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MicroRNA-200c is involved in proliferation of gastric cancer by directly repressing p27^{Kip1}

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ABSTRACT

P27^{Kip1}, also known as Cyclin-dependent kinase inhibitor 1B, is an important check-point protein in the cell cycle. It has been identified that although as a tumor suppressor, P27^{Kip1} is expressed in different cancer cell types, which shows the therapeutic potential in tumor genesis. In this study, we examined the upstream regulatory mechanism of P27^{Kip1} at the microRNA (miRNA) level in gastric carcinogenesis. We used bioinformatics to predict that microRNA-200c (miR-200c) might be a direct upstream regulator of P27^{Kip1}. It was also verified in gastric epithelial-derived cell lines that overexpression of miR-200c significantly inhibited the expression levels of P27^{Kip1}, whereas knockdown of miR-200c promoted P27^{Kip1} expression in AGS and BGC-823 cells. Furthermore, we identified the direct binding of miR-200c on the P27^{Kip1} 3′ -UTR sequence by luciferase assay. MiR-200c could enhance the colony formation of cells by repressing P27^{Kip1} expression. In addition, the negative correlation between P27^{Kip1} and miR-200c in human gastric cancer tissues and matched normal tissues further supported the tumor-promoting action of miR-200c *in vivo*. Our finding suggested that miR-200c directly regulates the expression of P27^{Kip1} and promotes cell growth in gastric cancer as an oncogene, which may provide new clues to treatment.

1. Introduction

Gastric cancer is one of the most common and aggressive forms in China [1]. Patients with gastric cancer are often given a poor prognosis due to the difficulty of early diagnosis with highly growth and high rate of recurrence [2,3].

It has been suggested that the abnormal expression of check point genes can always be found in tumorgenesis, including TP53, P27^{Kip1}, P21^{Cip2}, P16^{Ink1} and C-Myc [4–8]. P27^{Kip1}, also known as Cyclin-dependent kinase inhibitor 1B, binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1 stage [9]. It is always thought the inhibitor of cell cycle with a tumor suppressor [10,11]. In cancers it is often inactivated *via* impaired synthesis, accelerated degradation, or mislocalization [12]. We previously reported that FoxM1, the main positive regulator of cell cycle, can regulate gastric cancer cells proliferation and senescence through inhibition of P27^{Kip1} [13,14]. These observations support that P27^{Kip1} is strongly negatively correlated with gastric carcinogenesis. It is important to better understand the regulation of P27^{Kip1} expression in tumorgenesis for anti-cancer therapy.

Currently, microRNAs (miRNAs) has been thought one of the most important regulators in tumorgeneis [15–18]. MiRNAs are the small non- translated RNA molecules with approximately 18–24 nucleotides in length. Every miRNA can directly bind to the complementary sequence on the 3′-untranslated region (3′-UTR) of many possible target mRNAs, which thus regulates the genes expression by post-transcriptional gene silencing, producing sequence specific mRNA cleavage, or translational repression [19,20]. MiRNAs can be the tumor suppressors or oncogenes which are determined by the functions of their target genes [21,22]. There are only a few research on the miRNAs regulation on P27^{Kip1} expression in tumorgenesis, including miR-221/222 [23,24]. We still need more evidence on specific miRNA regulation on P27^{Kip1} and its function in gastric carcinoma.

In this study, we demonstrated that miR-200c is an oncogene in gastric carcinoma which can directly inhibit the expression of

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$P27^{Kip1}$ *in vivo* and *in vitro*. To our knowledge, this is the first time to explore the relationship between miR-200c and $P27^{Kip1}$ in gastric carcinoma. We propose that antagomiRNAs of miR-200c might be the potential for an anti-cancer therapy by reactivation of $P27^{Kip1}$.

2. Materials and methods

2.1. Cell lines and culture

The gastric epithelial-derived cancer cell lines AGS and BGC-823 were obtained from the cell repository for Academia Sinica (Shanghai). AGS cells were grown in Ham's F12 (Gibco, USA). BGC-823 cells were grown in RPMI1640 medium (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (Gibco, USA). Both the cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. RNA extraction and QRT-PCR

Total RNA was extracted by use of Trizol reagent (Invitrogen, USA). For qRT-PCR of $P27^{Kip1}$, total RNA was reverse transcribed by use of the RevertAid First Strand cDNA synthesis Kit (Fermentas, Canada). Real-time PCR was involved by the SUBY Green mixture (Takara, Japan) with the Biorad Sequence Detection System. Gene expression was normalized to that of β-actin. Results were calculated by the $2^{-\Delta\Delta Ct}$ method. The level of mature miR-200c expression was analyzed by TaqMan miRNA Assay (Applied Biosystems). cDNA was synthesized from total RNA samples by use of the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers. Real-time PCR involved TaqMan miRNA Assay primers with the TaqMan Universal PCR Master Mix. The reactions were run in the Biorad Sequence Detect System. The relative level of miRNA expression was normalized to that of U6 small noncoding RNA and the fold change for miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences for $p27^{Kip1}$, 5′-ATGTCAAACGTGCGAGTGTCTAA-3′, and antisense, 5′-TTACGTTTGACGTCTTCTGAGG-3′, and β-actin, sense, 5‘-AGTTGCGTTACACCCTTTCTTG-3′, and antisense, 5‘- CACCTTCACCGTTCCAGTTTT-3′.

2.3. Western blotting

The protein level of $P27^{Kip1}$ was determined by Western blotting which was normalized by β-actin. In brief, the cells were lysed in RIPA buffer. Then the lysate was spun down and the supernatant was harvested. The concentration of the proteins was detected by BCA assay. Equal amounts of proteins were separated by SDS polyacrylamide gels and transferred onto membranes (Millipore), which were blocked with 5% non-fat milk protein for 1 h, then incubated with primary antibodies overnight at 4 °C. The antibodies used were for $P27^{Kip1}$ (1:300, Santa Cruz Biotechnology) and β-actin (1:5,000, Sigma). The secondary antibodies were horseradish peroxidase-conjugated goat-anti-rabbit IgG (1:5,000, Santa Cruz Biotechnology). Immune complexes were detected by use of the Chemiluminescent HRP Substrate Kit (Millipore).

2.4. Luciferase assay

The mimics and the inhibitor of miR-220c were purchased from Ruibo (Guangzhou, China). The special fragment of the $P27^{Kip1}$ 3′-UTR containing the miR-200c predicted target sites was synthesized by Invitrogen (USA). Then the fragment was cloned into the multiple cloning sites of the luciferase reporter pMIR-REPOTR (Applied Biosystems, USA), designated as pMIR-REPORT-P27-3′-UTR, which was also used in PCR to generate pMIR-REPORT-P27-3′-UTRmut plasmid with mutation of the binding sites on the 3′-UTR of $P27^{Kip1}$. For the transfection of the palsmids, cells were seeded into 6-well plates (3×10^5 cells/well) for 18–24 h. Then the plasmids were transfected by the use of Lipofectamine 3000 (Invitrogen, USA). To examine the direct conjugation of miR-200c to the 3′-UTR of $P27^{Kip1}$, pMIR-REPORT-P27-3′-UTR and pMIR-REPORT-P27-3′-UTRmut were co-transfected into AGS cells with miR-200c mimics. pMIR-REPORT β-gal plasmid was used as a negative control. Luciferase activity in the cell lysates was determined by a single luciferase reporter assay (Promega, USA) 48 h after transfection, and target promoter-driven firefly luciferase activity was normalized to that of β-gal.

2.5. Clone formation assay

BGC-823 cells were incubated in 6-well plates for 18–24 h, which were transfected with the corresponding mimics/inhibitor for 48 h. Single cells were seeded on 6-well plates (300 cells/well). After 10 days of incubation, plates were stained with Giemsa for 20 min. The number of colonies with more than 50 cells was counted.

2.6. Patients and tissue specimens

Resected pairs of cancer tissue and distal normal gastric tissue (> 5 cm from the margin of the tumor) from 15 patients with gastric cancer were harvested during surgery at Qilu Hospital of Shandong University 2014–2015. None of the patients had received adjuvant chemotherapy before surgery. The diagnosis of gastric cancer was histopathologically confirmed. The general information for patients is in Table 1. The study was approved by the ethics committee of Shandong University School of Medicine.

2.7. Immunohistochemistry

The expression of $P27^{Kip1}$ in human species was determined by Immunohistochemistry. Resected tissue pairs were embedded with paraffin and sliced into 5- mm pieces, which were deparaffinized and dehydrated with xylene and a graded series of alcohol. Antigen retrieval was treated in 0.1 M citrate buffer at pH 6.0 with heating. Then 3% H_2O_2 was used to block the endogenous peroxidase activity. The slides were incubated with goat serum for 30 min, then with rabbit anti-human $P27^{Kip1}$ (Santa Cruz, USA) overnight at 4 °C. The results were detected with Diaminobenzidine (DAB) staining (Vector Laboratories, USA) which were calculated with the microscope images (Olympus BX60, Tokyo, Japan).

Table 1
Association of miR-200c and p27 expression in human gastric cancer tissues.

Category	No. of patients	miR-200c			P27		
		high	low	*P*	high	low	*P*
Age				> 0.05			> 0.05
< 60 years	6	4	2		1	5	
≥ 60 years	9	6	3		4	5	
Sex				> 0.05			> 0.05
Male	9	6	3		3	6	
Female	6	4	2		2	4	
Differentiation				> 0.05			> 0.05
Well	5	4	1		2	3	
Poor	10	6	4		3	7	
Tumor size				> 0.05			> 0.05
≤ 5 cm	8	5	3		3	5	
> 5 cm	7	5	2		2	5	

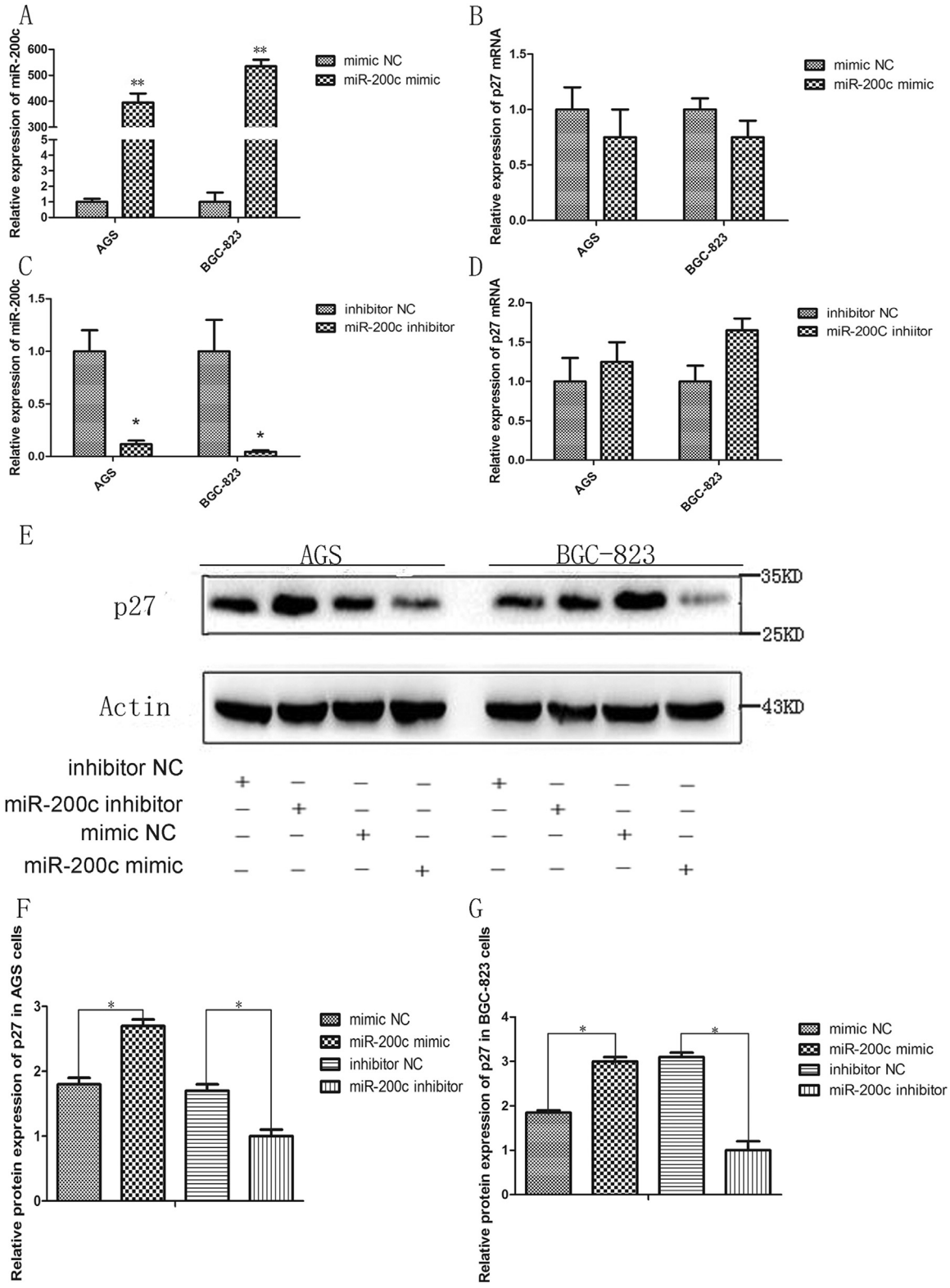

Fig. 1. MiR-200c downregulated the expression of $P27^{Kip1}$ in gastric cancer cell lines. QRT-PCR analyses of (A) miR-200c and (B) $P27^{Kip1}$ mRNA level in control and miR-200c mimics-transfected AGS, BGC-823 cell lines after 48 h. $^{*}P < 0.05$ and $^{**}P < 0.01$. (C) miR-200c and (D) $P27^{Kip1}$ mRNA level in control and miR-200c inhibitors-transfected AGS, BGC-823 cell lines after 48 h. $^{*}P < 0.05$ and $^{**}P < 0.01$. (E)Western blot analyses of $P27^{Kip1}$ protein levels in gastric cancer cells treated with control and miR-200c mimics or inhibitors. (F,G) Western blot analyses of $P27^{Kip1}$ protein levels. $^{*}P < 0.05$ and $^{**}P < 0.01$. Data are mean ± SEM of 3 independent experiments.

2.8. Statistical analysis

Quantitative data are expressed as mean ± SEM. Statistical analysis was in the use of SPSS 15.0 (SPSS, Inc., Chicago, IL) by two-tailed Student's *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

MiR-200c downregulated the expression of $P27^{Kip1}$ at the protein level in gastric cancer cell lines.

The bioinformatics analysis with miRanda (http://www.microrna.org/microrna), TargetScan (http://www.targetscan.org), and miRBase (http://www.mirbase.org) predicted $P27^{Kip1}$ as an downstream target of miR-200c which could directly bind to the 3′-UTR of $P27^{Kip1}$. To determine the correlation between miR-200c and $P27^{Kip1}$ mRNA expression level, we transfected the miR-200c mimics into AGS and BGC-823 cells for 48 h. The high expression of miR-200c was detected by QRT-PCR (Fig. 1A), which repressed the mRNA level of $P27^{Kip1}$ without significant difference (Fig. 1B). Similarly, the transfection of miR-200c inhibitor (Fig. 1C) also did not obviously increase the mRNA expression of $P27^{Kip1}$ (Fig. 1D). Then Western blotting was checked to determine the effect of miR-200c on the expression of $P27^{Kip1}$ protein. The results showed that with miR-200c overexpression, the $P27^{Kip1}$ protein level decreased. While with the inhibition of miR-200c, the $P27^{Kip1}$ protein level recovered to a higher level (Fig. 1E, F and G). Therefore, miR-200c inhibited $P27^{Kip1}$ expression in the gastric cancer cells by degrading the protein level of $P27^{Kip1}$.

3.1. $P27^{Kip1}$ is the direct target of miR-200c

The complementary sequence of miR-200c was found on the 3′-UTR site of $P27^{Kip1}$ (Fig. 2A). Then the direct regulation of miR-200c on $P27^{Kip1}$ expression by the binding of the special 3′-UTR site was determined by luciferase report assay. AGS cells were cotransfected with miR-200c mimics and $P27^{Kip1}$ wild-type or mutant-type 3′-UTR plasmids. Cotransfection of miR-200c and wild-type 3′-UTR plasmid reduced the luciferase activity by approximately 60% relative to the control, whereas mutant 3′-UTR cotransfection almost restored the luciferase activity (Fig. 2B). Thus, miR-200c directly targeted the binding site located at $P27^{Kip1}$ 3′-UTR and $P27^{Kip1}$ is one of direct targets of miR-200c.

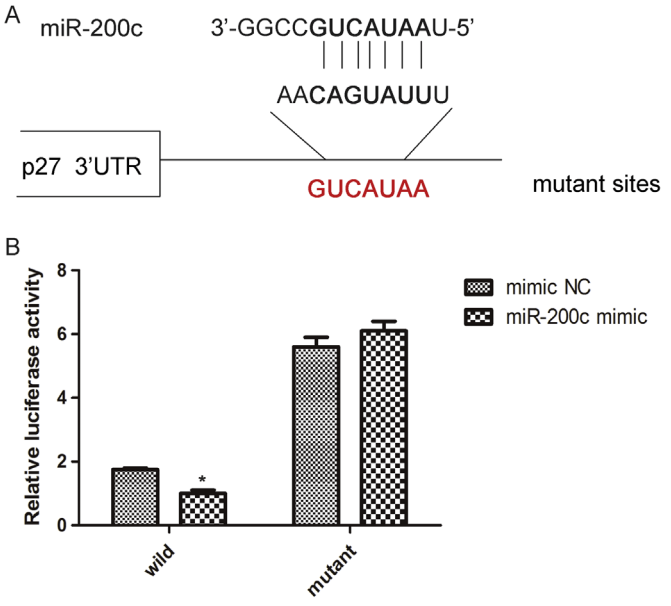

Fig. 2. $P27^{Kip1}$ is the direct target of miR-200c. (A) The wild sequence on 3′-UTR of $P27^{Kip1}$ that could be bound by miR-200c and the corresponding mutant sequence. (B) Luciferase activity assay with pMIR-REPORT-p27-3′-UTR and pMIR-REPORT-p27-3′-UTR mutant co-transfected with miR-200c mimics or the negative control in gastric cancer cells for 48 h. $^{*}P < 0.05$ *vs.* con. Data are mean ± SEM of 3 independent experiments.

3.2. MiR-200c was involved in the proliferation of gastric cancer cells

Colony formation assay in BGC-823 cells revealed that the expression level of miR-200c truly affected the proliferation of gastric cancer cells (Fig. 3A). The enforced expression of miR-200c enhanced the clone formation of cells, while the knockdown of miR-200c significantly inhibited the number of colonies (Fig. 3B). Thus, miR-200c could work as an oncogene in human gastric cancer cells to activate cell proliferation.

3.3. MiR-200c expression was enhanced in human primary gastric cancer

We determined the expression of miR-200c in human primary gastric cancer specimens and matched normal tissues from 15 patients. HE staining showed the pathology of the samples (Fig. 4A). QRT-PCR and IHC results showed that $P27^{Kip1}$ protein expression was inhibited in human gastric cancer specimens which was the same as the research before (Fig. 4A, B and C). At the same time, the level of miR-200c expression was enhanced in human gastric cancer samples (Fig. 4B), which supported the oncogenetic role of miR-200c *in vitro*. Therefore, miR-200c and $P27^{Kip1}$ might be negative covalent *in vivo* (Fig. 4D). We found no association of miR-200c or $P27^{Kip1}$ expression and patient age, gender or tumor size (Table 1).

4. Discussion

In this study, we showed that miR-200c played an oncogenetic role in human gastric carcinogenesis by directly downregulated the expression of $P27^{Kip1}$ and promoted the gastric cancer cell proliferation.

Gastric cancer (GC) is one of the most common human malignant diseases and the second leading cause of tumor-related deaths worldwide [1,2]. The incidence and mortality rate are particularly high in eastern Asia [3]. Despite recent advances in surgical techniques and adjuvant therapy after surgery, the 5-year survival rate is still low [25]. Carcinogenesis is a multi-factor, multi-phase and long-term interaction process on malignant transformation of normal cells. In this process, the abnormal regulation of cell cycle is involved in all the stages [26]. In previous studies, we have identified $P27^{Kip1}$ is an important target which could be regulated by different factors in the proliferation of gastric cancer cells, including *H. pylori* infection, the escape of cancer cells senescence, epigenetics, et al. [13,14,27]. We also find that $P27^{Kip1}$ can express in different cancer cell lines, which means it might be a valuable target for the diagnosis and treatment of gastric cancer.

In most cancers, reduced levels of $P27^{Kip1}$ are correlated with increased tumor size, increased tumor grade, and a higher propensity for metastasis [28]. There might be different mechanisms by which levels of $P27^{Kip1}$ are regulated in different types of cancers. The expression level of $P27^{Kip1}$ can be regulated from the different steps of gene expression, such as transcription, translation and proteolysis [29]. $P27^{Kip1}$ can also be regulated by changing its subcellular location [30]. Both mechanisms act to reduce the inhibition effect of $P27^{Kip1}$ on the cell cycle, allowing for the activation of Cdk1 and Cdk2, which can begin the process of the cell cycle. We have identified that FoxM1 can inhibit the promoter

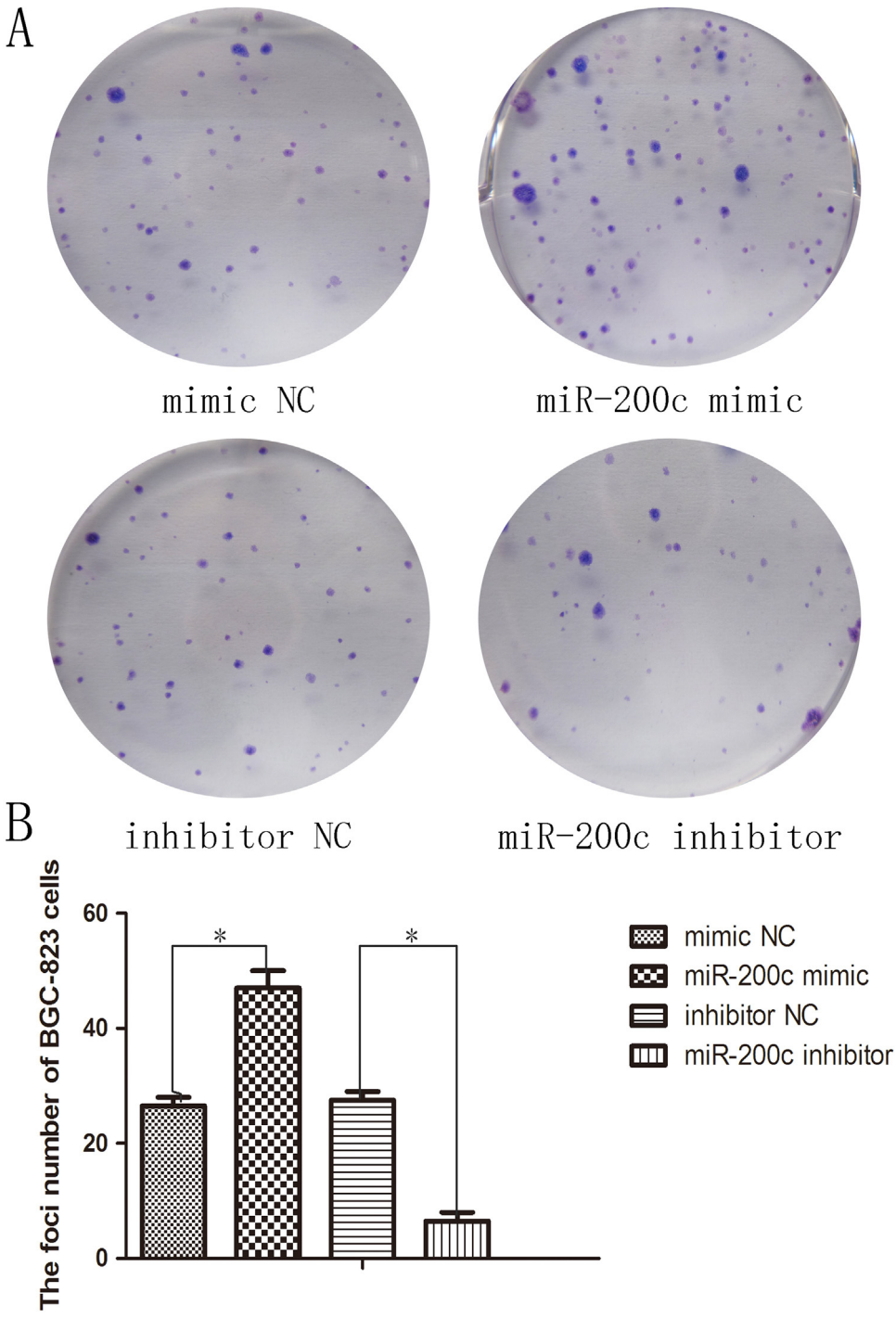

Fig. 3. MiR-200c was involved in the proliferation of gastric cancer cells. (A) Colony formation ability in gastric cancer cells with overexpression and knockdown of miR-200c and (B) quantification. $^{*}P < 0.05$. Data are mean ± SEM of 3 independent experiments.

activity of $P27^{Kip1}$ and thus is involved in gastric carcinoma. Since $P27^{Kip1}$ levels can also be moderated at the post transcriptional level, it has been proposed that $P27^{Kip1}$ may be regulated by miRNAs.

MiRNAs have recently been discovered as one of the crucial players in gastric carcinogenesis through posttranscriptional regulation of tumor suppressor and oncogenes [31–33]. A substantial number of deregulated miRNAs have been revealed in gastric cancer and the biological significance of those miRNAs has been confirmed in multiple functional experiments. Only a few researches focus on the cell cycle inhibitors. In this study, we first predicted miR-200c as the up-stream regulator of $P27^{Kip1}$ by bioinformatics. Then the negative regulative effect of miR-200c was determined in gastric cancer cell lines only at the translational level, which means that miR-200c does not affect the $P27^{Kip1}$ mRNA cleavage, but only repressed $P27^{Kip1}$ translation. Luciferase assay further suggested that $P27^{Kip1}$ was the direct target of miR-200c. The oncogenetic role of miR-200c was identified by the increase of clone number with the overexpression of miR-200c in gastric cancer cells. The higher level of miR-200c and reduced

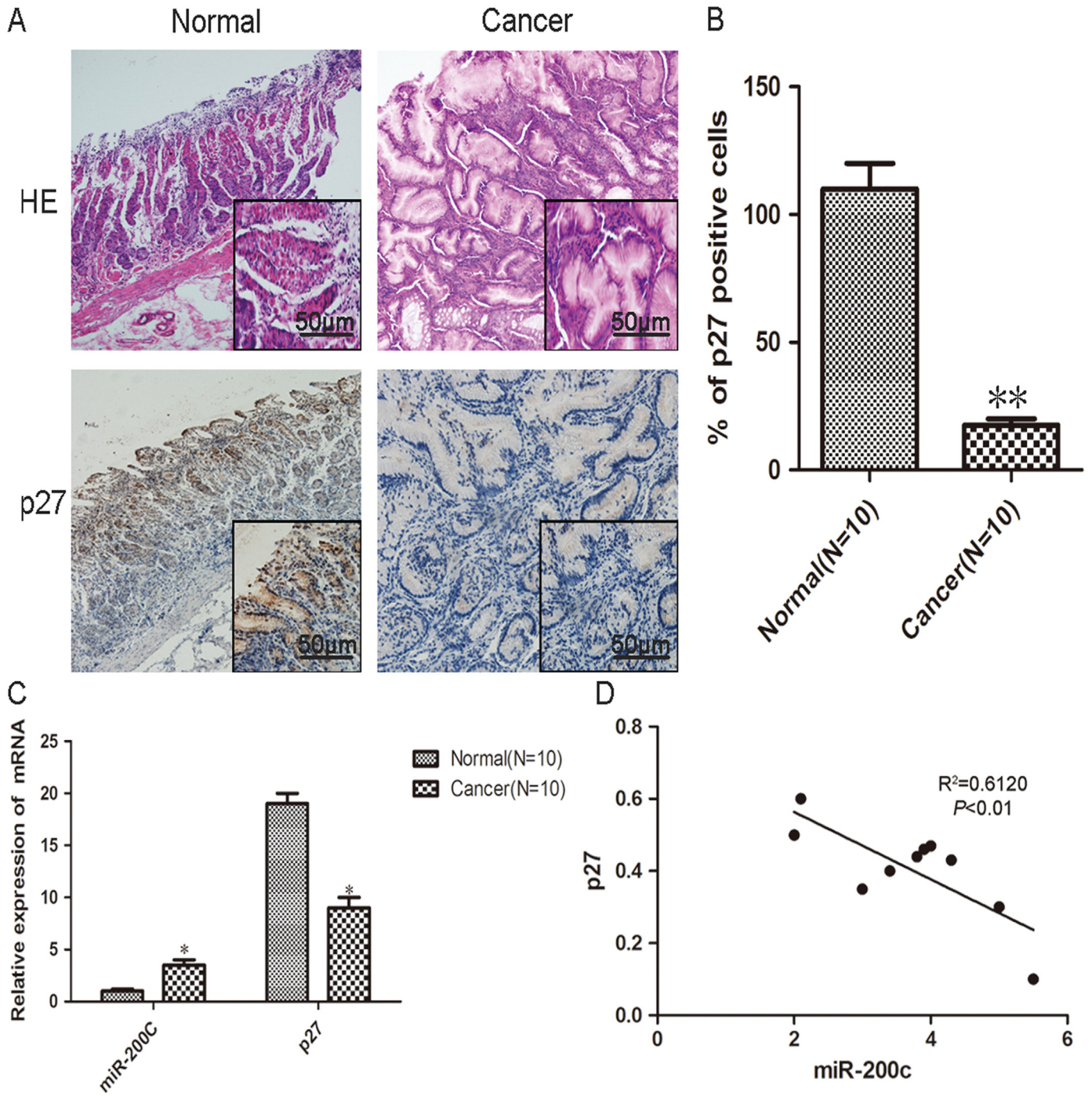

Fig. 4. MiR-200c expression was enhanced in human primary gastric cancer. (A) HE staining and Immunohistochemical staining of expression of $P27^{Kip1}$ in human normal (left panel) and cancerous (right panel) gastric tissues. (B) Percentage positive cells by immunohistochemistry for $P27^{Kip1}$ in human normal and cancerous gastric tissues. $^{**}P < 0.01$. (C) QRT-PCR analyses of miR-200c and $P27^{Kip1}$ mRNA in normal and cancerous human gastric tissues. $^{*}P < 0.05$. (D) Correlation of miR-200c and $P27^{Kip1}$ levels in human gastric cancer tissues after standardization with matched normal tissues. Data are mean ± SEM of 3 independent experiments.

expression of $P27^{Kip1}$ in human primary gastric cancer samples proved their negative correlation *in vivo*. $P27^{Kip1}$ has been showed prognostic value in ovarian cancer in which $P27^{Kip1}$ negative tumors progressed in 23 months compared to 85 months in $P27^{Kip1}$ positive tumors [19]. Similar studies have correlated low levels of p27 with a worse prognosis in breast cancer [20]. The correlations were also observed in patients with non-small cell lung cancer, colorectal cancer, and prostate cancer [21–23]. So $P27^{Kip1}$ can also be correlated with treatment response and work as a therapeutic target in cancer. In this study, we also identified the inhibition of $P27^{Kip1}$ expression in gastric carcinoma with the negative control of miR-200c. The antagomiRs of miR-200c that can block the activity of the miR-200c and allow for $P27^{Kip1}$ cell grow inhibition to take place might be a new way for gastric cancer treatment.

In summary, our results showed that miR-200c, which could negatively regulate the expression of $P27^{Kip1}$, was an important oncogene involved in the cell proliferation of gastric cancer. These findings may provide further information miR-200c to be useful as

an anti-cancer therapeutic agent in gastric cancer.

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