Hsa_circ_0001696 modulates cell proliferation and migration in colorectal cancer

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Abstract. Circular RNAs (circRNAs) are a novel class of endogenous non-coding RNA molecules that are extensively expressed in a variety of species. Recently, increasing evidence suggests that circRNAs have vital functions indifferent types of human cancer, such as gastric cancer, papillary thyroid cancer and lung cancer. However, the roles of circRNAs in the development of colorectal cancer (CRC) remain unclear. The present study aimed to determine the molecular mechanism underlying hsa_circ_0001696 on the proliferation and migration of CRC cells. Reverse transcription-quantitative PCR analysis was performed to detect hsa_circ_0001696 expression in 18 paired CRC tissues and matched adjacent normal tissues. RNA interference was also performed to decrease hsa_circ_0001696 expression, and its biological effects were further assessed via flow cytometry, wound healing, colony formation and western blot assays. The results demonstrated that hsa_circ_0001696 expression was significantly lower in CRC tissues compared with adjacent normal tissues. Furthermore, hsa_circ_0001696 knockdown promoted cell proliferation and migration, and the number of cell colonies significantly increased. In addition, western blot analysis demonstrated that the protein expression levels of cyclin-dependent kinase 4 (CDK4), cyclin D, cyclin E and matrix metalloproteinase 9 (MMP9) increased. Taken together, the results of the present study demonstrated that hsa_circ_0001696 expression was downregulated in CRC tissues, and inhibition of hsa_circ_0001696 promoted cell proliferation and migration by regulating the levels of CDK4, cyclin D, cyclin E and MMP9.

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Abbreviations: circRNA, circular RNA; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; CCK-8, Cell Counting Kit-8; PBS, phosphate buffered saline; CDK4, cyclin-dependent kinase 4; MMP9, matrix metalloproteinase 9

Key words: colorectal cancer, hsa_circ_0001696, proliferation, migration, CDK4, MMP9

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor, and it has high rates of global morbidity and mortality. There were over 1.8 million newly diagnosed CRC cases worldwide in 2018, and there were estimated to be over 880,000 mortalities (1). Affected by the western diet and lifestyle, the incidence and mortality rates of CRC have continued to increase in China, rising to the 3rd and 5th highest, respectively (2). The prognosis of CRC is closely associated with early diagnosis (3,4). As reported in 2012, the 5-year survival rate of early cancer was reported as 90%, and for the late stage, it was <10% (5). Thus, it remains crucial to identify accurate and non-invasive molecular biomarkers for CRC diagnosis and treatment.

Circular RNAs (circRNAs), a class of newly discovered endogenous non-coding RNAs that are formed by reverse splicing (6-9), are characterized by high expression levels (10,11). Their expression is often specific to tissues or developmental stages (8,12). Thus, circRNAs are considered effective biomarkers for the diagnosis and prognosis of different types of cancer due to their abundance and stability (13-17). Increasing evidence suggests that circRNAs have a large number of miRNA binding sites and serve as miRNA sponges to indirectly regulate gene expression (6,18,19). CircRNAs play an important role in human diseases (20-22), particularly in tumors, such as gastric cancer (23), papillary thyroid cancer (24) and lung cancer (25). However, research on circRNAs in human CRC is limited.

In the present study, circRNA databases (http://circbase.org) andcirc2Traits (http://gyanxet-beta.com/circdb) were used to screen two human circRNAs (hsa_circ_0001696 and hsa_circ_0001695) that are associated with CRC. The results demonstrated that hsa_circ_0001696 expression was downregulated in CRC tissues compared with adjacent normal tissues, while no significant differences were observed in hsa_circ_0001695 expression between the two groups. Based on these results, human hsa_circ_0001696 was identified as a candidate circRNA. Hsa_circ_0001696 is 347 nucleotides in spliced sequence length, its gene is located at chr7:35707043-35712888 and the gene symbol is HERPUD2. Taken together, the results of the present study suggest that

suppressing hsa_circ_0001696 expression may affect the proliferation and migration of CRC cells.

Materials and methods

Patients and clinical specimens. A total of 18 paired CRC tissues and matched adjacent normal tissues (5 cm from the edge of CRC) were collected from resected surgical specimens at the Ningbo First Hospital, Ningbo Hospital of Zhe Jiang University (Zhejiang, China), between August 2017 and February 2018. The study included 7 men and 11 women (mean average age, 63 years; age range, 47-81). All tissue samples were immediately frozen in RNA-fixer reagent (Bioteke Corporation) following collection and stored at -80˚C until RNA extraction.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from CRC tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA integrity was assessed using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Inc.), and RNA concentrations were determined using a NanoDrop ND2000 (Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed into cDNA using the GoScript RT System kit, with random primers (Promega Corporation; cat. no. A5001). The following thermocycling conditions were used: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 15 min. qPCR was subsequently performed using the SYBR-Green master mix (Promega Corporation; cat. no. A6001) and Mx3005P real-time PCR System (Stratagene; Agilent), according to the manufacturer's protocols. The following primer sequences were used for qPCR:hsa_circ_0001696 forward, 5'-GGAAGCAGTCTG CCCGAATA-3' and reverse, 5'-CCAAGCACAGAGTCACCA GT-3'; and GAPDH forward, 5'-TCGACAGTCAGCCGCATC TTCTTT-3' and reverse, 5'-ACCAAATCCGTTGACTCCGAC CTT-3'. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (26-28) and normalized to the internal reference gene GAPDH. All experiments were performed in triplicate.

Cell culture and transient transfection. The human CRC cell lines, HCT116 and SW620, were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (both purchased from Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified atmosphere with 5% CO_2. The small interfering (si)RNA and stable negative control (NC) sequences (Table I) were synthesized by Shanghai GenePharma Co., Ltd. For siRNA and NC transfection with the 95% purity and 20 uM initial concentration, cells were seeded into 6-well plates at 60% density and subsequently transfected at room temperature for about 60 min with 80, 100 or 120 nM siRNA after 24 h of cultivation, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequent experiments were performed 24 h post-transfection.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8) assay was performed to assess cell proliferation (TransGen Biotech, Co., Ltd.), according to the manufacturer's protocol. Transfected CRC cells were seeded into 96-well plates at a density of 2,000 cells/well and maintained in DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), and incubated for 24, 48, 72 and 96 h at 37˚C in a humidified atmosphere with 5% CO_2. Subsequently, cells were incubated with CCK-8 solution (10 μl) for 2 h at 37˚C and cell proliferation was analyzed at a wavelength of 450 nm, using a microplate reader (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Colony formation assay. Transfected CRC cells were seeded into 6-well plates at a density of 100 cells/well for 2 weeks to form colonies, and DMEM was changed every 3 days. After 14 days, the supernatant was discarded and cells were washed twice with phosphate-buffered saline (PBS). Cells were fixed with 2 ml methanol (Geneslscherical industry, Jinan, Shangdong, China, https://www.chembk.com/cn/chem/67-56-1) at room temperature for 20 min and subsequently stained with 0.5% crystal violet at room temperature for 15 min. Cells were re-washed with PBS and the number of cell colonies were observed using an inverted fluorescent light microscope (Olympus Corporation, CX33; magnifications, 40x10).

Wound healing assay. Once the HCT116 and SW620 cells reached 95% confluence, the monolayers were scratched (recorded at 0 h) and the transfected CRC cells were cultured with serum-free medium for 24 h. Cells were observed under an inverted fluorescent light microscope at 0 and 24 h (magnifications, 40x10). Image pro plus 6.0 software (Media Cybernetics, Inc.) was used to measure the distance at 0 and 24 h. GraphPad Prismv8.3.0.538 (Graph Pad Software, Inc.) was used to perform statistical analysis.

Western blotting. Total protein was extracted from CRC cells using cell lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), and the BCA method was used to detect the protein. Total protein (30 μg/lane) was separated by 12% SDS-PAGE and the separated proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore). The PVDF membranes were blocked in blocking solution (Beyotime Institute of Biotechnology) for 60 min in a shaker at room temperature and incubated with primary antibodies against: Cyclin-dependent kinase 4 (CDK4; cat. no. ab137675), cyclin D (cat. no. ab62151), cyclin E (cat. no. ab33911), matrix metalloproteinase 9 (MMP9; cat. no. BA0573) and β-actin (cat. no. 4ab010745) overnight at 4˚C on a shaker, all of which used primary antibody dilution buffer and were purchased from Beyotime Institute of Biotechnology. Following the primary incubation, membranes were incubated with HRP-conjugated affinipure rabbit anti-Goat IgG (H+L) (1:5,000; cat. no. SA00001-1; ProteinTech Group, Inc) at room temperature for 60 min. Protein bands were visualized using the bright ECL kit (Advansta, Inc. K-12045-D20, https://advansta.com/products/western-blot-substrate-WesternBright-ECL) and protein bands were detected using an Odyssey Infrared Imaging system (LI-COR Biosciences).

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the

Table I. Primer sequences used for cell transfection.

Name	Primer sequence (5'-3')
siRNA-1 (hsa_circ_0001696)	Forward: CCCGAAUACACCAAUCUCUTT Reverse: AGAGAUUGGUGUAUUCGGGTT
siRNA-2 (hsa_circ_0001696)	Forward: CCUCAAUCCUUCUGAGGAATT Reverse: UUCCUCAGAAGGAUUGAGGTT
siRNA-1 (CDK4)	Forward: GCAUGUAGACCAGGACCUATT Reverse: UAGGUCCUGGUCUACAUGCTT
siRNA-2 (CDK4)	Forward: GCAGCACUCUUAUCUACAUTT Reverse: AUGUAGAUAAGAGUGCUGCTT
siRNA-1 (MMP9)	Forward: CCACCACAACAUCACCUAUTT Reverse: AUAGGUGAUGUUGUGGUGGTT
siRNA-2 (MMP9)	Forward: GCGCUGGGCUUAGAUCAUUTT Reverse: AAUGAUCUAAGCCCAGCGCTT
NC	Forward: UUCUCCGAACGUGUCACGUTT Reverse: ACGUGACACGUUCGGAGAATT

siRNA, small interfering RNA; CDK4, cyclin-dependent kinase 4; MMP9, matrix metalloproteinase 9; NC, negative control.

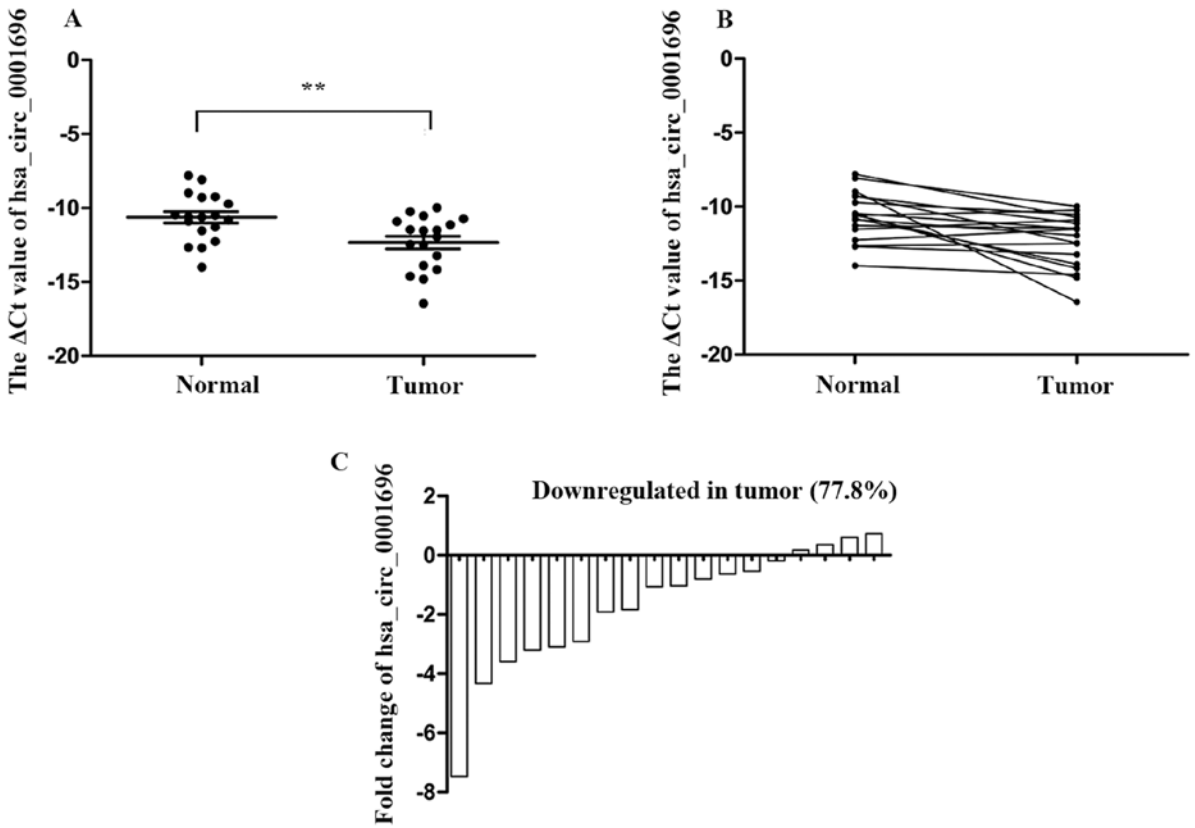

Figure 1. Diagnostic value of hsa_circ_0001696. (A and B) Hsa_circ_0001696 expression was downregulated in colorectal cancer tissues. (C) Expression patterns among 18 pairs of tissue samples. Data are presented as the mean ± standard deviation (n=3). **P<0.01. Higher ΔCt correlates to lower hsa_circ_0001696 expression.

mean ± standard deviation. Differences between paired groups were estimated by two-tailed Student's t-test. One-way analysis of variance followed by Tukey's post hoc test were used to compare differences between unpaired multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Hsa_circ_0001696 is downregulated in CRC tissues. RT-qPCR analysis was performed to detect hsa_circ_0001696 expression in paired CRC tissues. The results demonstrated that hsa_circ_0001696 expression was significantly lower in

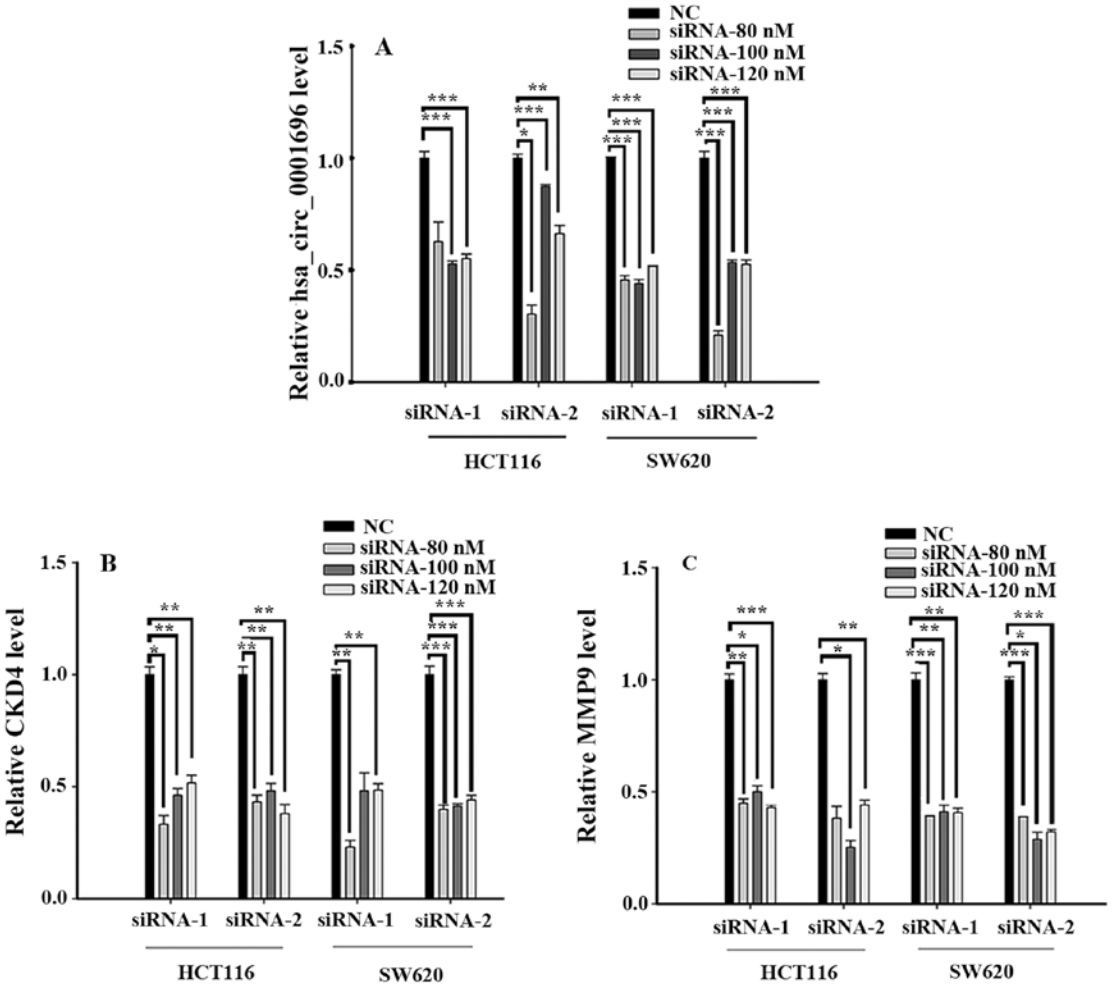

Figure 2. Transfection effect of siRNA. Cells were transfected with siRNA to knockdown (A) hsa _circ_0001696, (B) CDK4 and (C) MMP9 expression. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001. NC, negative control; si, small interfering; CDK4, cyclin-dependent kinase 4; MMP9, matrix metalloproteinase 9.

CRC tissues compared with adjacent normal tissues (P<0.01; Fig. 1A and B). Furthermore, among the 18 paired CRC tissues, 14 samples (77.8%) exhibited significantly downregulated hsa_circ_0001696 expression in the cancerous tissues (Fig. 1C).

Transfection effect of siRNA. A total of two siRNAs were used to silence hsa_circ_0001696, CDK4 and MMP9 expression in HCT116 and SW620 cells (Fig. 2). The results demonstrated that the transfection efficiency of siRNA-2 was higher, and siRNA-80 nM was used for subsequent experimentation, in which hsa_circ_0001696 was transfected into HCT116 and SW620 cells. With regards to CDK4, the transfection efficiency of siRNA-2 was higher, and siRNA-80 nM was also used for subsequent experimentation, whereas siRNA-100 nM of siRNA-1 was used for MMP9.

Silencing hsa_circ_0001696 promotes CRC cell proliferation. To investigate the effect of hsa_circ_0001696 on the biological behaviour of CRC cells, the CCK-8 and colony formation assays were performed. The results of the CCK-8 assay demonstrated that hsa_circ_0001696 knockdown significantly promoted HCT116 and SW620 cell proliferation compared with the NC group (Fig. 3A and B). The results of the colony formation assay demonstrated that hsa_circ_0001696 knockdown significantly increased the number of CRC cell colonies (Fig. 3C and D).

Silencing hsa_circ_0001696 promotes CRC cell migration. The wound healing assay was performed to assess the effect of hsa_circ_0001696 on CRC cell migration. The results demonstrated that the migratory ability of HCT116 and SW620 cells enhanced following transfection with si-circRNA, compared with the control group (Fig. 4A and B). These results suggest that hsa_circ_0001696 knockdown may promote the migration of CRC cells.

Mechanism of hsa_circ_0001696 in CRC. To further investigate the molecular mechanism of hsa_circ_0001696 on CRC cell proliferation and migration, the expression levels of cell cycle-related proteins (CDK4, cyclin D and cyclin E) and MMP9 were detected. RT-qPCR and western blot analyses demonstrated that the expression levels of CDK4, cyclin D, cyclin E and MMP9 significantly increased following transfection with si-circRNA (Fig. 5).

Silencing CDK4 and MMP9 inhibits CRC cell proliferation and migration, respectively. CDK4 and MMP9 expression levels were suppressed to determine whether they attenuated

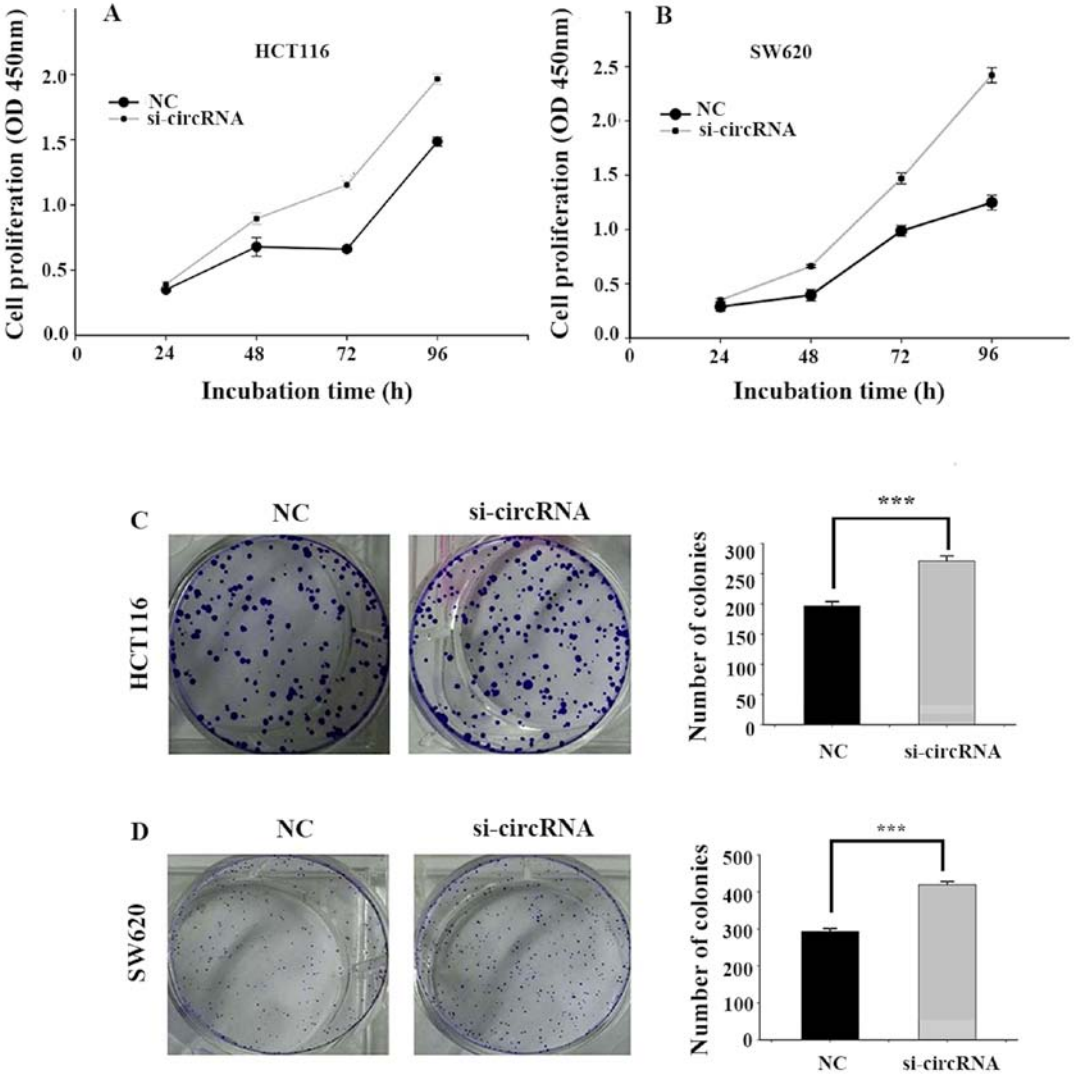

Figure 3. Hsa_circ_0001696 knockdown promotes cell proliferation. Downregulation of hsa_circ_0001696 expression promoted (A) HCT116 and (B) SW620 cell proliferation. Downregulation of hsa_circ_0001696 expression increased the number of colonies formed by (C) HCT116 and (D) SW620 cells. Data are presented as the mean ± standard deviation (n=3). ***P<0.001. NC, negative control; si, small interfering; circ, circular; OD, optical density.

the effect of hsa_circ_0001696 on cell proliferation and migration. The results demonstrated that silencing CDK4 expression inhibited CRC cell proliferation, while silencing MMP9 expression inhibited CRC cell migration (Fig. 6).

Discussion

CRC is one of the most common tumors of the digestive system, and its mortality and morbidity rates rank third worldwide (1). There were over 1.8 million newly diagnosed CRC cases worldwide in 2018, and there were estimated to be over 880,000 mortalities (1). Although radical tumor resection and adjuvant chemoradiotherapy have been extensively used in clinical practice, the recurrence rate remains high at 25-40% (29), and 80% of recurrences occur within 3 years of radical surgery (30). Thus, it remains critical to identify novel biomarkers for accurate and non-invasive identification of CRC recurrence, diagnosis and treatment.

CircRNAs are a class of novel non-coding RNAs (6,7). Compared to other non-coding RNAs, circRNAs have a unique 'back-splicing' structure, which is generated from the joining of an upstream 3' splice acceptor to a downstream 5' splice donor (31). This unique structure can be resistant to exonucleases, such as RNase, which makes circRNAs more stable in the human body compared with linear RNA isoforms (7). Thus, circRNAs are ideal molecular diagnostic markers that are more advantageous than linear RNA (11,16). Similar to long non-coding RNAs, there are subset of circRNAs called ceRNAs (32-34) that act as miRNA sponge molecules to regulate gene expression by adsorbing miRNAs (21,35,36). Recently, a number of studies have demonstrated that circRNAs exert a critical function in the progression of human CRC (35,37-39). However, little is known about the role of hsa_circ_0001696 in the development of CRC.

In the present study, circRNA databases and bioinformatics analysis demonstrated that hsa_circ_0001696 (gene symbol is HERPUD2) was significantly downregulated in CRC tissues compared with adjacent normal tissues. These results suggest that hsa_circ_0001696 may act as a suppressor gene in CRC progression.

To further investigate the molecular mechanisms involved in the regulation of hsa_circ_0001696, its expression was

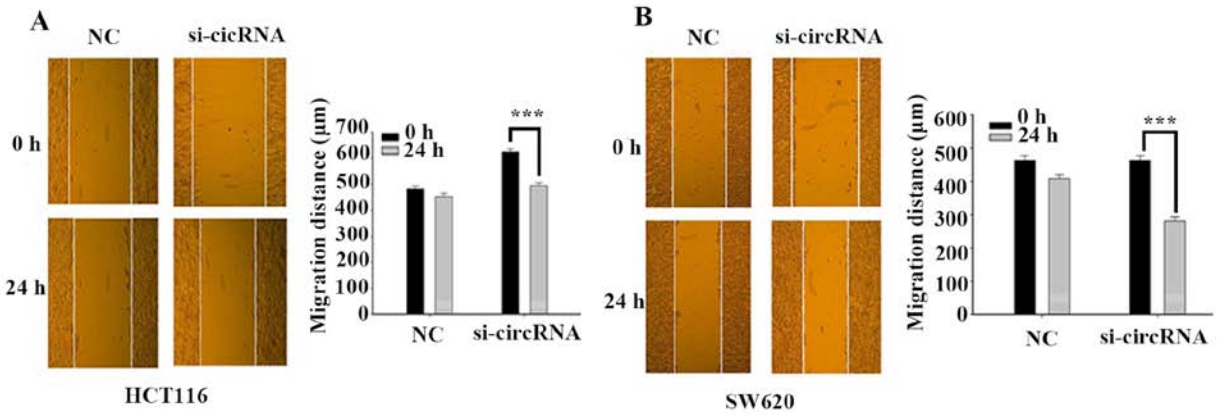

Figure 4. Hsa_circ_0001696 knockdown promotes cell migration. Downregulation of hsa_circ_0001696 expression promoted the migratory ability of (A) HCT116 and (B) SW620 cells. Data are presented as the mean ± standard deviation (n=3). ***P<0.001. NC, negative control; si, small interfering; circ, circular.

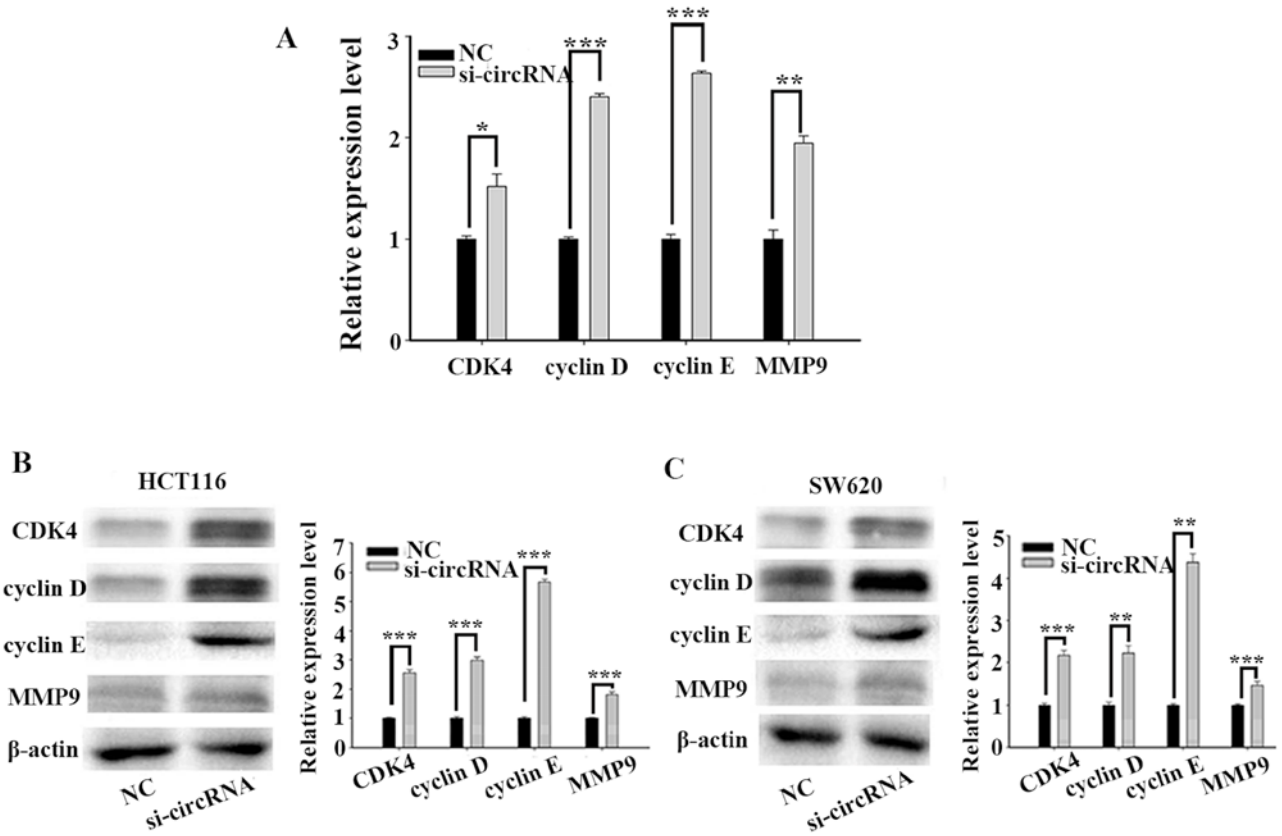

Figure 5. Expression levels of cell cycle-related and migration-related proteins in colorectal cancer cells. The results of (A) reverse transcription-quantitative PCR and (B and C) western blot analyses. Representative western blots from HCT116 and SW620 cells. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001. NC, negative control; si, small interfering; circ, circular; CDK4, cyclin-dependent kinase 4; MMP9, matrix metalloproteinase 9.

assessed in two CRC cell lines, HCT116 and SW620. To detect the effect of abnormally expressed hsa_circ_0001696 on CRC, cells were transfected with siRNA to knockdown hsa_circ_0001696 expression. The results demonstrated that hsa_circ_0001696 knockdown promoted CRC cell proliferation and migration, and the formation of cell colonies. In addition, CDK4, cyclin D, cyclin E and MMP9 mRNA and protein expression levels increased in cells transfected with siRNA. Furthermore, suppressing CDK4 and MMP9 expression inhibited cell proliferation and migration, respectively.

The results of the present study identified a novel association between hsa_circ_0001696 and CRC, whereby hsa_circ_0001696 may act as a potential novel molecular diagnostic marker. The results demonstrated that abnormal hsa_circ_0001696 expression affected CRC cell proliferation and migration, and the formation of cell colonies by interfering with the protein expression levels of CDK4, cyclin D, cyclin E and MMP9.

The present study is not without limitations. First, the number of clinical samples was limited. The results indicated

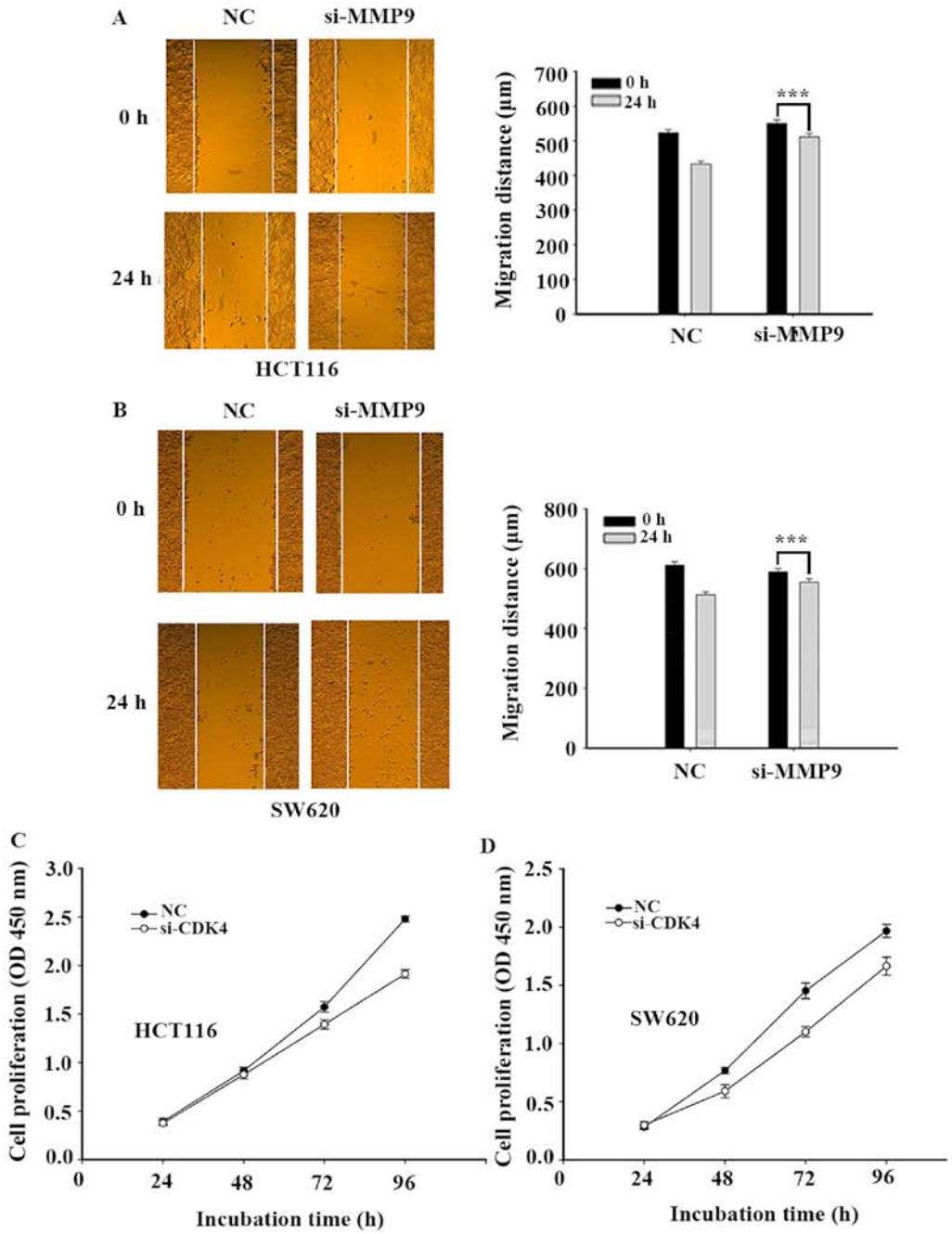

Figure 6. Knockdown of MMP9 and CDK4 expression inhibits cell migration and proliferation, respectively. Downregulation of MMP9 expression inhibited the migratory ability of (A) HCT116 and (B) SW620 cells. Downregulation of CDK4 expression inhibited (C) HCT116 and (D) SW620 cell proliferation. Data are presented as the mean ± standard deviation (n=3). ***P<0.001. NC, negative control; si, small interfering; MMP9, matrix metalloproteinase 9; CDK4, cyclin-dependent kinase 4; OD, optical density.

that hsa_circ_0001696 was expressed at significantly lower levels in 18 paired CRC tissues compared with adjacent normal tissues. However, the number of clinical samples was not sufficient to conclude that hsa_circ_0001696 can be used as a diagnostic marker. Thus, prospective studies should focus on collecting samples from multiple centres. Secondly, due to the structure of hsa_circ_0001696, the present study only designed two siRNAs. The interference effect may have been better if more siRNAs were designed. CircRNAs serve as miRNA sponges to indirectly regulate gene expression (14,21,22,40). However, the present study failed to assess whether hsa_circ_0001696 also has ceRNA functions or if it directly binds proteins. In addition, the specific miRNAs that interact with hsa_circ_0001696 to regulate the expression of miRNA target genes remain unknown, and the protein binding partners have not yet been investigated. Thus, these limitations will be the focus of prospective studies.

In conclusion, the results of the present study demonstrated that hsa_circ_0001696 expression was downregulated in CRC tissues compared with adjacent normal tissues, thus it may act as a molecular diagnostic marker. In addition, hsa_circ_0001696 knockdown promoted CRC cell proliferation and migration by regulating the expression levels of CDK4, cyclin D, cyclin E and MMP9.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contribution

LX and XYD designed the present study. PFL drafted the initial manuscript and performed statistical analysis. PFL, ZXZ and XY performed the experiments. HJS helped perform statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Human Research Ethics Committee of Ningbo First Hospital (Ningbo, China; approval no. 2019-R019) and performed in accordance with the World Medical Association Declaration of Helsinki (41). Written informed consent was provided by all patients prior to the study start.

Patient consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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