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Original Article

MiRNA-491-5p inhibits cell proliferation, invasion and migration via targeting JMJD2B and serves as a potential biomarker in gastric cancer

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Abstract: Our previous work discovered that the histone demethylase JMJD2B (KDM4B) plays oncogenic roles in gastric carcinogenesis, but the regulatory mechanism of JMJD2B in gastric cancer has not been well defined. It has been revealed that microRNAs function as gene regulators by binding to the 3'UTR of mRNAs to inhibit gene expression. In this study, we found that miR-491-5p suppressed cell proliferation, invasion and migration by directly targeting the JMJD2B 3'UTR in gastric cancer. Moreover, miR-491-5p was decreased in GC tissues compared with adjacent normal tissues, and JMJD2B had the inverse expression pattern. In contrast to healthy individuals, GC patients had lower miR-491-5p expression in serum (P<0.0001). Our data indicate that miR-491-5p serves as a tumor suppressor in GC and might be a novel potential biomarker for the detection of gastric cancer.

Keywords: Gastric cancer, miR-491-5p, JMJD2B

Introduction

Gastric cancer (GC) ranks as one of the most common malignancies in the world, with 951,000 new cases each year, accounting for 6.8% of all cancers. Furthermore, GC-related fatalities account for 8.8% of total cancer deaths, amounting to 723,000 deaths, which made it the third leading cause of cancer-related death (in both males and females) worldwide in 2012. Its incidence is higher in Asia than in Europe and South America [1, 2]. The occurrence and progression of gastric cancer are closely related to *Helicobacter pylori* (*H. pylori*) infection and dietary, lifestyle and metabolic factors [3]. Due to a lack of favorable early diagnostic biomarkers, the five-year survival rate of GC patients is less than 30% [4]. Early stage diagnosis of GC can avoid this deterioration and improve the survival rates of GC patients. To ameliorate the prognosis of GC patients, more effective early diagnostic biomarkers are needed [5].

MicroRNAs (miRNAs) are short, non-coding RNAs with a length of 18-22 nucleotides causing mRNA cleavage and subsequent degradation by binding to the complementary 3' untranslated region (UTR) of the mRNA. There is regulation between miRNA and mRNA at multiple levels that affects epigenetics, RNA stability and translation [6]. miRNAs play significant roles in cell proliferation, metastasis, differentiation, apoptosis and development. It has been demonstrated that miRNAs are aberrantly expressed in cancer tissues compared with normal tissues. In the past decades, sufficient evidence has been obtained to indicate that miRNAs can serve as biomarkers for diagnosis and therapeutics in cancer [7]. Our former research has shown that the histone demethylase JMJD2B (also named KDM4B) is overexpressed in GC tissues compared to adjacent normal tissues and that high levels of JMJD2B induce GC cell proliferation, survival, invasion and metastasis [8-10]. Nevertheless, the mechanism by which JMJD2B is regulated in GC

progression is unclear. Considering the regulation of gene expression by miRNA, we explored whether miRNA regulates JMJD2B expression in GC development. Therefore, we searched for potential miRNAs in four target databases: miRDB, miRanda/microRNA.org, Microcosm Targets and DIANA-microT v3.0. In the search results, miR-491-5p was coincidentally predicted in all databases. It has been indicated that miR-491-5p is a metastasis suppressor through the down-regulation of GIT1 and a significant biomarker for OSCC (oral squamous cell carcinoma) [11]. miR-491-5p suppresses NSCLC cell proliferation, migration, and invasion and accelerates apoptosis by targeting IGF-2BP1 [12]. Similarly, miR-491-5p prohibits cervical cancer cell proliferation by targeting hTERT [13]. Moreover, miR-491-5p induces pancreatic cancer cell apoptosis by targeting both TP53 and Bcl-XL through a mitochondria-mediated pathway [14]. Up to now, there has been no report on the role of miR-491-5p in gastric tumorigenesis.

It has been revealed that peripheral circulating miRNAs, such as miR-223, miR-21, miR-218 and miR-25, among others, are closely associated with the tumorigenesis or metastasis of GC and may serve as candidate biomarkers for GC [15-17]. Since serum and/or plasma are comparatively easy to obtain and miRNAs are relatively stable in peripheral circulating body fluids, they could be potential diagnostic biomarkers. The function and expression of miRNAs differ in various diseases, such as lung cancer, prostate cancer, breast cancer, ovarian cancer, liver cancer, myocardial diseases, and neurodegenerative diseases [18]. In our experiments, we found that miR-491-5p inhibited the expression of JMJD2B at the post-transcriptional level, and we further investigated the biological role of miR-491-5p in GC cells. miR-491-5p was down-regulated in the serum of GC patients compared with that of healthy people, which indicated the possibility of its application as a diagnostic biomarker of GC.

Materials and methods

Cell lines

The human gastric cancer cell lines BGC823, MGC803, SGC7901, and HGC27 and the immortalized epithelial cell line GES-1 were obtained from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, PR China). GES-1, BGC823, and SGC7901 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), and MGC803 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA). All the media above were supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). All cells were incubated at 37°C in 5% CO_2.

Tissue and serum samples

All tissue specimens (GC and adjacent normal tissues) were gathered from patients of the General Surgery Department (QiLu Hospital, Shandong University). All tissue samples were incubated in RNAlater RNA Stabilization Reagent (QIAGEN) at 4°C overnight and then stored at -80°C for later inspection.

Blood specimens were acquired from 52 healthy people from the Health Examination Center and 84 GC patients at the Department of Clinical Medical Laboratory with histologically confirmed GC who had not received radiotherapy, chemotherapy or surgery (QiLu Hospital, Shandong University). The serum was isolated from 5 ml of venous blood within 2 hours in a two-stage centrifugation: 1) to separate serum from whole blood: 3000 rpm for 10 min at 4°C; 2) to obtain pure serum from cellular debris: 16000 *g* for 10 min at 4°C. Finally, the upper serum was collected in 0.5 ml RNAase-free EP tubes and stored at -80°C before use. Every time the samples are thawed, the sera should be centrifuged at 16000 *g* for 10 min at 4°C before use.

The research was approved by the Ethics Committee of the Cheeloo College of Medicine, Shandong University.

RNA extraction and quantitative real-time PCR (QRT-PCR)

TRIzol reagent (Invitrogen, USA) was used for purifying RNA from tissue samples and cells. The MiRNeasy Mini Kit (Qiagen, Valencia, CA) was used for purifying RNA from serum samples according to the manufacturer's instructions. The has-miR-491-5p primer was synthesized by RiBoBio (Guangzhou, China). QRT-PCR (quantitative real-time PCR) was perfor-

med with SYBR Premix Ex Taq (TaKaRa) using the Bio-Rad CFX96TM Real-Time PCR System (Bio-Rad). The miRNA expression in tissue samples and cells was normalized using the endogenous U6 snRNA (RiBoBio, Guangzhou, China). Spike-in RNA (cel-miR-39) was used as a serum control. The QRT-PCR reaction program was as follows: 95°C for 20 s; 95°C for 10 s and 60°C for 20 s for 40 cycles; and 70°C for 10 s. The data were obtained using the formula $2^{-\Delta\Delta Ct}$.

MiRNA transfection

When MGC803 and SGC7901 cells reached 50%~70% confluence, 5 nmol of miR-491-5p mimics and negative control (NC) (RiBoBio, Guangzhou, China) were transfected by Lipofectamine™ 2000 (Invitrogen, USA). Total RNA and protein were extracted after 48 h/72 h and then subjected to QRT-PCR and western blot analysis.

Colony formation assay, cell invasion assay and scratch wound healing assay

1) MGC803 and SGC7901 cells were transfected with 5 nmol mimics and mimic controls for 48 h. Meanwhile, MGC803 and SGC7901 cells were co-transfected with 500 ng of pcDNA-JMJD2B and 5 nmol miR-491-5p mimics. Then, the cells were seeded into 6-well plates (300 cells/well) and cultured for 2 weeks. Colonies were fixed with methanol and then dyed with crystal violet. Colonies with more than 50 cells were counted.

2) Cell invasion was tested using Transwell insert chambers (Costar, Cambridge, MA, USA) with Matrigel (BD Biosciences, Bedford, MA). Twenty-four hours after transfection, 1×10^5 MGC803 and SGC7901 cells were seeded into the upper chambers with serum-free medium. Then, RPMI 1640 or DMEM containing 20% FBS was added to the lower chambers. After incubation for 16 h (MGC803) or 18 h (SGC7901), cells in the upper chambers were removed with a cotton swab. The cells that invaded to the basement membrane of the insert were fixed in 100% methanol for 10 min, stained with 0.5% crystal violet for 20 min, rinsed in PBS, and subjected to microscopic inspection.

3) Wound-healing tests were used to evaluate GC cell motility. A total of 1×10^6 cells/well were seeded in 6 well plates and cultured overnight, followed by transfection with miR-491-5p mimics or NC or co-transfection with mimics and pcDNA-JMJD2B. Eight hours later, the confluent cell monolayer was scratched with a sterilized pipette tip, and the plates were washed twice with PBS before adding fresh serum-free medium and being cultured for 24 h. Photo images of the plates were taken under a microscope.

Western blot

MGC803 and SGC7901 cells were transfected with miR-491-5p mimics or control for 72 h. The transfected cells were lysed with RIPA lysis buffer containing PMSF (Solarbio) for 10 min before measuring the concentration. The protein concentration was calculated with a BCA protein assay kit (Beyotime). Equal amounts of protein (80 μg) were separated by 10% SDS-PAGE and then transferred to a PVDF membrane, which was blocked in 5% non-fat milk. Next, the membrane was incubated with primary antibodies against JMJD2B (Bethyl Laboratories, Montgomery, USA) or β-actin (Santa Cruz Biotechnology, Santa Cruz, USA) for 12 h at 4°C. The membranes were washed 3 times by TBS-T and incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h (Santa Cruz Biotechnology, Santa Cruz, USA). The protein band was exposed with the enhanced chemiluminescence method (ECL, Millipore) by a multifunctional protein imaging system (Cell biosciences, AlphaInnotech, USA).

Luciferase activity assay

Three binding sites were predicted in the JMJD2B 3'UTR through the public database Targetscan 6.0. The wild-type vector containing 3 binding sites (pmiR-RB-REPORT_h-KDM4B wt) and four mutant vectors of the JMJD2B 3'UTR were constructed by RiBoBio (Guangzhou, China). The mutant vectors included all three mutation sites (pmiR-RB-REPORT_h-KDM4B all mut) and 3 single mutations (pmiR-RB-REPORT_h-KDM4B mut1, mut2, or mut3). The mutant sequence of the JMJD2B 3'UTR vector was designed based on the wild-type JMJD2B

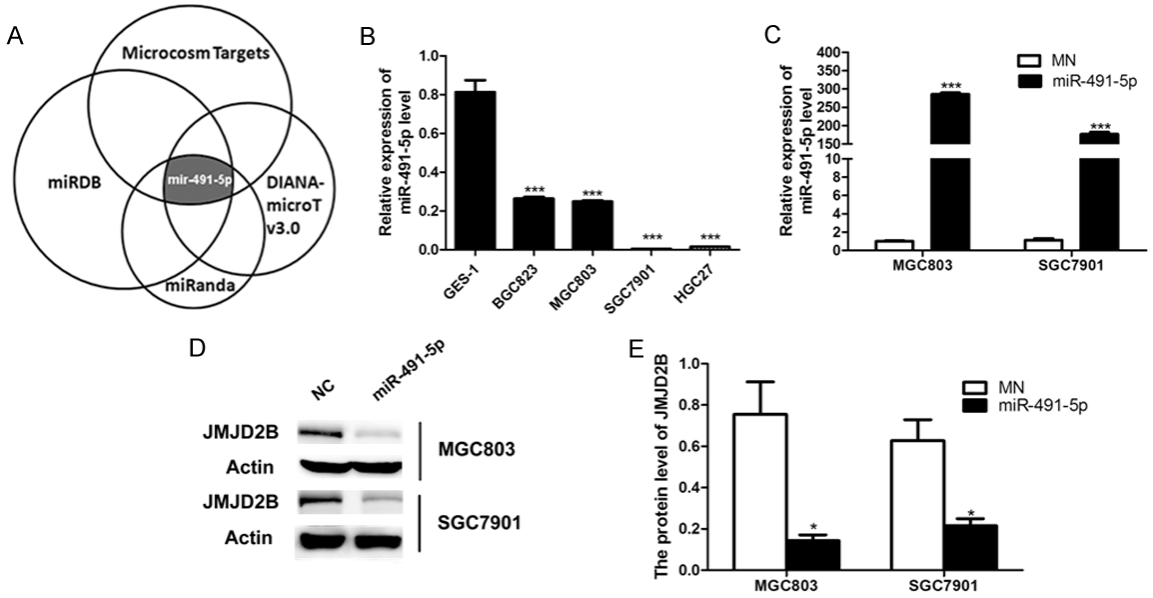

Figure 1. MiR-491-5p down-regulates JMJD2B in GC cell lines. A. MiR-491-5p was predicted to be the candidate miRNA targeting JMJD2B by four databases. B. MiR-491-5p expression in GC cell lines and the immortalized human gastric epithelial cell line GES-1 was assessed by QRT-PCR. C. Transfection of miR-491-5p mimics significantly enhanced its expression in MGC803 and SGC7901 cells. D, E. MiR-491-5p over-expression decreased the protein level of JMJD2B which was detected by western blot, and the bands were quantified. (*P<0.05; ***P<0.001).

3'UTR (human) and synthesized by PCR. The three targeting seed sequences are TCCCCAC (1244-1250), TCCCCAC (1928-1934) and TCCCCAC (1995-2001). The TCCCCAC sequence was mutated to AGGGGTC.

MGC803 cells were seeded in 24-well plates (1×10^5 cells/well) in triplicate. Twenty-four hours later, 5 nmol miR-491-5p mimics or NC mimics and 400 μg of the above pmiR-RB-REPORT_h-KDM4B vectors (wt, all mut, mut1, mut2, or mut3) were co-transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luciferase activity was measured at 36 h after transfection by the Dual-Glo luciferase assay system (Promega, USA). HRluc was the fluorescence reporter, and hluc was the internal reference.

Statistical analysis

All the experiments were done at least 3 independent times. Data was expressed as the mean ± SD. The differences were considered statistically significant at P<0.05. Student's t-test was performed for comparisons between two groups. All the analyses were two-tailed and performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and the ROC curves were generated by IBM SPSS Statistics 23.

Results

miR-491-5p down-regulates JMJD2B in GC cell lines

First, we searched four public databases to look for potential miRNAs targeting the JMJD2B 3'-UTR. We found that miR-491-5p showed up in all prediction databases (**Figure 1A**). To test the expression of miR-491-5p in different GC cell lines, QRT-PCR was done and revealed that miR-491-5p had lower expression in the GC cell lines BGC823, MGC803, SGC7901 and HGC27 than in GES-1 cells (immortalized epithelial cell line) (**Figure 1B**). Then, we transfected miR-491-5p mimics into MGC803 and SGC7901 cells and QRT-PCR verified the transfection efficiency (**Figure 1C**). Western blot was performed to ascertain whether miR-491-5p regulates JMJD2B expression. After transfection of 5 nmol miR-491-5p mimics and NC in MGC803 and SGC7901 cells for 36 h, the protein level of JMJD2B decreased significantly, as quantified by Photoshop (**Figure 1D**, **1E**).

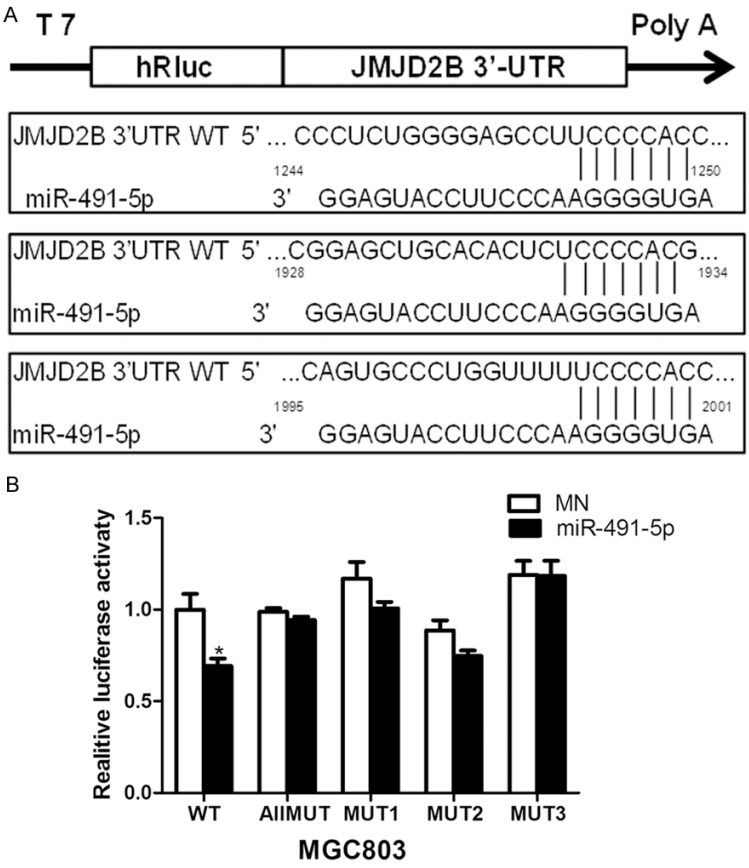

Figure 2. JMJD2B is the direct target of miR-491-5p. A. Three binding sites of miR-491-5p in the JMJD2B 3'UTR were predicted by Targetscan 6.0. B. Luciferase activity of the wild-type JMJD2B 3'UTR reporter vectors was decreased by overexpression of miR-491-5p in MGC803 cells, while it was not significantly decreased with all binding sites mutation vector and the individual mutation vectors compared with the negative control. (*P<0.05).

Consequently, miR-491-5p negatively regulated JMJD2B expression in the GC cell lines.

JMJD2B is the direct target of miR-491-5p

To study the mechanism by which miR-491-5p reduces the JMJD2B level in GC cell lines, we used TargetScan to predict the potential binding sites of miR-491-5p in the JMJD2B 3'UTR and found 3 binding regions (**Figure 2A**). Accordingly, we constructed 5 luciferase reporter vectors, including a wild-type vector containing 3 binding sites, an all-mutant vector in which 3 binding sites were mutated, and 3 single mutant vectors with each site mutated individually. Our results showed that the luciferase activity was significantly reduced after co-transfection of pmiR-RB-REPORT_h-KDM4B wt and miR-491-5p mimics compared with negative control (P<0.05). However, the reduction in luciferase activity was abolished by transfecting the mutant vector containing all 3 mutations. These data indicated that miR-491-5p could directly target the 3'UTR of JMJD2B mRNA in GC cells. To further confirm which binding site plays a vital role in this process, we transfected cells with the 3 single mutation vectors individually. Our data showed that the luciferase activity was not significantly decreased with the individual mutation vectors compared with the negative control (**Figure 2B**). Taken together, JMJD2B is the direct target of miR-491-5p, and the three binding sites are essential for the regulation of JMJD2B expression.

miR-491-5p inhibits cell proliferation, invasion and migration

To determine the biological function of miR-491-5p in human GC cell lines, a series of experiments were carried out. As miR-491-5p had low expression in more malignant GC cell lines, we transfected miR-491-5p mimics or the NC mimic into MGC803 and SGC7901 cells in 6-well plates to test its effect in a clonogenesis assay. The colony formation assay showed that the colony numbers were significantly decreased by ectopic expression of miR-491-5p in contrast to the NC while the reduction in colony number was reversed when cells were co-transfected with miR-491-5p mimics and pcDNA-JMJD2B. Colony numbers were counted and analyzed by Student's t-test (**Figure 3A**). Next, we determined the role of miR-491-5p in GC cell invasion and migration. The transwell test showed that miR-491-5p suppressed the invasion of MGC803 and SGC7901 cells (**Figure 3B**). Wound healing experiments revealed that over-expression of miR-491-5p hindered the motility of MGC803 and SGC7901 ce-

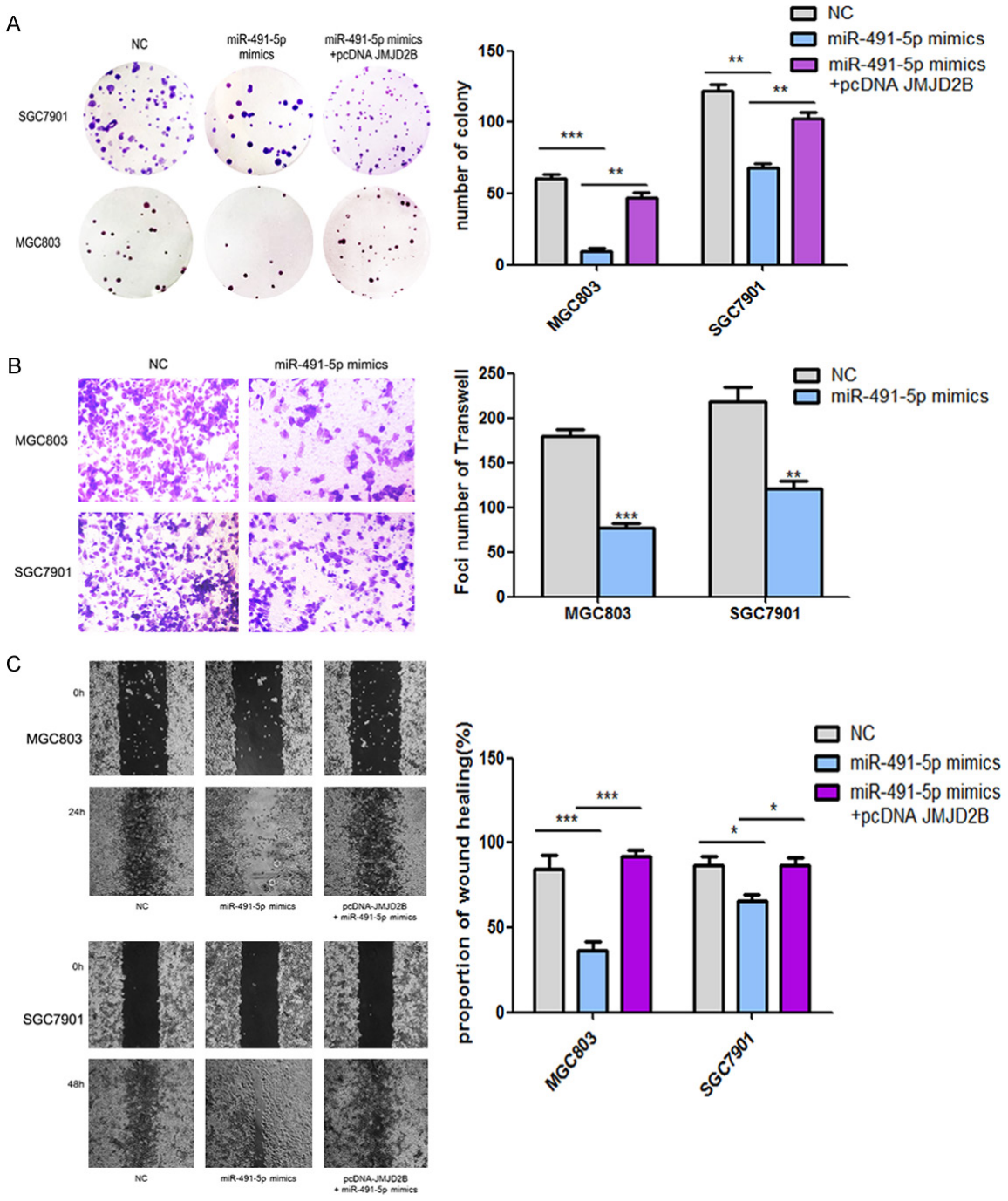

Figure 3. MiR-491-5p inhibits cell proliferation, invasion and migration. A. Colony formation assay of MGC803 and SGC7901 cells showed that the colony numbers were significantly decreased by ectopic expression of miR-491-5p in contrast to the NC, while the reduction in colony numbers was reversed when cells were co-transfected with miR-491-5p mimics and pcDNA-JMJD2B. B. Transwell assays illustrated that the invasion ability of MGC803 and SGC7901 cells was inhibited by miR-491-5p. Representative image are shown at 100×, and quantitative analysis of transwell assays was performed. C. Wound-healing assays showed the migration of MGC803 and SGC7901 cells after transfection of miR-491-5p mimics was hindered and this repression of motility was reversed by the up-regulation of JMJD2B (*P<0.05, **P<0.01, ***P<0.001).

lls, and this repression of motility was reversed by the up-regulation of JMJD2B (**Figure 3C**). To summarize, the above findings suggested that aberrant expression of miR-491-5p suppressed GC cell proliferation, migration and invasion by inhibiting JMJD2B expression in vitro.

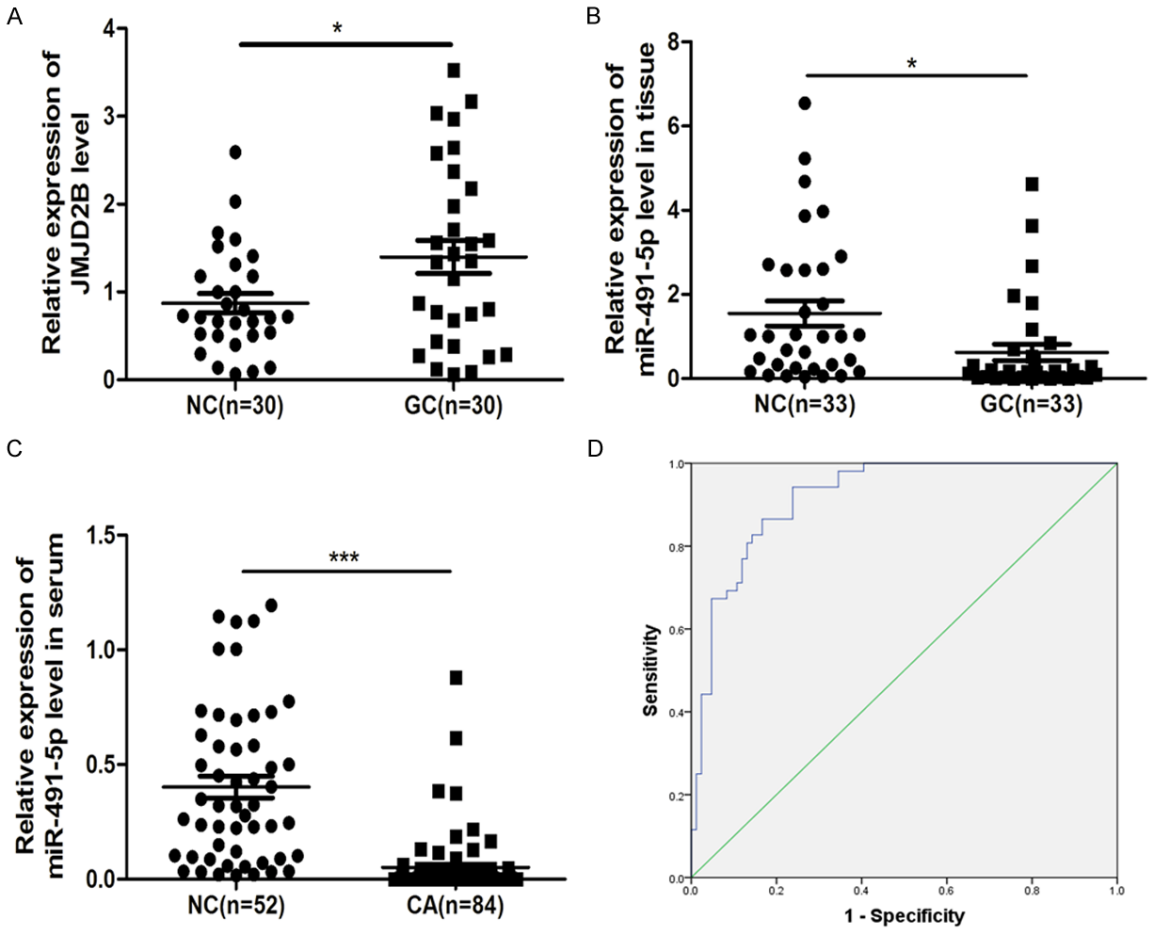

Figure 4. miR-491-5p is down-regulated in GC tissues and GC patient serum samples compared with normal controls. A. JMJD2B was over-expressed in GC specimens compared to adjacent non-tumor specimens. B. The median expression of miR-491-5p was significantly decreased in GC samples compared with normal controls. C. Circulating miR-491-5p levels were significantly lower in the serum of GC patients compared with that of healthy controls. D. ROC curves demonstrated a sensitivity of 94.2% and a specificity of 76.2%, with an AUC of 0.919 (95% CI, 0.874-0.963). (*P<0.05, ***P<0.0001).

miR-491-5p is down-regulated in GC tissues and GC patients' serum compared with normal control

To analyze whether the above findings have any clinical relevance, we determined the RNA level of JMJD2B in 30 GC patients collected from the Department of General Surgery and found that JMJD2B expression was increased in GC tissues compared with adjacent normal tissues (**Figure 4A**). Next, we used QRT-PCR to examine the expression of miR-491-5p in 33 GC tissues. The results demonstrated that miR-491-5p was reduced in GC tissues compared with adjacent normal tissues (**Figure 4B**). Based on the above findings, we assumed miR-491-5p might be a candidate biomarker for the diagnosis of GC. Then, we examined the levels of miR-491-5p in the serum by QRT-PCR. We recruited 52 healthy people and 84 GC patients without any treatment before the test. The results indicated that the level of miR-491-5p was significantly decreased in the serum of GC patients compared with healthy individuals (**Figure 4C**). The ROC curve analysis showed a sensitivity of 94.2% and a specificity of 76.2%, with an AUC of 0.919 (95% CI= 0.874-0.963) (**Figure 4D**), which indicated that miR-491-5p might serve as a diagnostic biomarker for GC detection.

Discussion

With a lack of typical symptoms and specific biomarkers in early stages, gastric cancer is often diagnosed at advanced stages with a high

fatality rate. Bryan A. Chan [19] stated that to further advance GC clinical practice, there is an urgent need for consensus in the molecular classification system to provide better predictive and prognostic biomarkers.

Plenty of studies have reported the mechanisms by which miRNAs act as cancer suppressors or oncogenes [20-22]. miRNAs can be up-regulated or down-regulated in GC tissues compared with adjacent normal tissues. It was elucidated that miRNAs might be novel molecular markers and treatment targets for the metastasis and prognosis of gastric cancer [23]. In our previous study, we investigated the mechanism of an important histone demethylase JMJD2B in promoting the progression of gastric cancer, while the regulation of JMJD2B in gastric cancer was not well defined. Considering the regulation of gene expression by miRNAs, we searched four public databases described above for candidate microRNAs that target JMJD2B. miR-491-5p was found in all the databases, which aroused our interest. In this paper, we investigated the expression of miR-491-5p in gastric cancer and its regulation of JMJD2B expression.

It was discovered that miR-491-5p is down-regulated in some human malignancies, such as OSCC, lung cancer, cervical cancer and pancreatic cancer [24-26]. The expression of miR-491-5p in GC has not been reported. In our study, we first demonstrated that miR-491-5p expression was suppressed in GC tissues. Its expression in GC cell lines, including BGC823, MGC803, SGC7901 and HGC27 cells, was generally lower than that in immortalized human gastric epithelial cells, GES-1. All the above results indicated that miR-491-5p might be a tumor suppressor in gastric carcinogenesis. To prove the regulation of JMJD2B expression by miR-491-5p, we transfected miR-491-5p mimics in GC cells to enhance its level and found that JMJD2B was inhibited by miR-491-5p at the post-transcription level. Recently, it has been reported that miR-491-5p suppresses JMJD2B in ERα-positive breast cancer tissues and cell lines. They found one binding site for miR-491-5p in the JMJD2B 3'UTR at 1995 to 2001, which was also validated in our experiment [27]. In addition to the previously confirmed site at 1995 to 2001 in the 3'UTR of JMJD2B, we found another two potential binding sites from 1244 to 1250 and from 1928 to 1934. To investigate whether these two binding sites are essential for the regulation of JMJD2B by miR-491-5p, we constructed vectors with all the sites mutated and three single mutated vectors. The luciferase activity assay showed that JMJD2B was a direct target of miR-491-5p and that all three binding sites played vital roles in the regulation.

The biological function of miR-491-5p was also studied in GC cells. miR-491-5p over-expression noticeably prohibited the clonogenic, invasion and migration capabilities in GC cells. Consistent with the studies in NSCLC and breast cancer, miR-491-5p also acted as a tumor suppressor in GC by inhibiting JMJD2B expression.

Currently, serum biomarkers, including carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), are used clinically for GC diagnosis with poor sensitivity and specificity. Therefore, the precise diagnosis of GC is still based on invasive endoscopic examination. Several studies have illustrated that serum miRNAs might be promising non-invasive biomarkers for the early diagnosis of GC and for disease progression surveillance [28, 29]. We detected miR-491-5p by QRT-PCR and found that the expression of miR-491-5p was down-regulated in the serum of GC patients, which was in accordance with its expression in GC tissues. ROC curve analysis showed a sensitivity of 94.2% and a specificity of 76.2%, with an AUC of 0.919 (95% CI=0.874-0.963), which indicated that miR-491-5p might serve as a potential biomarker in GC diagnosis. A large cohort may be required for further validation.

In our recently published paper, we investigated the role of *H. pylori* infection on JMJD2B up-regulation in gastric carcinogenesis and its underlying mechanism [8-10]. *H. pylori* infection plays an important carcinogenetic role in GC and lymphoma of mucosa-associated lymphoid tissue (MALT). Generally, *H. pylori* colonizes approximately one-half of the world's population, with a variable prevalence in different countries. Plenty of studies have discovered that *H. pylori* infection can affect miRNA expression in host cells, which is involved in the pathological development of gastric cancer [30]. miR-141 was significantly down-regulated in *H. pylori*-positive tissues [31]. Ectopic expression of let-7c accompanies the *H. pylori*-

related carcinogenic process and could be a promising noninvasive biomarker in GC [32]. Ectopic expression of miR-101 is significantly decreased in *H. pylori*-positive tissues and cells. Over-expression of miR-101 dramatically suppresses GC cell proliferation and colony formation by targeting SOCS2 [33]. To further explore whether miR-491-5p is involved in the modulation of JMJD2B by *H. pylori* infection, we detected the expression of miR-491-5p and JMJD2B in MGC803 and BGC823 GC cells after *H. pylori* infection. The results showed that miR-491-5p decreased over time upon *H. pylori* infection (data not shown). Future studies must focus on the regulation of miR-491-5p by *H. pylori* and its biological activity in *H. pylori*-induced gastric pathogenesis.

In conclusion, our work demonstrated that miR-491-5p inhibited gastric carcinogenesis by suppressing JMJD2B expression and that miR-491-5p was down-regulated in gastric tissues and serum of GC patients. miR-491-5p is a potential biomarker for GC diagnosis and may suggest a possible therapeutic strategy for the intervention of gastric cancer.

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Disclosure of conflict of interest

None.

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