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MiR-29c suppresses cell invasion and migration by directly targeting CDK6 in gastric carcinoma

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Abstract. – OBJECTIVE: Gastric carcinoma (GC) is one common malignant tumor with high morbidity and mortality rates all over the world. Recently, numerous studies have showed that the microRNAs (miRNAs) dysregulation was implicated in GC carcinogenesis. This research aimed to explore the potential associations between miR-29c and cyclin-dependent kinase 6 (CDK6) in GC.

PATIENTS AND METHODS: GC tissues and corresponding normal tissues were collected from 54 GC patients who underwent surgery at the Second Hospital of Anhui Medical University between 2015 and 2017. We measured the expressions of CDK6 and miR-29c in GC tissues using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). We next investigated the functions of miR-29c in GC cells by performing transwell assays. To further determine the correlation between miR-29c and CDK6 in GC cell invasion and migration, the rescue experiments were performed by co-transfecting miR-29c inhibitor and CDK6 siRNA into AGS cells.

RESULTS: MiR-29c expressions were significantly declined in GC tissues and cells. Additionally, functional assays showed that the miR-29c over-expression suppressed the invasion and migration capacities of GC cells. According to TargetScan and dual luciferase reporter assays, CDK6 was identified as a new miR-29c target. Moreover, the knockout of CDK6 had similar effects as the miR-29c over-expression in GC cells. The current research indicated that miR-29c over-expression could inhibit tumor behaviors in GC partially *via* down-regulating CDK6.

CONCLUSIONS: We revealed that miR-29c down-regulated in GC tissues and cells. MiR-29c over-expression effectively suppressed the GC cell invasion and migration. Moreover, CDK6 was identified as a direct functional target of miR-29c in GC. The current study provides new insights for the GC treatment and suggests that miR-29c/CDK6 axis is a therapeutic candidate target for GC patients.

Key Words:
Gastric carcinoma, MiR-29c, Invasion, Migration, CDK6.

Introduction

Gastric carcinoma (GC) is a common human malignancy with high mortality and morbidity worldwide[1]. In recent decades, even though the GC incidence and mortality rates have been steadily declining thanks to the advanced medical treatment, it is considered that over 950,000 new patients with GC emerged per year, leaving approximately 720,000 mortalities. This makes GC the third primary cause of tumor-related deaths and the fourth most common cancer in the world[2]. However, the majority of GC patients are usually diagnosed with clinically poor characteristics in the advanced stage[3]. As GC development is a multi-step and multi-factorial process, the investigation of the intrinsic mechanisms underlying GC development seems to favor the effective therapies of GC[4]. Recently, microRNAs (miRNAs) have been identified as promising biomarkers and attractive therapeutic targets of human cancers[5]. MiRNAs are a variety of non-coding RNAs, 20-24 nucleotides in length, and they have been found to be able to inhibit the translation *via* binding to the 3'-UTRs of target mRNAs[6]. Aberrant miRNA expressions are strongly connected with the progression of multiple tumors, such as glioblastoma[7], and colorectal cancer[8]. Moreover, a growing body of evidence supports that miRNAs serve as oncogenes or tumor suppressors in tumors[9]. For example, miR-92a was found to accelerate the hepatocellular carcinoma cell proliferation and invasion *via* targeting forkhead box A2 (FOXA2)[10]; Wan et al[11] found that miR-767-3p suppressed lung adenocarcinoma cell growth and migration *via* regulating claudin18 (CLDN18); Tang et al[12] reported that miR-106a promoted the human endometrial adenocarcinoma growth by targeting Bcl-2-like protein 11

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(BCL2L11). However, the expressions and underlying functions of miR-29c in GC still need to be studied, which is of great significance to explore novel biomarker and therapeutic strategy for patients with GC.

Onco-protein is an attractive therapeutic target due to its causal relationship with the development of tumors. In addition, tumor cells often rely on onco-proteins to continue to proliferate and survive[13]. As the core parts of cell cycle regulation, one kind of such onco-proteins called cyclin-dependent kinases (CDKs) contained more than 20 members, which either directly or indirectly had essential functions in all eukaryotic organisms, such as the major cell-cycle transitions[14]. CDK6 is one CDK family member, the oncogenic capacity of which has been investigated in several studies[15]. Moreover, previous studies showed that specific inhibitors of CDK6 had antitumor functions in multiple tumors[16,17]. Nevertheless, the functions and regulatory mechanisms between CDK6 and miR-29c in GC are little-known by people. Therefore, investigating the underlying mechanism may contribute to develop more effective therapeutic strategies for GC.

Patients and Methods

Cell Lines and Tissue Specimens

GC tissues and the corresponding normal tissues were collected from 54 GC patients who underwent surgery at the Second Hospital of Anhui Medical University between 2015 and 2017. All tissue samples were put into liquid nitrogen for storage within 10 min after separation and reserved for further assays. Written informed consent was obtained from all GC patients enrolled in this study. This investigation was approved by the Ethics Committee of the Second Hospital of Anhui Medical University.

The human GC cell line AGS and gastric epithelial cell line GES-1 were obtained from the cell repository for the Academia Sinica (Shanghai, China). All the above cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) which contained penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an atmosphere with 5% of CO_2 at 37°C.

Cell Transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to transiently transfect the miR-29c mimics, inhibitor or CDK6 siRNA into GC cells in line with the manufacturer's instructions. The GC cells with different transfections were used for subsequent assays.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNAs from the above-mentioned tissue samples and cultured GC cells in line with the manufacturer's instruction. Then, the TaqMan MicroRNA Reverse Transcription kit (Thermofisher Scientific, Waltham, MA, USA) was used for the synthesis of complementary DNA (cDNA). The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted by a SYBR Premix Ex Taq II kit (TaKaRa, Otsu Shiga, Japan). The PCR reaction conditions were 95°C (3 min), denaturation for 40 cycles at 95°C (15 sec) followed by an annealing step at 60°C (30 sec). The relative expressions of miR-29c and CDK6 mRNA were normalized to U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expressions of genes. The primer sequences were listed in Table I.

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-29c forward	5'-GCCTAGCACCATTTGAAATCG -3'
miR-29c reverse	5'-GTGCAGGGTCCGAGGT -3'
U6 forward	5'- CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
CDK6 forward	5'-GGACTTTCTTCATTCACACCG -3'
CDK6 reverse	5'- GACCACTGAGGTTAGGCCA-3'
GAPDH forward	5'- ACCTGACCTGCCGTCTAGAA -3'
GAPDH reverse	5'- TCCACCACCCTGTTGCTGTA-3'

U6: small nuclear RNA, snRNA; CDK6: cyclin dependent kinase 6; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Western Blots

The total proteins were extracted by lysing cells with the radioimmunoprecipitation assay (RIPA) buffer which contained protease and phosphatase inhibitors (Beyotime, Shanghai, China). The protein concentrations were measured with a bicinchoninic acid protein (BCA) assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After the separation with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), the proteins were then transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF was pre-incubated in Tris-Buffered Saline and Tween (TBST) with 5% of skimmed milk for 2 h at room temperature, followed by incubation with the appropriate antibody overnight at 4°C. Then, it was incubated with Anti-Rabbit IgG (1:4000; ab191866; Abcam, Cambridge, MA, USA) for 2 h. The enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA) was applied to observe the results of the antigen-antibody complex on PVDF. The primary anti-bodies were as follows: anti-CDK6 (1:10000; ab222395; Abcam, Cambridge, MA, USA); anti-GAPDH (1:500; ab181603; Abcam, Cambridge, MA, USA). Protein levels were measured by an enhanced chemiluminescent detection system (Beyotime, Shanghai, China). GAPDH was an internal reference.

Transwell Assays

The invasion and migration abilities of the treated GC cells were assessed by transwell assays using transwell chambers (Corning, Corning, NY, USA) with or without Matrigel (Clontech, CA, USA) coated. Firstly, GC cells with different transfections were seeded into the top chambers in the serum-free medium. In the meantime, the medium containing 10% of FBS was added into the bottom chambers. Having been cultivated for 48 h, cells stayed on the top chambers were removed carefully with cotton swabs, while the invasive cells on the bottom chamber were subsequently fixed and stained, respectively with formaldehyde (4%) and crystal violet (0.1%). The difference between the migration assay and the invasion assay was that the transwell chambers were not coated with Matrigel. An inverted microscope (Olympus, Tokyo, Japan) was used to measure and count the invasive and migratory cells in five randomly selected fields.

Luciferase Reporter Assay

The amplified wild-type (WT) or mutant (MUT) CDK6-3'-UTR was synthesized chemically and respectively cloned into the pGL3 luciferase vectors (Promega, Madison, WI, USA). Then, the GC cells were seeded in 96-well plates and co-transfected with CDK6-3'UTR-WT or CDK6-3'UTR-MUT together with miR-29c mimics. Subsequently, the Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA) was used to detect the relative luciferase activities 48 h after the transfections.

Statistical Analysis

All the above experiments were conducted at least three times. All the data were shown as the mean ± SD (standard deviation). The GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) together with the Statistical Product and Service Solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA) were applied to assess the statistical analysis. The Student's *t*-test was used to determine the statistically significant differences, $p<0.05$ was considered to be statistically significant.

Results

MiR-29c was Down-Regulated and CDK6 was Up-Regulated in GC

We measured the expressions of CDK6 and miR-29c in GC tissues and the corresponding normal tissues using qRT-PCR. Results showed that the miR-29c expressions in GC tissues were decreased significantly when compared to the corresponding normal tissues (Figure 1A). In the meantime, we also measured the miR-29c expression in GC cells. It was found that, compared to the normal gastric epithelial cell GES-1, the GC cell line AGS presented a significant lower miR-29c expression (Figure 1B). We next detected the mRNA expression level of CDK6 in GC. The results inversely revealed that the CDK6 mRNA expression in the GC cell line AGS was markedly up-regulated in contrast with that in normal gastric epithelial cell GES-1 (Figure 1C). Additionally, we further explored the relationship between CDK6 and miR-29c expressions in GC tissues to fully understand the underlying mechanisms. The results showed that miR-29c expressions were negatively correlated with CDK6 expressions in GC (Figure 1D).

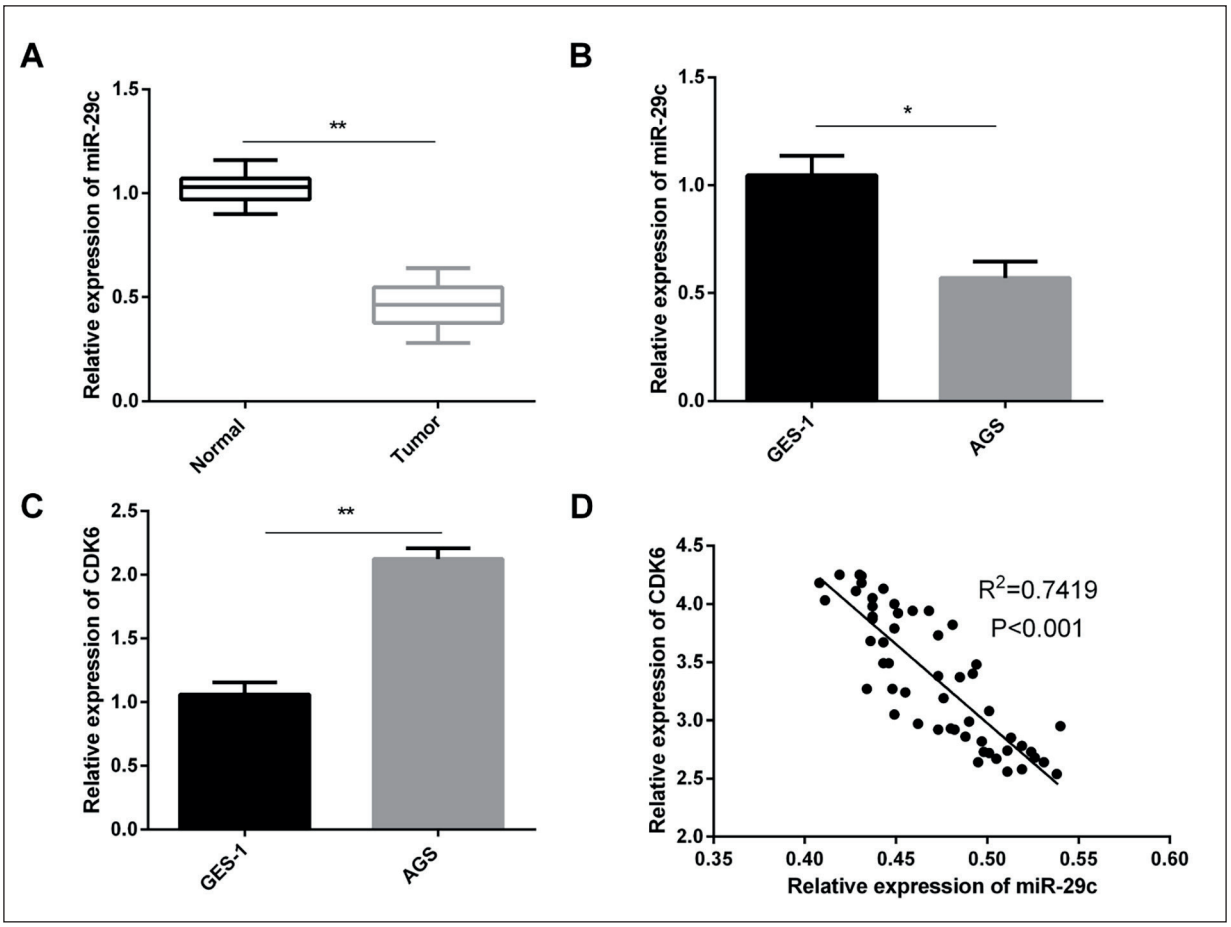

Figure 1. MiR-29c expression was decreased and CDK6 expression was increased in GC. **A,** The qRT-PCR analysis was applied to detect the miR-29c expressions in GC tissues (n=54) and matched normal tissues (n=54) (**p<0.01). **B,** The miR-29c expressions in GC cells were determined using qRT-PCR (*p<0.05). **C,** The CDK6 expression in GC cells was measured using the qRT-PCR (**p<0.01). **D,** Correlation between miR-29c and CDK6 expressions in GC tissues were analyzed.

MiR-29c Repressed GC Cell Migration and Invasion

We next investigated the functions of miR-29c in GC invasion and migration by performing transwell assays. Firstly, we transfected miR-29c mimics or inhibitor into GC cell lines to induce or inhibit the miR-29c expressions. Then, qRT-PCR was conducted to verify the transfection efficiencies (Figure 2A). Subsequently, we performed transwell assays to determine the invasion and migration capacities of GC cell line AGS which were transfected with miR-29c mimics or inhibitor. As shown in Figure 2B, the miR-29c over-expression significantly suppressed while the miR-29c down-regulation prominently enhanced cell invasion ability when compared to the control group. Furthermore, we examined the migration capacity of AGS cells which were treated with different transfections. It was found that the migration ability of miR-29c-overexpressed AGS cells was significantly inhibited when the migration of the miR-29c-inhibited AGS cells was markedly promoted (Figure 2C and 2D).

MiR-29c Inversely Modulated the CDK6 Expressions via Directly Targeting its 3'UTR

Bioinformatics analysis by TargetScan indicated that CDK6 was a functional target of miR-29c. The target sequences of miR-29c in CDK6 3'UTR or the mutant sequences were inserted into the luciferase reporter vectors (Figure 3A). Subsequently, the luciferase reporter assay was carried out to confirm whether CDK6 was a

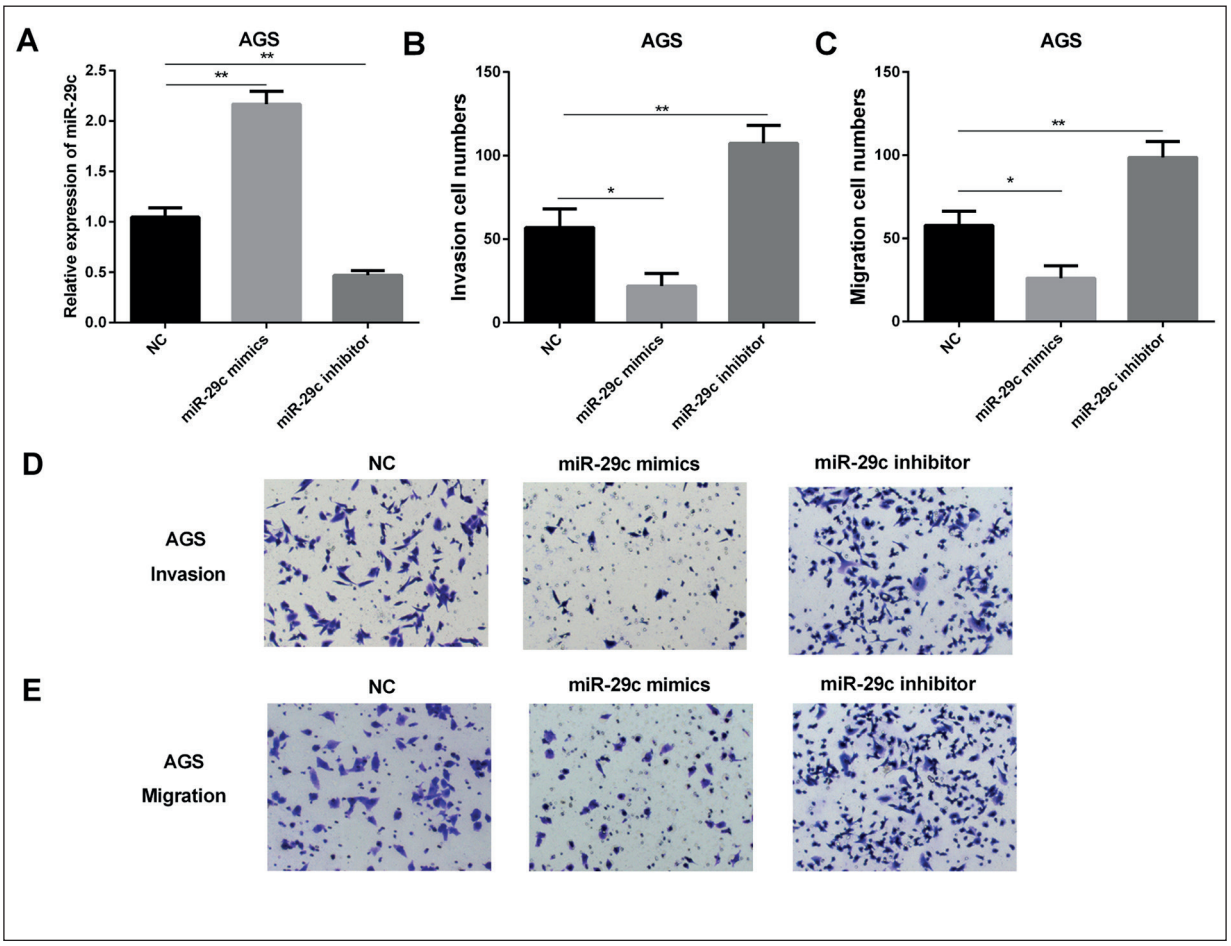

Figure 2. MiR-29c inhibited GC cell invasion and migration. **A,** The miR-29c expressions in GC cells transfected with miR-29c mimics or inhibitor were detected using the qRT-PCR (**p<0.01). **B,** The invasion cell numbers of GC cells were counted (*p<0.05, **p<0.01). **C,** The migration cell numbers of GC cells were counted (*p<0.05, **p<0.01). **D,** Cell invasion was observed by the transwell assay in transfected GC cells (magnification: 40×). **E,** The transwell assays were conducted to detect cell migration in transfected GC cells (magnification: 40×).

direct target of miR-29c. The results revealed that the AGS cells with transfections of miR-29c mimics significantly reduced the luciferase activity of CDK6-3'-UTR-WT; on the other hand, the luciferase activity of CDK6-3'-UTR-MUT was not notably affected by the miR-29c mimics (Figure 3B), suggesting that the interaction between CDK6 and miR-29c was specific. Moreover, we next examined the effects of miR-29c on the mRNA expressions and protein expressions of CDK6 using qRT-PCR and Western blots. Data demonstrated that the miR-29c over-expression in the AGS cells resulted in depressed CDK6 mRNA and protein expressions; on the contrary, the miR-29c down-regulation contributed to an increase in CDK6 mRNA and protein expressions (Figure 3C and 3D).

Knockdown of CDK6 Reversed the MiR-29c-Mediated Inhibitory Effects on GC Cell Invasion and Migration

To further determine the synergistic functions between miR-29c and CDK6 in GC cell invasion and migration, the rescue experiments were performed by co-transfecting with the miR-29c inhibitor and CDK6 siRNA into AGS cells. qRT-PCR and Western blots results showed that, compared to the cells with transfection of miR-29c inhibitor, CDK6 siRNA was sufficient to inhibit CDK6 mRNA and protein expressions in the AGS cells co-transfected with the CDK6 siRNA and miR-29c inhibitor (Figure 4A and 4B). Subsequently, transwell assays were used to determine the functions of CDK6 siRNA in the GC cell invasion and migration. The results indicated that

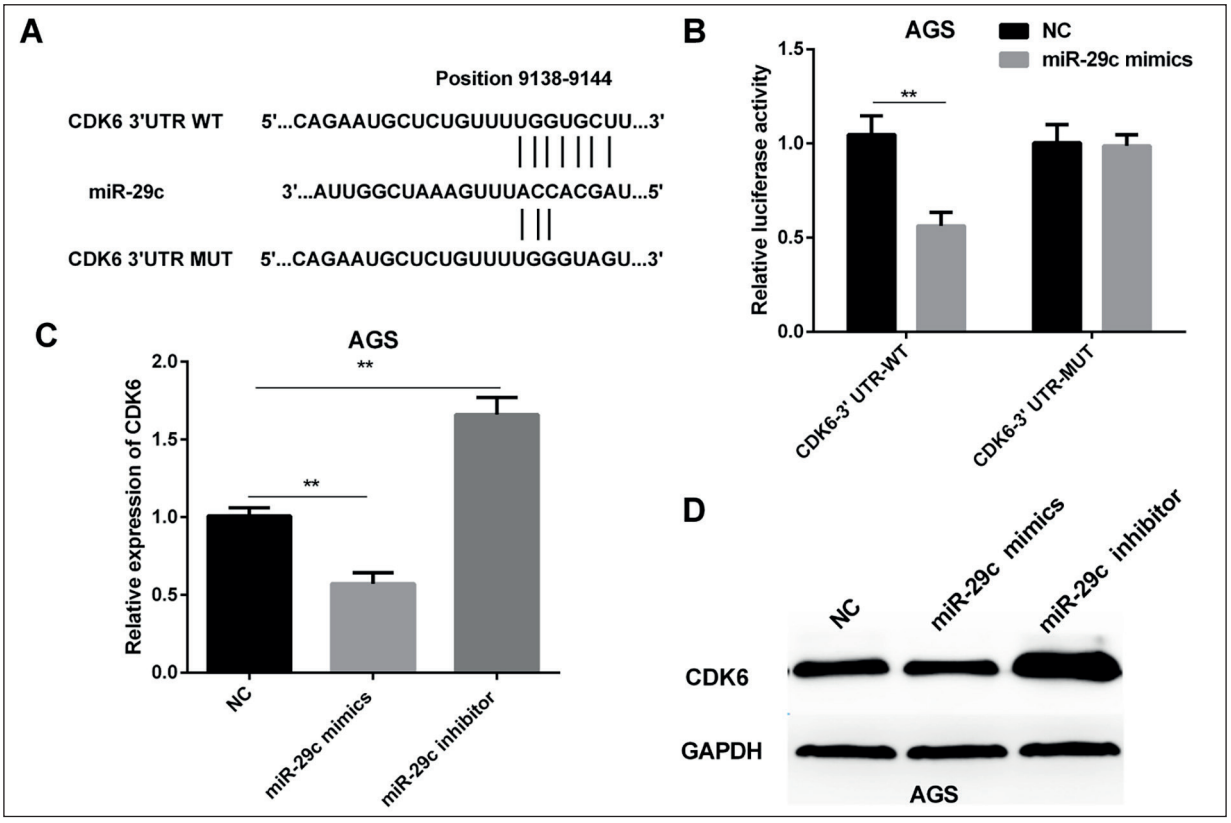

Figure 3. MiR-29c regulated CDK6 expression *via* directly binding its 3'-UTR. **A,** The binding sequences of miR-29c in the CDK6 3'-UTR were shown. **B,** The luciferase reporter gene assay was carried out to show the correlation between miR-29c and CDK6 (**p<0.01). **C,** The qRT-PCR analysis was used to determine the regulatory functions of miR-29c in the CDK6 mRNA levels in GC cells (**p<0.01). **D,** Western blots were performed to detect the CDK6 expressions in GC cells with different transfections.

cell migration and invasion were promoted when the miR-29c inhibitor was transfected into AGS cells; however, the invasion and migration were both partially reversed by co-transfecting with miR-29c inhibitor and CDK6 siRNA. Thus, these results suggested that CDK6 was implicated in miR-29c-mediated suppression functions in GC cell invasion and migration (Figure 4C and 4D).

Discussion

As one common malignancy globally, GC remains a huge burden and serious threat for human health[18]. Recently, although remarkable progress has been made in GC diagnosis and treatment, the recurrence and metastasis make the prognosis of patients with GC still dismal[19,20]. In recent years, a number of studies have shown that GC is a kind of multi-stage processes caused by the accumulation of epigenetic and genetic alterations, especially the aberrant expressions of miRNAs[21]. Therefore, accurate determinations of miRNA expressions in cancer tissue are important parameters to understand the key functions of miRNAs in various biological processes, including development, apoptosis, metastasis, and differentiation[22]. Hence, the tumor-related miRNAs have been studied to fully understand GC carcinogenicity and explore new biomarkers.

MiRNAs exert important functions in the regulation of GC pathogeny and progression[23]. A large amount of studies have revealed that different types of miRNAs, which participate in GC carcinogenesis, alter the expression profile in GC. For example, Tang et al[24] revealed that miR-182, through targeting zinc finger, AN1-type domain 4 (ZFAND4, also known as ANUBL1), inhibited GC proliferation and miR-107 could promote cell proliferation in GC *via* targeting cyclin-dependent kinase 8[25]; in addition, Rao et al[26] indicated that miR-122 inhibited the GC cell invasion and proliferation through targeting cAMP responsive element binding protein 1 (CREB1). In the current

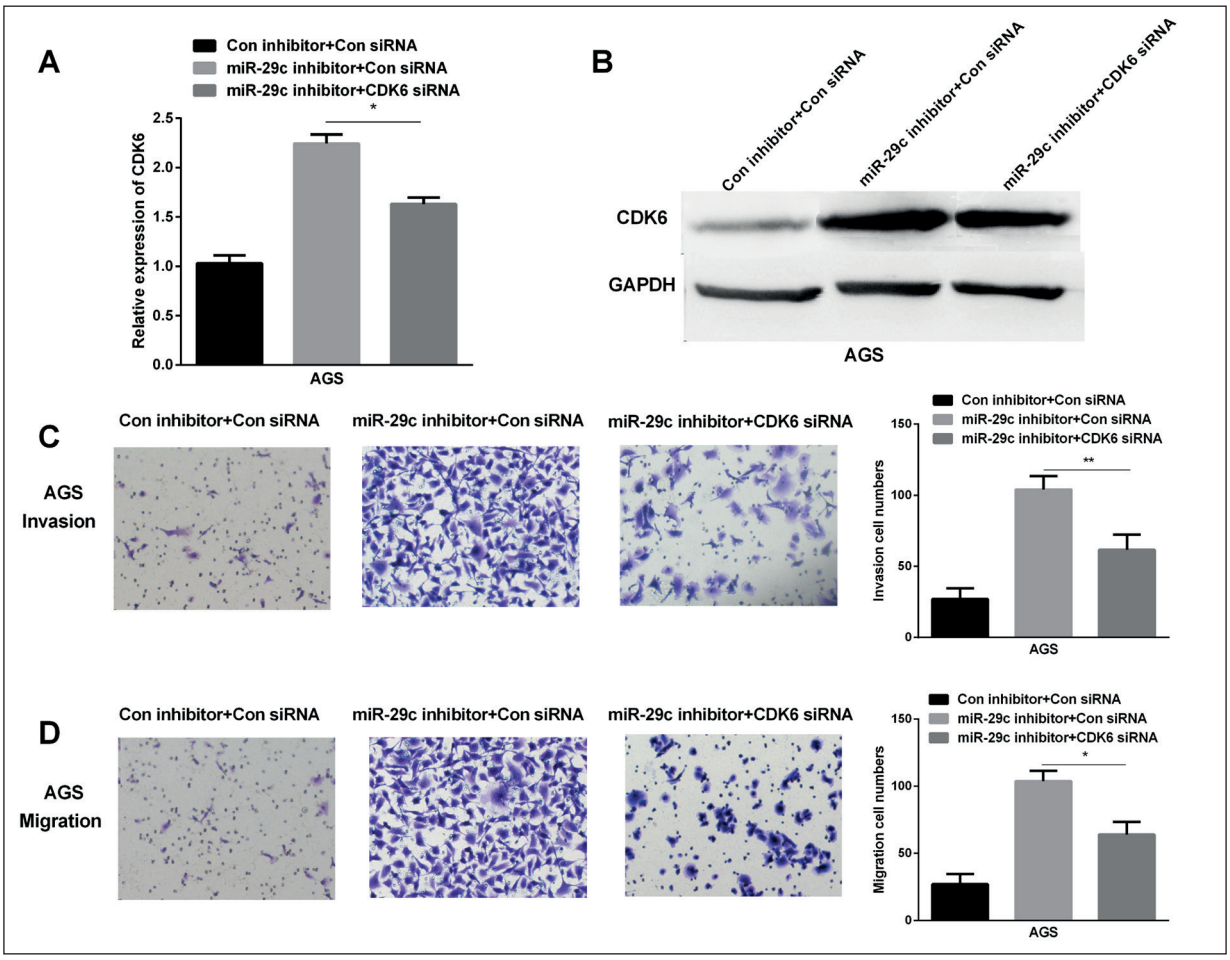

Figure 4. Knockdown of CDK6 abrogated the inhibition function mediated by miR-29c in GC cell invasion and migration. **A-B,** The mRNA or protein expression level of CDK6 was measured by qRT-PCR or Western blots in GC cells which were co-transfected with the CDK6 siRNA and miR-29c inhibitor (*p<0.05). **C-D**, Transwell assays were conducted to detect migration and invasion ability in GC cells co-transfected with CDK6 siRNA and miR-29c inhibitor (*p<0.05, **p<0.01) (magnification: 40×).

study, miR-29c was shown to be down-regulated in GC and miR-29c over-expression could inhibit the GC cell invasion and migration, suggesting that miR-29c functioned as a tumor suppressor in GC. These results supported the findings of the previous researches[27,28].

In the current research, CDK6 was identified as a new functional target of miR-29c. CDK6 belongs to the family of serine-threonine kinases[29], the activation of which could facilitate cell cycle[30]. CDK6 has been regularly reported to be over-expressed in various cancers, including hepatocellular carcinoma[31], non-small cell lung cancer[32], and osteosarcoma[33]. In line with this evidence, our data showed an increased expression level of CDK6 in GC, which was negatively correlated with miR-29c expressions. Western blots and the luciferase reporter assays were also conducted in the experiments of GC cells. The results also demonstrated that the CDK6 down-regulation could restore the miR-29c effects in GC cells.

Conclusions

MiR-29c expressions were reduced in GC tissues and cells. MiR-29c over-expression effectively suppressed GC cell invasion and migration. Moreover, CDK6 was identified as a direct functional target of miR-29c in GC. The current work provides new insights into the GC development and suggests that the miR-29c/CDK6 axis may be a therapeutic candidate target for GC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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