol, stained with 0.1% crystal violet and counted.

**Apoptosis Detection**

Apoptosis was assessed using Annexin V-FITC/PI double staining kit (Beyotime, Jiangsu, China). Cell samples were analyzed by flow cytometry (FACScan, BD Biosciences, Franklin Lakes, NJ, USA). Each sample was assayed in triplicate.

**In Vitro Cell Transwell Assays**

For the invasion assay, 2×10^5 cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; 8 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with 500 μl Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and the upper chamber was filled with cell suspension. Following 24 h of incubation at 37°C, cells that invaded the matrigel to the lower membrane were stained with 0.1% crystal violet. Filtered cells counted under a microscope (DP50; Olympus Corporation, Tokyo, Japan). The migration assay is the same with invasion assay. However, Matrigel was not used and the permeating time for cells was 12 hours.

**In vivo Tumorigenicity**

Xenograft model was used to detect the miR-802 on the tumorigenicity. The procedure was described by previous study.

**Luciferase Reporter Assay**

A normal and a mutated 3’-UTR of RASA1 were constructed using PCR, and were then inserted into the multiple cloning sites in the psiCHECK-2 vector. GC cells were seeded into a 24-well plate (1×10^4 per well), and were co-transfected with a luciferase reporter construct (200
miR-802 suppresses gastric cancer oncogenicity

ng) and miRNA-802 mimics (50 nM) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). After transfection for 48 h, luciferase activity was measured using the dual luciferase reporter assay system. All experiments were performed in triplicate.

**Western Blot Analysis**

The cells were lysed on ice with RIPA lysis buffer (Applygen Technologies Inc., Beijing, China) supplemented with protease inhibitors. Equal sample volumes of proteins (30 μg) were loaded for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. The polyvinylidene fluoride (PVDF) membranes were blocked using 5% non-fat dry milk in a tris buffered sodium with tween-20 (TBST-20) solution at room temperature for 1 h, and incubated with monoclonal rabbit anti-RAB23 (1:1,000; Haiding, Beijing, China) overnight at 4˚C. Detection was performed with peroxidase-conjugated secondary antibodies using an enhanced chemiluminescence system (Applygen Technologies Inc., Beijing, China). The blot was photographed by FluorChem imaging system (Alpha Innotech Corp., San Leandro, CA, USA). GADPH was used as a loading control.

**Statistical Analysis**

The data were expressed as mean ± SD (standard deviation, SD), and were analyzed using Student’s t-test between two groups in vitro and in vivo data. All data were analyzed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). p-values < 0.05 were considered to be statistically significant.

**Results**

**Expression of miR-802 in GC Tissues and Cells**

We firstly detected the expression levels of miR-802 in GC tissues and cells by RT-PCR. The results showed that the expression levels of miR-802 in GC tissues were lower than those in matched normal tissues (Figure 1A). Similarly, miR-26a was lower in all GC cells (SGC-7901, MGC-803, HGC-27, BGC-823) compared with normal gastric epithelial cell line (GES-1) (all p < 0.05, Figure 1B).

**miR-802 Overexpression Suppressed the GC Cell Proliferation, Colony Formation, and Induces Apoptosis**

To discover the effect of miR-802 on GC cell proliferation, miR-802 mimics were transfected into the human GC cell line HGC-27. The over-expression of miR-802 was confirmed by RT-PCR (Figure 2A). Then, colony formation assays and MTT were performed to detect the effect of miR-802 on GC cell proliferation. As shown in Figure 2B, we found that up-regulation of miR-802 in HGC-27 cells significantly inhibited cell proliferation (p < 0.05), compared with

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Figure 1. Up-regulation of expression of miR-802 in GC tissues and cell lines. (A) RT-PCT was used to detect expression of miR-802 in human GC tissues and normal gastric tissues. (B) RT-PCT was used to detect expression of miR-802 in GC cell lines (SGC-7901, MGC-803, HGC-27, BGC-823) and normal gastric epithelial cell line (GES-1). *p < 0.05 and **p < 0.01.
the NC transfection. Furthermore, similar findings were observed in colony formation assays (Figure 2C). Based on above results, we wondered whether miR-802 could influence HGC-27 apoptosis. Next, we performed flow cytometry assays and found that up-regulation of miR-802 inhibited apoptosis ($p < 0.05$, Figure 2D). These results suggested that miR-802 might be a tumor suppressor in GC.

**MiR-802 Suppressed GC Cell Metastasis in vitro**

In order to investigate whether the miR-802 played a critical role in GC cell metastasis, we performed transwell assays. As shown in Figure 3A, we observed that overexpression of miR-802 resulted in decreased migration of HGC-27 cells relative to controls. The similar findings were also observed in invasion assay (Figure 3B). These results indicated that miR-802 plays a suppressive regulator in GC cell metastasis. Then, we tried to explore the potential mechanism of miR-802 on regulating GC metastasis. Our attention focused on MMP-2 and -9, which play a critical role in the metastasis of GC. We detected the expression level of MMP-2 and -9 in HGC-27 cells by Western blotting, our data showed that the expression level of MMP-2 and -9 were decreased in GC cells transfected with miR-802 mimics (Figure 3C).

**Identification of RAB23 as a Target Gene of miR-802**

To explore how miR-802 affected malignant development of LSCC, we searched for potential regulatory targets of miR-802 by two prediction tools (miRanda and TargetScan). As shown in Figure 4A, Sp1 may be a direct target gene of miR-802, and revealed that RAB23 mRNA contained a miR-802-nucleotide seed match at position of the RAB23 3’-UTR. We constructed luciferase reporter vectors to identify whether RAB23 is a direct target of miR-802 by luciferase reporter as-
miR-802 suppresses gastric cancer oncogenicity

The results showed that overexpression of miR-802 mimics led to a reduction of luciferase activity when the reporter construct contained the RAB23 3'-UTR (Figure 4B). However, mutation of the miR-802 binding site from the RAB23 3'-UTR abolished this effect of miR-802 (Figure 4B). Moreover, miR-802 over-expression significantly down-regulated RAB23 mRNA and protein expression in HGC-27 cells (Figure 4C-D). Taken together, our results suggested that miR-802 modulates RAB23 expression by directly targeting its 3'-UTR.

**The Effect of miR-802 in GC Xenograft Growth**

The above experiment showed that miR-802 served as a tumor suppressor by inhibiting GC proliferation and metastasis. To confirm those results, we further examined the effects of miR-802 on tumor growth using a GC xenograft model. Mice were transplanted with HGC-27 cells overexpressing miR-802 or miR-NC. The results showed that overexpression of miR-802 significantly reduced xenograft tumor volume (Figure 5A) and tumor weight (Figure 5B). Thus, it was also demonstrated that miR-802 interference inhibited the tumor growth in vivo.

**Discussion**

MiR-802, a miRNA located on chromosome 21, has been reported to be involved in some tumors progression. For instance, Yuan et al. found that miR-802 exhibits its antitumor effect in breast cancer cells by regulating FoxM1. Wu et al. reported that elevated expression of miR-802 inhibited tongue squamous cell carcinoma cell viability and invasion through inhibiting MAP2K4 expression. Wang et al. indicated that overexpressed miR-802 significantly suppressed EMT, migration and invasion in prostate cancer cells by regulating Flot-2. All these studies suggested that miR-802 served as a tumor suppressor in various cancers. To our best knowledge, whether miR-802 played a similar role in GC remains unknown.

In the present work, we determined the expression of miR-802 in GC tissues and cell lines. As expected, our results showed that miR-802 expression was significantly down-regulated in GC tissues and cell lines, suggesting that miR-802 may exert a tumor-suppressive role in GC. To confirm our hypothesis, we used miR-802 mimic to up-regulate the expression levels of miR-802 in GC cells. Then, we performed in vitro and in vivo analysis to detect the role of miR-802 in GC.
cells proliferation, migration, invasion and apoptosis. Our data also confirmed our suggestion that over-expression of miR-802 suppresses GC progression. In order to explore how miR-802 regulated GC cell invasion and migration, we focused on MMP-3 and -9; it is now widely accepted that the overexpression of MMPs, especially MMP3, may accelerate the process of invasion\textsuperscript{18,19}. Our

![Figure 4](image_url)

**Figure 4.** miR-802 targets the RAB23 gene. (A) The predicted binding sites of miR-802 in the WT and MUT 3’-UTR of RAB23. (B) Luciferase reporter assay of HGC-27 cells transfected with the wide-type or mutant-type RAB23 reporter plasmid and miR-802 mimic or miR-NC. (C) RT-qPCR detected RAB23 mRNA expression in HGC-27 cells transfected with the miR-802 mimics. (D) Western blot analysis of RAB23 protein expression in HGC-27 cells transfected with the miR-802 mimics. *p < 0.05 and **p < 0.01.

![Figure 5](image_url)

**Figure 5.** miR-802 upregulation inhibited GC HGC-27 xenograft growth. (A) Growth curves of tumor volumes in xenografts of nude mice were established based on the tumor volume. (B) The weight of tumor tissues of different groups was measured. *p < 0.05 and **p < 0.01.
results showed that over-expression of miR-802 resulted in down-regulation of MMP3- and -9.

RAB23 is located in the pericentromeric region of chromosome 6. Increasing evidence suggests that RAB23 overexpression plays pivotal roles in malignancies. For example, Jiang et al. reported that overexpression of RAB23 resulted in increased cell growth and invasion by promoting NF-kB signaling. Jian et al. also reported that RAB23 promoted squamous cell carcinoma cell migration and invasion via integrin β1/Rac1 pathway. More importantly, Hou et al. found that RAB23 served as a tumor promoter in GC. On the other hand, previous studies showed that RAB23 is a target of several tumors. Importantly, Bin et al. confirmed that over-expression of miR-367 suppressed the invasion and metastasis of gastric cancer by directly targeting RAB23. All these evidence revealed the important value of miRNAs and RAB23. Thus, we searched the miRanda and TargetScan, and identified that RAB23 may be a target of miR-802. Furthermore, we demonstrated that RAB23 was a direct target of miR-802 in GC cells by luciferase reporter assay and Western blot analyses. We observed that overexpression of miR-802 repressed RAB23 expression at both mRNA and protein levels. Taken together, our results revealed an important role of miR-802 in regulating tumorigenesis possibly through regulating RAB23.

Conclusions

We demonstrated that miR-802 was frequently downregulated in GC tissues and serves as a tumor suppressor by directly targeting RAB23. Therefore, this research may provide a therapeutic strategy for patients with GC.

Conflict of interest

The authors declare no conflicts of interest.

References


