

Original Article

Tumor-suppressive microRNA-195-5p regulates cell growth and inhibits cell cycle by targeting cyclin dependent kinase 8 in colon cancer

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Abstract: MicroRNAs (miRNAs) are key regulators in gene expression. Dysregulation of them in cancer development have been attracting increasing attention. The purpose of this study was to investigate the potential role of miR-195-5p in colon cancer (CC) biology. Expression of miR-195-5p in CC specimens and adjacent normal tissues were measured by quantitative polymerase chain reaction (qPCR). Overexpression of miR-195-5p was established by transfecting mimics into SW480 CC cells. Following, MTT assays, wound healing assays, invasion assays and cell cycle assays were used to explore the potential function of miR-195-5p in SW480 cells. Dual-luciferase reporter assays were performed to validate the regulation of a putative target of miR-195-5p, in corroboration with qPCR and western blot assays. The expression of miR-195-5p in CC specimens was significantly lower than that of adjacent normal tissues ($P < 0.05$). Overexpression of miR-195-5p inhibited cellular growth, suppressed cellular migration and invasion, and led to cell cycle arrest at G1 phase *in vitro*. Dual-luciferase reporter assays showed that miR-195-5p binds the 3'-untranslated region (UTR) of CDK8, suggesting that CDK8 should be a direct target of miR-195-5p. Moreover, qPCR and western blot assays confirmed CDK8 mRNA and protein levels were reduced after overexpression of miR-195-5p. These findings are supportive of miR-195-5p as a novel tumor suppressor in CC, thus may serve as a new strategy for cancer treatment.

Keywords: MiR-195-5p, cell cycle, CDK8, SW480, colon cancer

Introduction

Colon cancer (CC) is one of the leading causes of death worldwide [1]. Improvement of diagnosis and treatment has resulted in improved long-term survival for patients with early CC. Therapy for advanced CCs may include a combination of surgery, chemotherapy or other effective therapeutic regimen, whereas the survival of advanced disease remains poor. Therefore, it is essential to develop more effective therapeutic methods.

MicroRNAs (miRNAs) are composed of approximately 20-25 nucleotide-non-coding RNAs, which can regulate gene expression at post-transcriptional level through inhibiting protein translation or degrading mRNA of target gene [2, 3]. Many studies [4, 5] have shown that miR-

NAs are involved in a variety of processes including tumor cell proliferation, differentiation, and apoptosis. Moreover, aberrant miRNA expression has been frequently observed in various types of human tumors. These reports suggest that miRNAs may function as either tumor-suppressor genes or oncogenes [6]. Subsequently, miRNA research has become a hot spot in CC research whereby miRNAs are believed to have broad prospects in terms of diagnosis and treatment of this disease [7]. Recent studies showed that miR-195 was significantly downregulated in CC [8, 9]. However, the role that miR-195-5p plays in development of CC is still largely unknown.

Cyclin dependent kinase 8 (CDK8), is a member of the CDK family, which is located on chromosome 13q12 and is found to be frequently dys-

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regulated in various human cancers, including gastric cancer [10], pancreatic cancer [11], and colorectal cancers [12]. CDK8 was identified as an oncogene involved in the proliferation and cell cycle regulation of CC cells [12, 13]. However, mechanisms for the regulation of CDK8 activity are not fully understood. The computer sequence analysis [14] (TargetScan and miRDB) suggested that the 3'-untranslated region (UTR) of CDK8 mRNA might represent a target of miR-195-5p, we speculate that miR-195-5p may play an important role by targeting CDK8 in CC.

In this study, we found that miR-195-5p was down-regulated in CC tissues when compared with that of adjacent normal tissues. Over-expression of miR-195-5p inhibited proliferation of SW480 CC cells, in association with inhibition of migration and invasion, and disrupted the cell cycle by targeting CDK8. Therefore, our findings demonstrated the role of tumour suppressor of miR-195-5p in CC progression and indicated that miR-195-5p might serve as a novel therapeutic target for CC.

Materials and methods

Specimens and ethics

A total of 42 CC specimens and matched adjacent normal colon tissues were surgically obtained from patients at the Department of Gastrointestinal Surgery of the Shanghai East Hospital. Collection of the patient specimens was approved by the Ethics Review Board of Tongji University, Shanghai, China. Informed consents were obtained from all patients. All tissue specimens were evaluated pathologically. No patients had received irradiation or cancer chemotherapy prior to resection.

Cell culture and transfection

SW480 CC cells and HEK293T cells were purchased from the Chinese Science Institute (Shanghai, China). Cells were maintained in incubator at 37°C in 5% CO₂ and 95% air. These cells were cultivated and refreshed every 3-4 days with Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 u/ml) and streptomycin (100 µg/ml) (Enpromise, China). Cells with over 95% viability as shown by trypan blue staining were qualified for further experiments.

miR-195-5p mimics and non-specific negative control (NC) oligos were purchased from GenePharma (Shanghai, China). The sequence of the miR-195-5p mimic was 5'-UAGCAGC-ACAGAAUAUUGGC-3' and the sequence of the NC mimic was 5'-UCACAACCUCCUAGAA-AGAGUAGA-3'. For transfection, cells (3×10^5) were added into each well of a 6-well plate and cultured with serum- and antibiotic-free DMEM. When the cell density achieved 30-40% confluence, Lipofectamine transfection reagent (Invitrogen, USA) was used to introduce the mimics according to the manufacturer's instructions. The ratio of mimics to Lipofectamine was 1 µg:3 µl.

MiRNA isolation and quantitative polymerase chain reaction (qPCR)

MiRNAs were extracted from tumor and adjacent normal colon tissues by using the miRcute microRNA isolation kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The expression level of miR-195-5p was detected by the One-Step qRT-PCR method (EzOmics SYBR qPCR kit). The miR-195-5p primer, U6 primer and EzOmics SYBR qPCR kit were purchased from Biomics Biotechnologies Inc (Jiangsu, China). The U6 primer used as an internal control was: 5'-GTCCTATCCAGTGCAGG-GTCCGAGGTGCACTGGATACGACAAAATATGGA-AC-3' (stem-loop primer) 5'-TGCGGGTGCTCGC-TTCGCAGC-3' (sense) and 5'-CCAGTGCAGGGT-CCGAGGT-3' (antisense). Briefly, for amplification of miR-195-5p, 100 ng RNA was used in a 25-µl reaction system containing 12.5 µl 2X Master Mix, 0.5 µl 50X SYBR-Green, 0.5 µl reverse transcription primer (10 µM), 0.5 µl sense and 0.5 µl antisense primers (10 µM). One Step PCR parameters for miRNA quantification were as follows: 37°C for 60 min for reverse transcription, 10 min at 95°C, and then 40 cycles of 20 sec at 95°C, 30 sec at 62°C and 30 sec at 72°C. Each sample was tested in triplicate.

Total RNAs were isolated from the cultured cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. For CDK8 mRNA detection, reverse transcription was performed using the PrimeScript RT-PCR kit (Takara, Shiga, Japan). Real-time PCR was performed using a 7900HT Fast RT-PCR instrument (Applied Biosystems,

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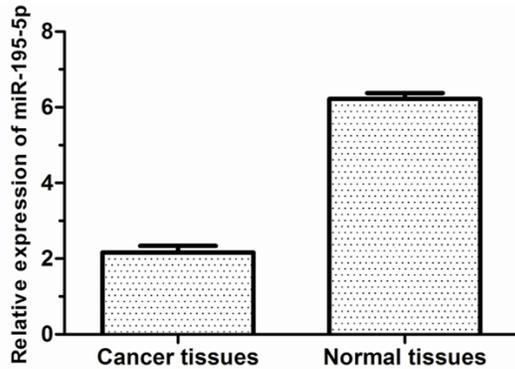


Figure 1. MiR-195-5p is significantly decreased in CC tissues. The graph represents the $2^{-\Delta\Delta Ct}$ values \pm SEM, *P < 0.05.

Singapore) using SYBR-Green. GAPDH mRNA levels were used for normalization. The primer sequences were as follows: CDK8, 5'-GGG-ATCTCTATGTCGGCATGTAG-3' (sense) and 5'-AAATGACGTTTGGATGCTTAA-3' (antisense); GAPDH, 5'-AAGGTCGGAGTCAACGGATT-3' (sense) and 5'-CTGGAAGATGGTGATGGGATT-3' (antisense). The PCR parameters for relative quantification were as follows: 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 57°C and 45 sec at 72°C. Each sample was tested in triplicate, and the relative quantification equation was used to calculate the relative expression [15].

Cell proliferation[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay

SW480 cells were plated at 3,000/well in 96-well plates (BD Biosciences, USA) and incubated at 37°C. When cells reached 30-40% confluence, they were transfected with either 50 or 100 nM miR-195-5p mimics or NC mimics. One group of cells was treated with lipofectamine alone as a mock control. We then assessed cell proliferation at 12, 24, 36 and 48 h post-transfection using the MTT assay. Briefly, 20 μ l (5 mg/ml) MTT (Sigma, USA) solution was added to each well. After a 4-h incubation at 37°C, the supernatant was removed and 150 μ l DMSO was added. After 10 min of agitation (100 rpm), the absorbance at 490 nm of each sample was measured by a microplate spectrophotometer. Each experiment was performed in triplicate and included 6 replicates.

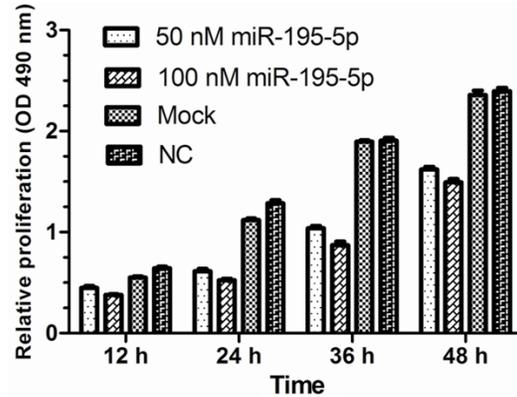


Figure 2. Overexpression of miR-195-5p inhibited cell proliferation. The proliferation of SW480 cells transfected with miR-195-5p (50 and 100 nM) was examined at various time points and compared with that of the mock and NC groups. Graph represents OD 490 nm \pm SEM, *P < 0.05.

Cell migration and invasion assay

Cell migration was evaluated by the wound healing assay. SW480 cells were transfected with miR-195-5p mimics (100 nM) or NC mimics, and when cells reached 90% confluence, a scratch was made through each well using a sterile pipette tip. Cells were monitored under the microscope (magnification, $\times 100$) for 0, 12, 24 and 48 h after wounding. Images of cells were captured at the same position before and after incubation to document the repair process. The experiments were repeated three times.

Transwell invasion assays were performed to evaluate invasion ability as follows: 600 μ l 10% FBS DMEM was added into a 24-well plate, and a transwell filter insert was placed into the well; the filter was filled with 5×10^4 cells in 200 μ l DMEM with 0.1% BSA, and the cells were cultured for 30 h at 37°C in 5% CO₂. Invasion ability was observed under an inverted microscope, and when the cells crossed into the basal well, the invasion was terminated. The matrigel was scraped off, and numbers of cells remaining in the basal well were analyzed. The filters were washed three times with PBS, fixed with 4% paraformaldehyde, stained by 0.1% Crystal Violet solution, and washed three times with water. Images of the stained cells were obtained. To quantify the number of invading cells, Crystal Violet-stained cells in 10 random

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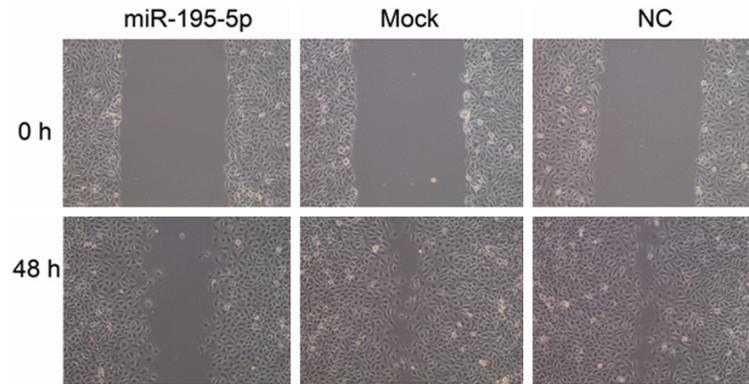


Figure 3. Overexpressing of miR-195-5p in SW480 cells showed impaired migration in wound healing assays. Images were obtained on an inverted microscope with x100 magnification.

visual fields were counted, to compute the mean cell numbers.

Cell cycle assay

MiR-195-5p (100 nM), mock and NC cells were harvested at 48 h after transfection, centrifuged at 1,200 rpm for 10 min and washed three times with cold PBS. Ice-cold 70% ethanol was subsequently added dropwise, and the cells were fixed at 4°C overnight. After a 30-min digestion in RNase (0.1 g/l), a total of 250 μ l (0.05 g/l) propidium iodide (PI) staining solution was added to each sample which was then incubated for 30 min at room temperature (RT) in the dark. Cell cycles were then analyzed by a flow cytometer.

Dual-luciferase reporter assay

HEK293T cells were seeded in 12-well plates (BD, USA) in complete medium and incubated at 37°C with 5% CO₂. CDK8 3'-UTR were cloned into the psiCHECK-2 vector, and co-transfected with miR-195-5p mimics (100 nM) or NC mimics when cells reached 80-90% confluence. Thirty-six hours after transfection, luciferase activity was measured by the dual-luciferase reporter assay kit (Promega, USA). Briefly, the cells were washed twice with PBS then lysed by incubation at RT for 15 min with passive lysis buffer (PLB). The supernatants were collected, and 20 μ l of the aliquots was added to 96-well plates. The firefly luciferase (FL) reporter was measured immediately after adding Luciferase Assay Reagent II (LAR II). Next, 100 μ l of Stop & Glo® reagent was added to each well to initiate

the Renilla luciferase (RL). The psiCHECK-2 vector that provided constitutive expression of FL was co-transfected as an internal control. All experiments were performed for three times.

Western blot assay

The proteins were resolved on an SDS denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. Antibody to CDK8 or β -actin was incubated with the membranes overnight at 4°C. Blots were then washed

and incubated for 1 h with secondary antibodies. After washing with PBST, immunoreactive protein bands were detected using the Odyssey scanning system (Li-Cor, Lincoln, NE, USA).

Statistical analysis

Data from at least three separate experiments are presented as mean \pm standard error of the mean (SEM). The two tailed *t*-test was used to draw a comparison between groups. Differences were considered significant for *P*-values less than 0.05. All data were assessed using the SPSS version 18.0 (SPSS, Chicago, IL, USA).

Results

miR-195-5p expression was decreased in CC specimens

The expression levels of miR-195-5p were measured in CC specimens and the adjacent normal tissues by real-time PCR (**Figure 1**). Compared with the adjacent normal tissues, miR-195-5p expression was significantly decreased in the CC specimens (*P* < 0.05).

Overexpression of miR-195-5p in SW480 CC cells inhibited cell proliferation

The viability of cells transfected with either 50 or 100 nM miR-195-5p mimics was measured by performing MTT assays. As depicted in **Figure 2**, overexpression of miR-195-5p groups (50 and 100 nM) showed significantly lower optical density (OD) values at 490 nm than the

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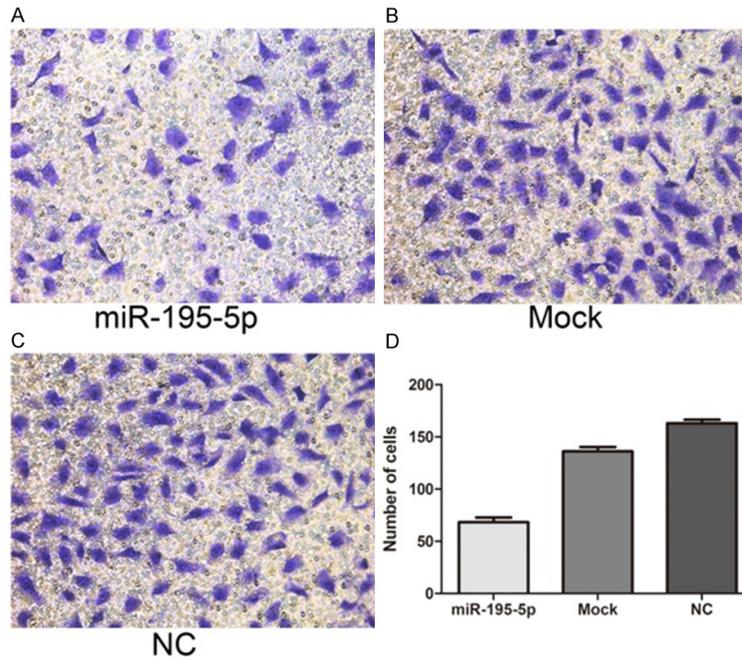


Figure 4. Overexpression of miR-195-5p inhibited cell invasion ability. A-C. Images were obtained on an inverted microscope with x200 magnification. D. Quantification of the number of crystal violet cells. Data represent mean + SEM, * $P < 0.05$.

mock and NC groups at 12, 24, 36 and 48 h. Thereby miR-195-5p at a concentration of 100 nmol/l showed the greatest inhibitory effect, 100 nM was used in the following experiments. These results demonstrated that overexpression of miR-195-5p suppressed the cellular proliferation of SW480 cells.

Overexpression of miR-195-5p in SW480 cells inhibited cell migration and invasion

The migratory and invasion ability of SW480 cells with and without transfection of miR-195-5p mimics was detected by wound healing assays and transwell assays. The wound healing assay results showed that the migration ability of the miR-195-5p groups was lower than either the mock or NC groups. As shown in **Figure 3**, the monolayer of miR-195-5p transfected cells still showed a clear gap in the scratched region compares with the mock and NC groups at 48 h. These results showed that overexpression of miR-195-5p in SW480 cells inhibited cellular migration.

In transwell invasion assays, the number of invaded cells stained with Crystal Violet of the

miR-195-5p (100 nM) groups (**Figure 4A**) was significantly less than the mock or NC groups (**Figure 4B and 4C**). Invasion rates of groups were determined by counting the number of cells that invaded through matrigel and confirmed the results observed by inverted microscope (**Figure 4D**). These results indicated that overexpression of miR-195-5p could suppress cellular invasion ability.

miR-195-5p disrupted the cell cycle of SW480 cells

Thirty-six hours after the transfection of miR-195-5p mimics (100 nmol/l), flow cytometry analysis revealed that the percentage of G0/G1 phase cells dramatically increased in the miR-195-5p groups, when compared with that of the mock and NC

groups ($P < 0.05$) (**Figure 5A**). While the proportion of S-phase and G2/M phase cells decreased in the miR-195-5p groups compared with that of the mock and NC groups ($P < 0.05$) (**Figure 5B and 5C**). These findings suggested that miR-195-5p could initiate G0/G1 phase arrest and that upregulation of miR-195-5p expression could lead to the reduction of S-phase and G2/M phase cells. The respective proportions of G0/G1, S and G2/M phase cells were shown **Figure 5D**.

miR-195-5p regulated CDK8 expression by targeting its mRNA in SW480 cells

The bioinformatic algorithms TargetScan and miRDB suggested that the 3'-UTR of CDK8 mRNA might represent a target of miR-195-5p. We found a potential binding site for miR-195-5p which was located 1525-1531 bp downstream from the 5' end of the CDK8 3'-UTR (**Figure 6A**). Next we constructed a psiCHECK-2/CDK8 3'-UTR vector, which contained the Renilla luciferase (RL) gene and the 3'-UTR region of CDK8. This vector was transfected into 293T cells together with either miR-195-5p or NC mimics, and the luciferase activity was

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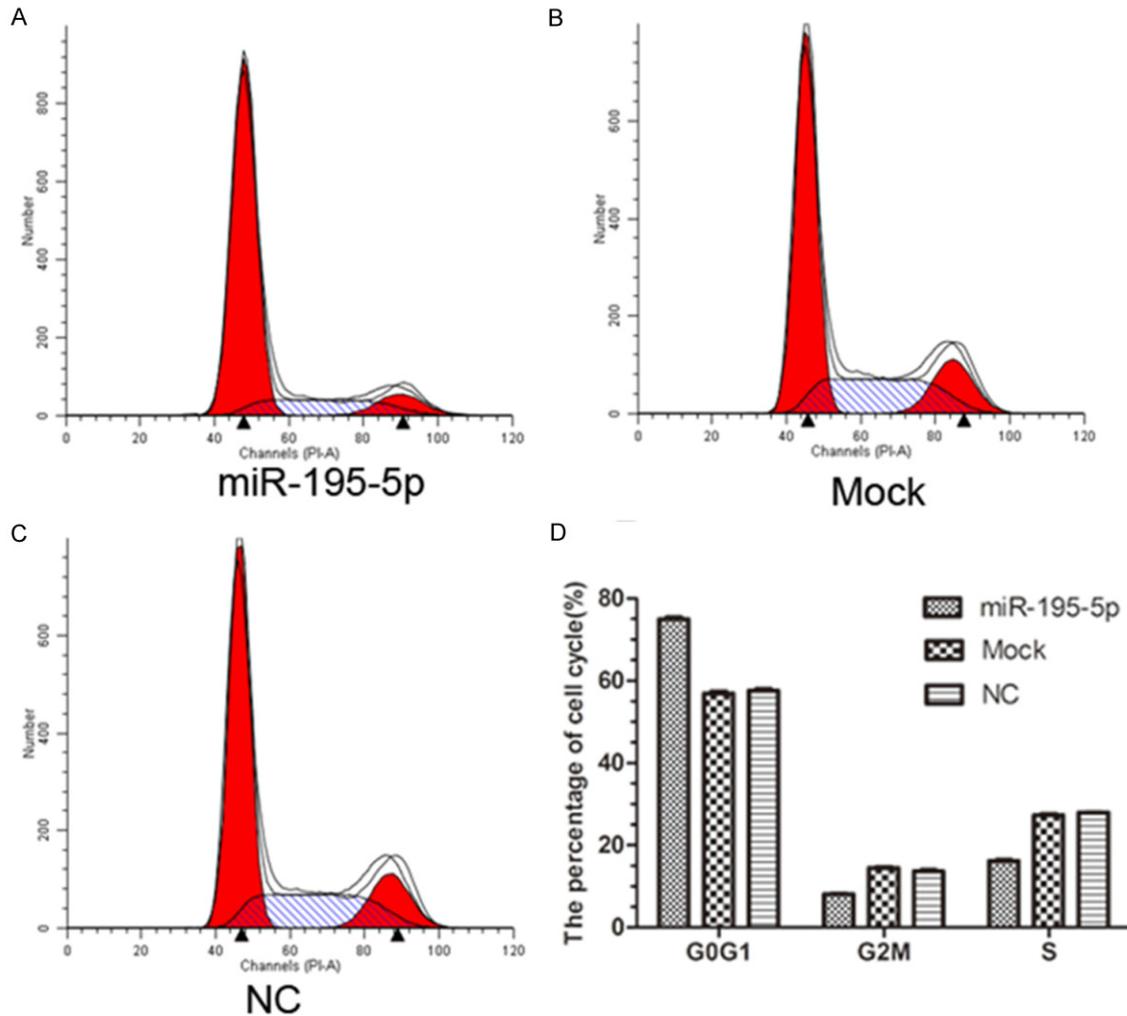


Figure 5. Overexpression of miR-195-5p disrupted the cell cycle distribution. A-C. Cell cycle distribution was analyzed by flow cytometry 36 h after transfection of SW480 cells. D. The respective proportion of cell cycle phase of miR-195-5p, mock and NC groups, * $P < 0.05$.

analyzed. The ratio of FL/RL was calculated, and showed that the miR-195-5p group had over 3-fold higher activity than that of the NC group ($P < 0.05$) (Figure 6B). These results suggested that miR-195-5p directly interacted with the CDK8 3'-UTR in the psiCHECK-2 reporter plasmid and led to the degradation of RL mRNA.

Finally, we performed qPCR and western blot analysis of CDK8 expression in SW480 cells with and without transfection of miR-195-5p mimics, or NC controls. We found that overexpression of miR-195-5p significantly decreased CDK8 expression at both the mRNA and protein levels (Figure 7A and 7B). These data further

indicated that CDK8 was a potential target of miR-195-5p.

Discussion

The occurrence of CC is a complex process, which includes the activation of oncogene and inactivation of tumor suppressor genes caused by genetic and environmental changes. MiRNAs represent a class of small non-coding RNAs that regulate the gene expressions at the post-transcriptional level and subsequently control some crucial physiological and pathological processes [16]. Many studies published in recent years have used miRNA expression profiles for cancer diagnosis and prognosis [17,

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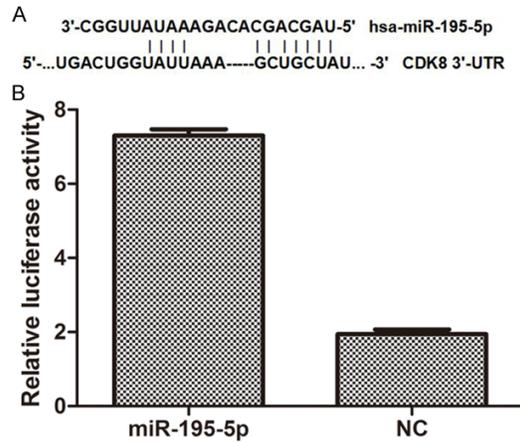


Figure 6. CDK8 was a direct target of miR-195-5p. A. The binding sites for miR-195-5p in the 3'-UTR of CDK8 mRNA. B. The relative luciferase activity (FL/RL) was measured in HEK293T cells after co-transfection of the CDK8 luciferase construct with either miR-195 or NC; *P < 0.05.

18]. The expression of miR-195 has been previously reported to be decreased in CC [8, 9, 19]. miR-195-5p and miR-195-3p belong to miR-195, and our study researched the carcinogenesis of miR-195-5p in CC.

In this study, we examined the expression of miR-195-5p in human CC tissues and its potential role in disease pathogenesis. Firstly, RT-PCR results showed that the expression level of miR-195-5p was significantly decreased in the CC specimens. This indicated that miR-195-5p might play an important role in the pathogenesis of CC. Next, we transfected miR-195-5p mimics into SW480 cells to generate its overexpression. This led to significant inhibition of cellular proliferation as measured by MTT assays, which implied that miR-195-5p repressed the growth of colon cells. Using the wound healing assays, we found that the overexpression of miR-195-5p in SW480 cells could suppress cellular migratory ability. We also found that miR-195-5p could suppress cell invasion ability by transwell assays. Furthermore, analysis of miR-195-5p mimic cells by flow cytometry showed that miR-195-5p induced G1 arrest compared with mock and NC cells. These findings suggested that miR-195-5p has an inhibitory role in CC and might be a promising therapeutic candidate for CC.

We further investigated the target genes of miR-195-5p by using prediction methods of

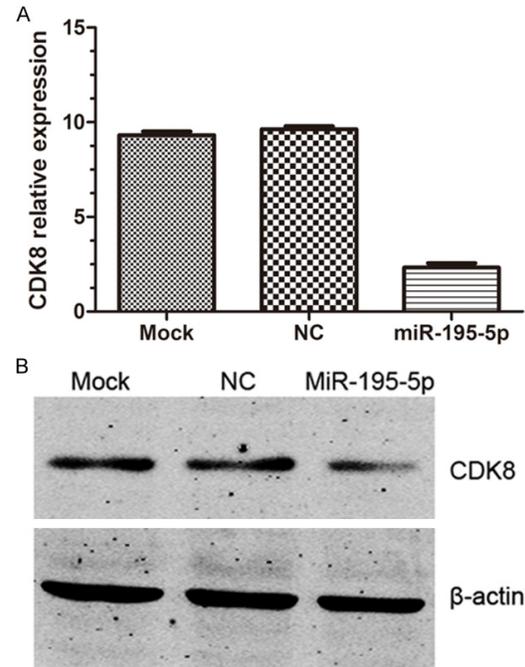


Figure 7. miR-195-5p inhibited CDK8 expression. qRT-PCR (A) and western blot (B) analysis of CDK8 mRNA and protein levels, respectively.

bioinformatics or literature. We found that the sequence 5'-UAGCAGC-3' of miR-195-5p could combine with the sequence 3'-UTR 5'-GCUGCUA-3' of CDK8 gene mRNA thus inhibiting the transcription and translation of CDK8 gene. CDK8 was an important proto-oncogene inhibiting apoptosis and its abnormal expression had close relationship with the occurrence and development in many tumors [12, 20]. Evolving understanding of CDK8 activity might allow development of a robust responder hypothesis and personalized clinical approach. Through luciferase assays, we demonstrated CDK8 as a direct target of miR-195-5p in CC cells. Additionally, we found that both the mRNA and protein levels of CDK8 were significantly lower in miR-195-5p than those in mock and NC groups. These findings support the prediction that CDK8 is a downstream target of miR-195-5p.

Collectively, our findings suggest that miR-195-5p can disrupt the cell cycle by targeting CDK8 in SW480 cells. We show that its overexpression can reduce cell proliferation and inhibit the migratory and invasion ability of cancer cells. The findings reveal that miR-195-5p is a tumor suppressor gene in CC. Moreover, the luciferase

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ase, qPCR and western blot assays illustrate CDK8 as a downstream target of miR-195-5p. The artificial upregulation of miR-195-5p using CDK8 as a therapeutic agent could offer a new promising direction for future CC treatment.

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Disclosure of conflict of interest

None.

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