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miR-194 inhibits gastric cancer cell proliferation and tumorigenesis by targeting KDM5B

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Abstract. – OBJECTIVE: MicroRNAs play critical roles in regulating gene expression and various cellular processes in human cancer malignant progression. The aim of the present study was to examine the expression pattern of miR-194 in gastric cancer (GC) and its biological role in tumor progression.

MATERIALS AND METHODS: Using quantitative RT-PCR, we detected miR-194 expression in GC cell lines and primary tumor tissues. The proliferation, migration, and invasion assays were performed to investigate the effect of miR-194 on the GC cells. The target of miR-194 was predicted by TargetScan and confirmed by luciferase reporter assay. KDM5B expression was detected by Western blot.

RESULTS: miR-194 was significantly down-regulated in GC tissues and cell lines. Over-expression of miRNA-194 could inhibit GC cell proliferation, migration, and invasion *in vitro*. Also, miR-194 inhibited tumor growth and progression in vivo. Dual luciferase-based reporter assay indicated direct regulation of KDM5B by miR-194.

CONCLUSIONS: Our findings suggested that miR-194 directly targeted KDM5B and thereby acted as a tumor promoter in GC progression.

Key Words:
miR-194; Gastric cancer; KDM5B.

Introduction

Gastric cancer (GC) is one of the most malignant tumors among men and women in the world. In 2008, it was reported that about 738,000 people died from gastric cancer[1,2]. Importantly, Chinese GC patients have a worse outcome than GC patients in the USA[3]. Although great efforts have been adapted to improve early diagnosis rate and provide synthesized and advanced treatments for patients with GC, the prognosis of GC patients remains poor as they often experience post-treatment cancer relapses and metastasis[4,5]. Therefore, a better understanding of the molecular mechanisms that involved in gastric cancer metastasis is essential for the development of novel therapeutic strategies.

MicroRNAs (miRNAs), a class of small non-coding RNA molecules, result in translational repression or degradation and contribute to the inhibition of gene expression[6,7]. Currently, many miRNAs has been confirmed to play important roles in carcinogenesis and cancer metastasis[8,9]. Chen et al[10] reported that miR-194 was significantly downregulated in GC tissues and tissues. Furthermore, they confirmed that upregulation of miR-194 could inhibit proliferation, migration, and invasion of GC cells, possibly by targeting RBX1. However, as a single miR may have numerous targets, whether other targets of miR-194 may also be involved in the development of GC remains to be elucidated.

KDM5B is a jmjc domain-containing histone demethylase, which belongs to KDM5 family. Lu et al[11] firstly reported that KDM5B expression was up-regulated in human breast-cancer cell lines and primary breast carcinomas. Recently, more and more studies reported that KDM5B played an important role in tumor progression. Enkhbaatar et al[12] reported that ectopic expression of KDM5B promotes epithelial-mesenchymal transition (EMT) of cancer cells. Nishida et al[13] reported that KDM5B knockdown resulted in the inhibition of EC cell growth, sphere formation and invasion ability. For gastric cancer, Wang et al[14] showed that KDM5B expression was significantly up-regulated in gastric cancer and over-expression of KDM5B could promote cell growth and metastasis in vitro. Those finding revealed that KDM5B might be a tumor promoter in GC. However, the regulatory mechanism of KDM5B in GC cells, as well as its association with miR-194, remains to be fully elucidated.

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In the present study, we provided evidence that miR-194 played a crucial role in the regulation of proliferation and invasion of GC cells. We also identified a key target of miR-194 that is involved in the GC progression.

Patients and Methods

Patients and Samples

Twenty-five paired of GC tissues and matched adjacent normal liver tissues were obtained from Cangzhou central hospital, these cases were histologically confirmed. The samples were obtained at the time of surgery and were immediately snap-frozen in liquid until use. The project was approved by the ethics committee of Cangzhou Central Hospital.

Cell Lines and Culture

Three GC cell lines (SGC7901, MGC-803, NCI-N87), and a normal gastric epithelium cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmid Transfection

Cells were transfected with the aforementioned vectors using Lipofectamine® 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The miR-194 overexpression vector (GenePharma, Shanghai, China) and its control mimic (GenePharma, Shanghai, China) were used at a final concentration of 500 ng/µl. After transfection, the cells were collected for future examination and the effects of miR-194 transfection were determined by quantitative real-time polymerase chain reaction (qRT-PCR) at 24 hours post-transfection.

Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from clinical tissues and cell lines using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The reverse-transcription reactions were carried out using a MiraMasTM Kit (MBI Fermentas, Burlington, ON, Canada), which contains poly (A) polymerase used for polyadenylation of miRNA. Real-time polymerase chain reaction (RT-PCR) was performed using an ABI 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each sample was examined in triplicate, and the relative expression level of miR-194 was normalized to that of GAPDH by the $2^{-\Delta\Delta CT}$ cycle threshold method. Primer sequences are as follows: miR-194 Forward 5′ TCCGAAGGTGTACCTCAAC 3′, Reverse 5′ GTGCAGGGTCCGAGGT 3′; GAPDH forward, 5′-ACAACTTTGGTATCGTGGAAGG-3′ and reverse 5′-GCCATCACGCCACAGTTTC-3′.

Western Blot

Western blotting analysis was performed according to standard methods. The membranes were probed with polyclonal rabbit antibodies against anti-KDM5B (1:500; Chemicon, Temecula, CA, USA). The membranes were stripped and re-probed with an anti-GAPDH mouse monoclonal antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA) as a loading control.

Luciferase Reporter Assays

For the luciferase assay, SGC7901 cells were cultured in 12-well plates and each was cotransfected with 400 ng of either pGL3- KDM5B or pGL3-KDM5B -mut, 50 ng of pRL-TK (Promega, Madison, WI, USA), and 50 nmol/L of miR-194 mimics or NC. The pRL-TK Renilla luciferase plasmid was used as an internal control to correct differences in both transfection and harvest efficiencies. After 48-h transfection, luciferase activities of reporters in the whole-cell lysate were detected and normalized based on luciferase activities of pRLTK with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Cell Proliferation, Transwell Migration and Invasion Assays

Cells were seeded into 96-well plates at a density of 5000 cells/well and cultured for the indicated times, followed by incubation with 10% CCK-8 reagent (Beyotime Institute of Biotechnology, Jiangsu, China) at 37°C following the manufacturer's instructions. Results are expressed as absorbance at 490 nm.

Twenty-four-well transwell chambers with an 8.0-µm pore size polycarbonate membrane were used to measure cell migration and invasion. In brief, 48 h after transfection, GC cells were resuspended in 200 ml serum-free 1640 medium were placed into the upper chamber of the insert with or without Matrigel. Medium with 5% fetal bovine serum (FBS) was added into the lower chambers

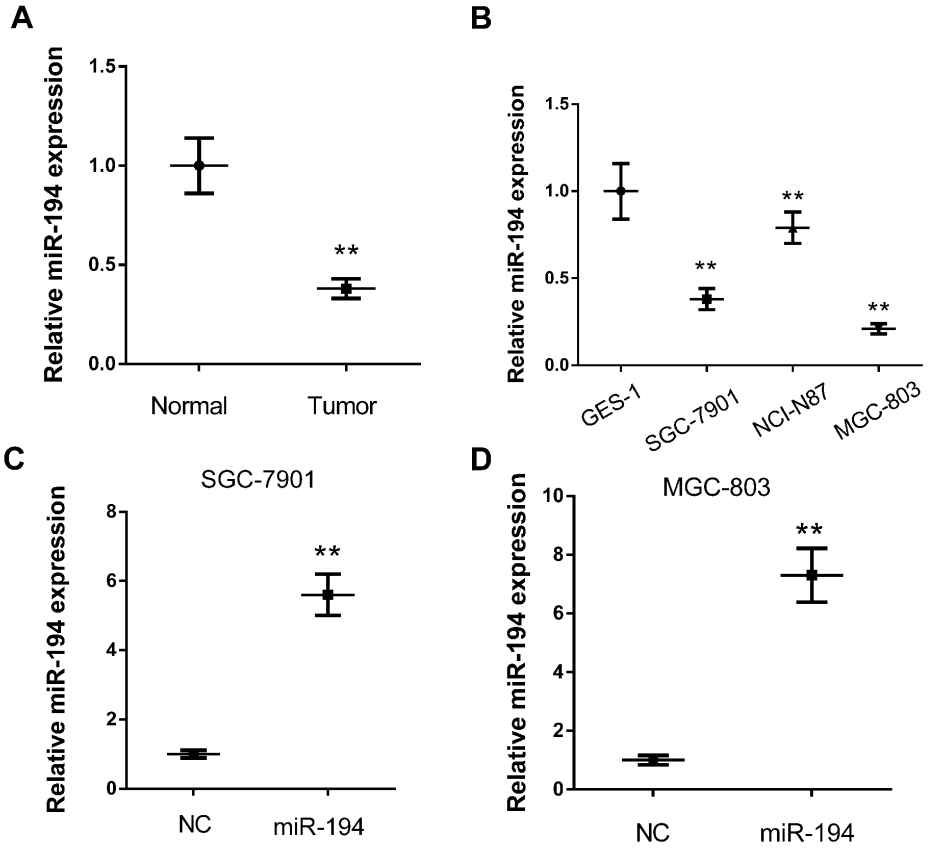

Figure 1. Expression of miR-194 in cell lines and tissues of GC. ***(A)*** Relative expression levels of miR-194 in GC tissues and adjacent non-tumorous tissues were evaluated by qRT-PCR. ***(B)*** Relative expression of miR-194 in three GC cell lines (NCI-N87, MGC-803, SGC-7901) and the normal gastric cell line GES-1. ***(C,D)*** MGC-803 and SGC-7901 cells that were transfected with corresponding miRNA vectors were subjected to qRT-PCR for miR-194. $^{*}p < 0.05$, $^{**}p < 0.01$.

as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were carefully removed. Cells that had migrated through the membrane were fixed with methanol and stained with 0.1% crystal violet (Biotime, Pudong, Shanghai, China), imaged and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Animal Experiments

The mouse experiments were conducted in the Animal Laboratory Center. GC cells treated with miR-194 mimics (miR-194) or negative control oligonucleotides (NC) were suspended in 100 ml serum free medium and injected subcutaneously into the left flank of 4- to 6-week old male BALB/c nu/nu nude mice. All mice were maintained in the pathogen-free (SPF) conditions. The in vivo volume of tumors was determined weekly (length x width x height). After 6 weeks, all mice were sacrificed by an overdose of sodium amobarbital for Ki-67 immunostaining (Sigma, Xuhui, Shanghai, China).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Values are expressed as the mean ± standard deviation of at least three independent experiments. Significant differences were confirmed using one-way analysis of variance. $^{*}p < 0.05$ was considered to be statistically significant and $^{**}p < 0.01$ extremely significant.

Results

Decreased Expression of miR-194 in Human GC Cells and Tissues

To determine the expression levels of miR-194 in GC, quantitative RT-PCR analysis was performed in 25 pairs of GC tissues and pair-matched adjacent noncancerous tissues. As shown in Figure 1A, the relative level of miR-194 expression in GC tissues was significantly downregulated, compared with that in corresponding noncan-

cerous tissues. We further analyzed miR-194 expression in GC cell lines. We found that miR-194 was significantly downregulated in the three GC cell lines (SGC-7901, NCI-N87, and MGC-803) compared with that of in normal GES-1gastric cells (Figure 1B). Expression of miR-194 was determined by qRT-PCR after transfection of miR-194 mimics or negative control oligonucleotides. The results showed that transfection of miR-194 restored its expression in SGC-7901 and MGC-803 cells (Figure 1C and 1D).

miR-194 Overexpression Restrained Cell Proliferation, Invasion and Migration in GC Cells

Next, we explore the function of miR-194 in GC. CKK8 assay showed that the rate of cell proliferation in the miR-194 mimics transfected group was significantly decreased compared with miR-NC groups ($p < 0.01$, Figure 2A). Furthermore, we investigated whether miR-194 could also inhibit migration and invasion of GC cells. We found that enforced expression of miR-194 inhibits tumor cell migration in MGC-803 and SGC-7901 cells compared with the miR-NC group (Figure 2B). At the same time, we also found that miR-194 markedly decreased the invasive capacity of MGC-803 and SGC-7901cells (Figure 2C). Collectively, miR-194 suppressed the migration and invasion of GC cells.

miR-194 Inhibited Tumor Growth and Progression

We further investigated whether miR-194 could inhibit the growth of GC cells *in vivo*. We used a BALB/C nude xenograft mouse model in which mice were transplanted with miR-194 and miR-NC transfected cells. Our findings showed that overexpression of miR-194 significantly reduced xenograft tumor volume (Figure 3A) and tumor weight (Figure 3B).

KDM5B is a Direct Target of miR-194

Using an online microRNA target database we found KDM5B to be a potential target of miR-194 with a complementary 3'UTR binding site for the seed sequence of miR-194 (Figure 4A). To determine the association between miR-194 and KDM5B, we performed a luciferase reporter assay to determine whether miR-194 directly targets the 3'UTR of KDM5B. The results showed that miR-194 significantly reduced the activity of KDM5B 3′-UTR, but not the binding motif mutant one (Figure 4B). Furthermore, KDM5B mRNA and protein levels were examined by RT-qPCR and western blot. KDM5B mRNA expressions were expressed lowly by miR-194 mimics in SGC-7901 and MGC-803 cells, respectively. Up-regulation of miR-194 in MGC-803 and SGC-7901 significantly decreased KDM5B protein expression, which indicated that miR-194 negatively regulates KDM5B (Figure 4C and 4D).

Discussion

In the present work, we observed that miR-194 expression was significantly down-regulated in GC cells and tissues. We demonstrated that miR-194 suppressed the migratory and invasive abilities of GC cells *in vitro* with SGC7901, MGC-803 cell lines. Further, function studies revealed that overexpression of miR-194 suppressed tumor growth in a nude mice model. This finding

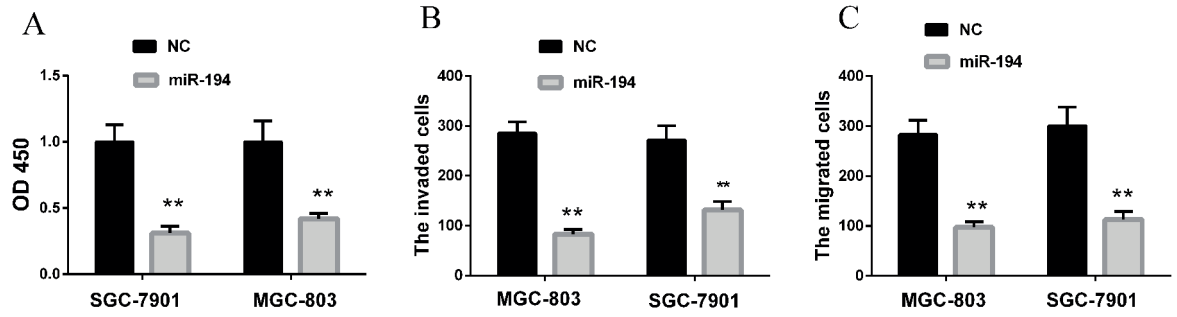

Figure 2. Over-expression of miR-194 inhibits GC cell proliferation, migration and invasion. ***(A)*** Cell proliferation was determined in MGC-803 and SGC-7901 cells after tranfected with miR-194 mimic or miR-NC using CCK8 assay. ***(B,C)*** Cell invasion and migration were determined in MGC-803 and SGC-7901 cells after tranfected with miR-194 mimic or miR-NC using transwell invasion assay. $^{*}p < 0.05$, $^{**}p < 0.01$.

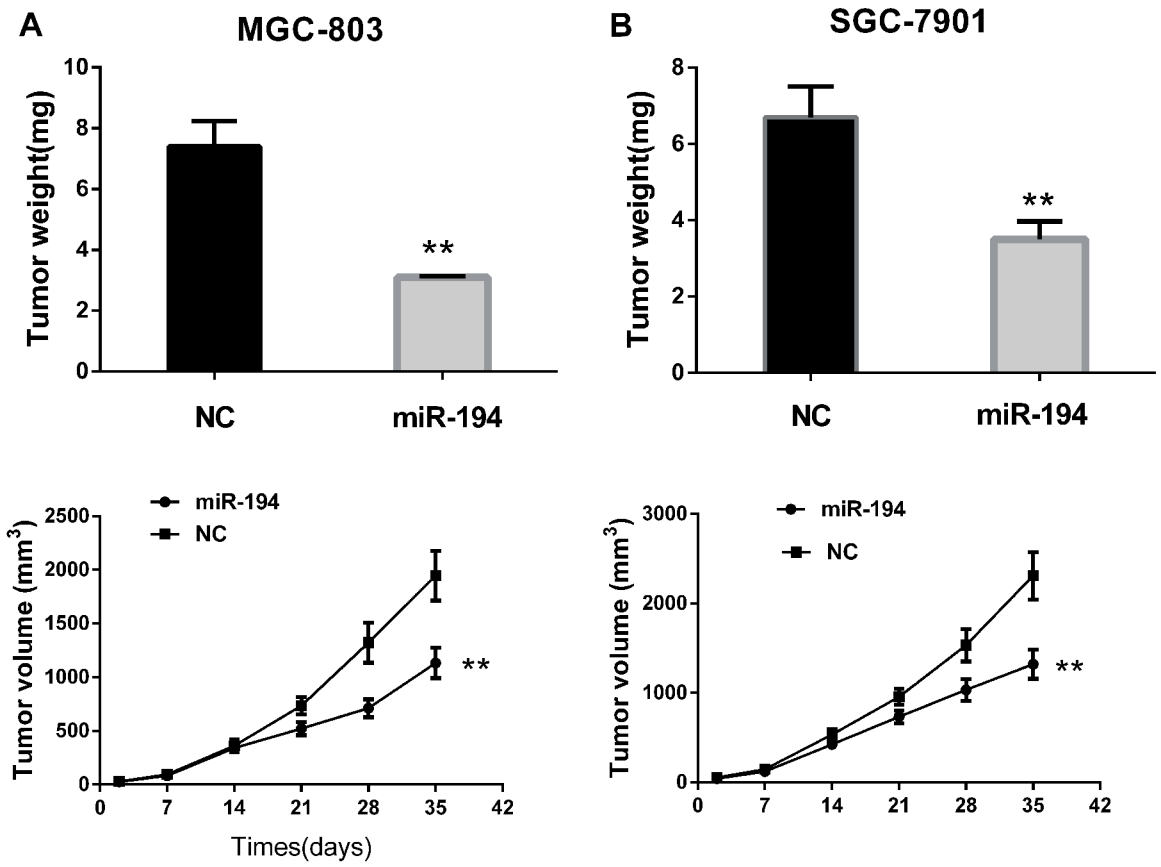

Figure 3. miR-194 inhibited tumor growth and progression. ***(A)*** Tumor xenograft model. The MGC-803 cells were injected into the hindlimbs of nude mice. And the tumor size and weight were observed and measured. ***(B)*** Tumor xenograft model. The SGC-7901 cells were injected into the hindlimbs of nude mice. And the tumor size and weight were observed and measured. $^{*}p < 0.05$, $^{**}p < 0.01$.

is consistent with a previous report showing that miR-194 is downregulated in several cancers[15,16]. Moreover, Bioinformatics analysis and luciferase activity assay demonstrated that KDM5B is a direct target genes of miR-194. The miR-194 mimics can significantly decrease protein expression level of KDM5B in the GC cells. Overall, our results showed that miR-194 functioned as a tumor suppressor gene by targeting KDM5B.

Roles of miR-194 have been suggested in a variety of types of human cancer. Zhang et al[17] found that miR-194 suppresses proliferation, migration and invasion by targeting RAP2B in human bladder cancer. Zhao et al[18] showed that miR-194 acted as a prognostic marker and regulated the progression of HCC through directly inhibiting the expression of MAP4K4. Liang et al[19] reported that miR-194 promoted the growth, migration, and invasion of ovarian carcinoma cells by targeting protein tyrosine phosphatase nonreceptor type 12. More importantly, Li et al[20] showed that Exogenous expression of miR-194 inhibited cell migration, invasion, and the epithelial-mesenchymal transition phenotype in gastric cancer cells. Furthermore, they confirmed that miR-194 served as a tumor suppressor by targeting FoxM1. Those data provided strong evidence that downregulation of miR-194 in the different tumor was associated with aggressive progression and poor prognosis.

KDM5B has been confirmed as an oncogene in several tumors. Many studies explored the potential mechanism of miR regulating KDM5B. For instance, Li et al[21] found that the enforced expression of miR-29a in prostate cancer cells inhibited proliferation, and induced apoptosis by repressing the expression of KDM5B. Nilsson et al[22] showed that miR-137 served as a tumor suppressor in prostate carcinogenesis and progression by targeting KDM5B. Bamodu et al[23] revealed that KDM5B-silencing in the breast cancer cells correlated with the upregulation

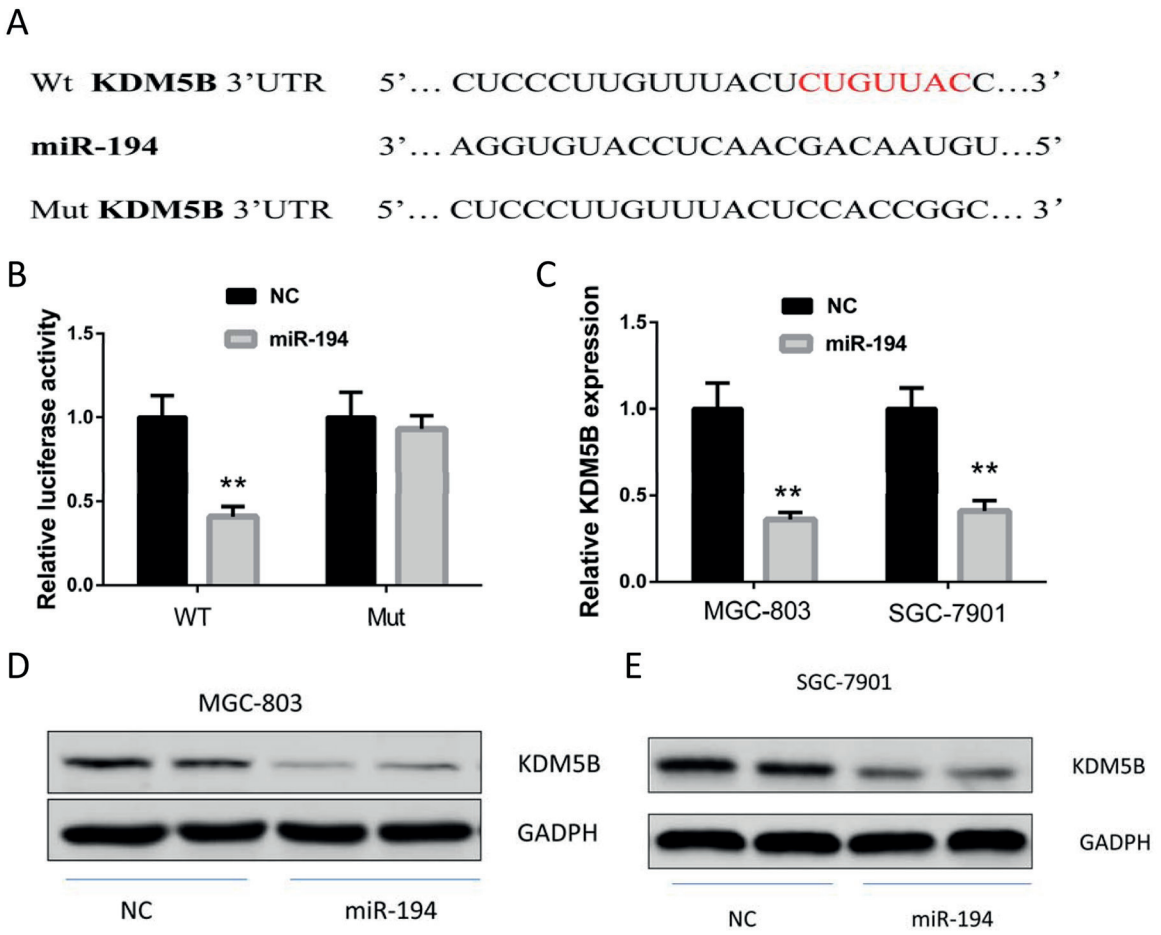

Figure 4. miR-194 suppressed KDM5B expression by directly targeting the KDM5B 3'-UTR. (A) TargetScan prediction programs indicated that miR-194 could bind to the 3'UTR of KDM5B. (B) SGC-7901 cells were co-transfected with miR-194 mimics or control and reporter plasmid or the mutant 3'UTR of KDM5B, together with the controls. At 48 h after transfection, the luciferase activity was measured. *(C)* Real-time RT-PCR data of KDM5B in MGC-803 and SGC-7901 cells transfected with the miR-194 or negative control mimics. Data were normalized to GAPDH mRNA. (D,E) Western blot analysis of KDM5B protein levels in SGC-7901 and MGC-803 cells 24 h after transfection. $^{*}p < 0.05$, $^{**}p < 0.01$.

of hsa-miR-448 and led to the suppression of MALAT1 expression with decreased migration, invasion and clonogenic capacity in vitro. The above findings suggested that KDM5B might be a target for many different miRNAs.

Conclusions

Our report demonstrates that miR-194 inhibits GC cell proliferation, migration, and invasion, and an important tumor promoter KDM5B is identified as a target of miR-194. miR-194 might be a potential therapeutic target for GC treatment in the future.

Conflicts of interest

The authors declare no conflicts of interest.

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