CircHMGCS1 is upregulated in colorectal cancer and promotes proliferation of colorectal cancer cells by targeting microRNA-503-5p

Introduction
Colorectal cancer (CRC) is one of the most prevalent tumors and the second leading cause of cancer-related mortality worldwide. Currently, the incidence of CRC is still rising, and surgery is the only possible cure method for CRC.\(^1,2\) However, the oncological outcome of CRC patients remains unsatisfactory. The 5-year survival rates markedly decline from about 90% in early-stage locally confined tumors to only about 5% in cases with metastatic disease, which is dependent on the tumor stage at a great extent.\(^3,4\) Therefore, the underlying mechanisms of CRC progression are necessary to further elucidated.

In recent decades, studies have shown that endogenous RNA plays an important regulatory role in various physiological processes of cells.\(^5,6\)

Abstract
This study aims to explore the regulatory mechanism of circHMGCS1/microRNA-503-5p (miR-503-5p) axis during colorectal cancer (CRC) development and progression. Real-time quantitative polymerase chain reaction (RT-qPCR) was applied to evaluate the expression of circHMGCS1 and miR-503-5p in CRC samples and their adjacent non-tumor specimen. Then, cell proliferation and cell apoptosis and migration and invasion of circHMGCS1-knocked down cells were further detected, using cell counting kit-8 (CCK-8), flow cytometry, Transwell assay, and western blotting assays. CircHMGCS1 was found to be significantly upregulated in CRC, and its high expression was closely correlated with the poor clinical parameter. In addition, the knockdown of circHMGCS1 could significantly inhibit CRC cells' growth promoting apoptosis, as suggested by the expression of apoptosis pathway-related proteins, which changed consistently. Furthermore, miR-503-5p inhibitors were able to reverse the suppression of cell proliferation induced by silencing circHMGCS1. Therefore, circHMGCS1 might serve as a promising bio-marker and treatment target for CRC.

Keywords
apoptosis, circHMGCS1, colorectal cancer, microRNA-503-5p, proliferation

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For example, micro-RNAs (miRNAs), long-non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) are widely found in eukaryotes, and they are tightly involved in the formation and progression of tumor.\(^7,8\) To date, circRNAs are one kind of endogenous RNA splicing products, which containing covalently bonded circular structure, but without 5′ caps and 3′ tails. Herein, circRNAs have very stable biological functions owing to its highly stable structure.\(^9,10\) Emerging evidence has elucidated that circRNAs participate in diverse signaling pathways, especially in the development of cancers.\(^2,11,12\) CircHMGCS1, also called circ_0072391, is a critical enzyme in mevalonate pathway, which has been suggested as a promising cancer therapeutic target.\(^13,14\) Besides, circHMGCS1 was dramatically overexpressed in hepatoblastoma cancer, while the depletion of circHMGCS1 could suppress cell growth in liver cancer cells, which indicates that circHMGCS1 takes a great role in hepatoblastoma progression.\(^15\) However, little is known of circHMGCS1 in the other cancers, including CRC. Consequently, we tried to explore circHMGCS1’s expression in CRC patients in our center, and the relationship between circHMGCS1 expression and clinicopathological characteristics post-operative survival were further studied. In addition, the potential role of circHMGCS1 on tumor proliferation and metastasis was further explored.

**Materials and methods**

**Tissues**

CRC samples and their paired non-tumor specimens were collected from 100 cases of patients with CRC. The inclusion criteria were that all patients had undergone radical excision without receiving any other treatment before surgery and been pathologically diagnosed with CRC between 2009 and 2015 in our center, while the specimens were frozen in liquid nitrogen after excision and stored at −80°C. All patients signed the informed consent before participating in this research, while the Ethics Committee of our center approved this study.

**Cell culture**

Five CRC cell lines (HT-29, LoVo, SW480, Caco-2, and SW680), and the immortalized intestinal epithelial cell line called fetal human colon (FHC), were obtained from Beina Company (China). Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), containing 20% fetal bovine serum (FBS). HT-29, LoVo, SW480, SW620, and FHC cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS. DMEM, RPMI, and FBS were obtained from Gibco (USA).

**RNA interference**

LoVo and SW480 cells were transfected with circHMGCS1-specific lentiviruses, to knock down the expression of circHMGCS1. To date, the multiplicity of infection (MOI) is 50 for LoVo and SW480 cells, while the transfected LoVo cells were screened by puromycin (5 μg/mL). Finally, we successfully generated circHMGCS1-knocked-down cell lines (si-circHMGCS1-1 and si-circHMGCS1-2 cells) and the negative control (NC; si-Control cells). To date, the target sequences of circHMGCS1 are as follows: si-circHMGCS1-1: 5′-UGGAAGCCUUGGGGCUUCGU-3′, si-circHMGCS1-2: 5′-GCCUGGAAGCCUUUUGGGCUU-3′, and si-Control: 5′-UUCUCCGAACGUUGACACGUUGUCACGUTT-3′. MiR-503-5p-specific mimics, inhibitors, as well as their NC were purchased from OBIO Biotechnology Company (China) and transfected into circHMGCS1-knocked-down cell lines or 293T cell lines, using Lipofectamine 3000 Transfection Reagent (Thermo Fisher, USA).

**Real-time quantitative polymerase chain reaction assays**

The extraction of RNA was applied with TRIzol Reagent (TaKaRa, China). Following that RNA was converted to complementary DNA (cDNA) with the PrimeScript RT Master Mix (TaKaRa), and then, real-time quantitative polymerase chain reaction (RT-qPCR) assays were applied to analyze the relative expression of circHMGCS1 and miR-503-5p, using the SYBR Premix Ex Taq II Kit (TaKaRa). The primer sequences of circHMGCS1 are follows: forward: 5′-TCTAGCTCGGATGTTGCTGA-3′ and reverse: 5′-TCAGGCTTGTAAAAATCATAGGC-3′. The primer sequences of miR-503-5p is as follows: RT: 5′-GTCGTATCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCAG-3′, forward: 5′-CGCGCCATGGATCTCCAG-3′, and reverse: 5′-AGT...
GCAGGGTCCGAGGTATTTCGGCCACATTGTGAACTT-3′.

**In vitro cell proliferation assays**

The cell counting kit-8 (CCK-8; Donjindo, Japan) assay was applied to analyze cell proliferation. Cells were plated onto 96-well plates at the concentration of $5 \times 10^3$ cells per well. Then, the CCK-8 was added to the wells, and cell viability was assessed at 1, 2, 3, and 4 days, using a microplate reader (Bio-Rad, USA).

**Flow cytometric analyses**

LoVo cells were pre-treated with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Keygen, New York, USA), and then, cell apoptosis was determined on a FACS Canto II flow cytometer (BD Biosciences, New York, USA).

**Transwell assays**

The metastasis of LoVo cell lines was detected using Transwell chambers. LoVo cells were incubated with 200 μL serum-free RPMI in the upper chamber and 500 μL McCoy’s 5A Medium with 10% FBS was added in the lower chambers. After 24h, the cells were fixed and stained, followed by analysis using a digital microscope. To date, Matrigel coating was applied in the upper Transwell chambers during the detection and the invasion of LoVo, but not used in migration assays.

**Western blotting assay**

Proteins were extracted from cells, separated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes. After that, the membranes were then treated with 5% skimmed milk (Solarbio), incubated with primary antibodies and secondary antibodies, and finally imaged with an enhanced chemiluminescent (ECL) kit. To date, the primary antibodies used (Cell Signaling Technology; USA) were all at a concentration of 1:1000.

**Dual-luciferase reporter assays**

The 293T cells were transfected with pmirGLO-circHMGCS1-WT or pmirGLO-circHMGCS1-MUT plasmid, with miR-503-5p mimics or miR-503-5p NC. After 48 h, passive lysis buffer (Promega, China) and the Dual-Luciferase Reporter Assay (Promega, USA) were used to calculate the relative luciferase activity through normalizing the firefly luminescence to Renilla luminescence.

**Statistical analyses**

All data were shown as mean ± standard error of mean (SEM). The chi-square test, two-tailed Student’s t-test, Mann Whitney U test, analysis of variance (ANOVA), and Kruskal–Wallis test were applied to evaluate the data. $P < 0.05$ was considered statistically significant.

**Results**

**Upregulation of circHMGCS1 predicted poor prognosis of CRC**

To detect the relationship between the circHMGCS1’s expression and clinical parameters in CRC people, RT-qPCR was performed to analyze the circHMGCS1 expression in CRC tissues and adjacent normal tissues. From Figure 1(a), the results displayed that the overexpression of circHMGCS1 accounted for 69% (69/100) of CRC specimens. Meanwhile, circHMGCS1 was dramatically upregulated in CRC samples (Figure 1(b)). Then, we divided the tissue samples into two groups (high expression group and low expression group) according to the median of the relative circHMGCS1 expression. Our results revealed that the expression of circHMGCS1 was highly associated with tumor size ($P = 0.002$), histologic grade ($P = 0.045$), pTNM stage ($P = 0.009$), and pN status ($P = 0.009$; Table 1). Consistently, our data also showed that CRC patients with high expression level of circHMGCS1 were more likely to have larger tumor size (Figure 1(c)), low histologic grade (Figure 1(d)), later pTNM stage (Figure 1(e)), lymph node metastasis (Figure 1(f)), and shorter overall survival time (Figure 1(g)). Together, these results revealed that circHMGCS1 was upregulated in CRC tissues, and this upregulation was negatively correlated with clinical outcome.

**Downregulation of circHMGCS1 inhibits proliferation of CRC cells**

The RT-qPCR results showed that circHMGCS1 is significantly upregulated in CRC cell lines (HT-29, LoVo, SW480, Caco-2, and SW620 cells)
Figure 1. Upregulation of circHMGCS1 predicted poor prognosis of CRC. (a) CircHMGCS1 is significantly overexpressed in 69% CRC patients. (b) CircHMGCS1 showed higher expression in CRC samples, compared to normal samples, detected using RT-qPCR assay. (c) The correlation between the expression of circHMGCS1 and tumor size. (d) The correlation between the expression of circHMGCS1 and histologic grade. (e) The correlation between the expression of circHMGCS1 and pTNM stage. (f) The correlation between the expression of circHMGCS1 and pN stage. (g) Higher CircHMGCS1 indicated a worse disease-free survival revealed by Kaplan–Meier analysis (*P < 0.05, ***P < 0.01, ****P < 0.001).

compared with that in the normal cell (FHC). Meanwhile, LoVo cells showed the highest expression (Figure 2(a)). Then, we depleted the expression of circHMGCS1 in LoVo and SW480 cells by the transfection of circHMGCS1-specific siRNAs. The results indicated that siRNAs could dramatically decrease the expression of circHMGCS1 in LoVo cells (referred to si-circHMGCS1-1 and si-circHMGCS1-1 groups), compared to the NC group (si-Control; Figure 2(b)). Subsequently, these circHMGCS1-knocked down cells were further applied for proliferation experiments.
Table 1. Relationship between circHMGCS1’s expression and clinical parameters in CRC.

| Variable                      | Cases | CircHMGCS1 |          |          |
|-------------------------------|-------|------------|----------|----------|
|                               |       | High expression | Low expression |    |          |
|                               |       | Sex          | P value   |          |
|                               |       | Male         | 0.529    |          |
|                               |       | Female       | 0.529    |          |
|                               |       | Tumor size (cm) |          |          |
|                               |       | ⩽6           | 0.002    |          |
|                               |       | >6           | 0.002    |          |
|                               |       | Histologic grade (WHO) | 0.045 |          |
|                               |       | Low          | 0.045    |          |
|                               |       | High         | 0.045    |          |
|                               |       | pTNM stage   | 0.009    |          |
|                               |       | I–II         | 0.009    |          |
|                               |       | III–IV       | 0.009    |          |
|                               |       | pN status    | 0.009    |          |
|                               |       | N0           | 0.009    |          |
|                               |       | N1–N2        | 0.009    |          |
|                               |       | CA19-9 level | 0.081    |          |
|                               |       | Normal       | 0.081    |          |
|                               |       | Upregulate   | 0.081    |          |
|                               |       | CEA level    | 0.398    |          |
|                               |       | Normal       | 0.398    |          |
|                               |       | Upregulate   | 0.398    |          |

CRC: colorectal cancer; pTNM: pathological tumor–node–metastasis; CEA: carcinoembryonic antigen; WHO: World Health Organization.

The bold value means that the differences between the circHMGCS1’s high expressions and the low expression group have statistical significance.

Figure 2. Downregulation of circHMGCS1 inhibits proliferation of CRC cells. (a) The expression of circHMGCS1 in CRC cell lines (HT-29, LoVo, SW480, Caco-2, and SW680), and the immortalized intestinal normal cells are called as FHC. (b) Successful construction of circHMGCS1-downregulated LoVo cells by a lentivirus-based method. (c) Successful construction of circHMGCS1-downregulated SW480 cells by a lentivirus-based method. (d) The cell growth of circHMGCS1-downregulated LoVo cells, revealed by CCK-8 assay (**p < 0.01, ***p < 0.001). (e) The cell growth of circHMGCS1-downregulated SW480 cells, revealed by CCK-8 assay (**p < 0.01, ***p < 0.001).
Our results showed that circHMGCS1 depletion effectively suppressed the proliferation of LoVo CRC cells, as the lower optical density (OD) value was observed in si-circHMGCS1-1 and si-circHMGCS1-2 groups (Figure 2(d)). Consistently, similar tendency was observed in SW480 cells, after the depletion of circHMGCS1 (Figure 2(c) and (e)). Furthermore, the downregulation of circHMGCS1 induced cell apoptosis at a greater extent compared with that in the control group (Figure 3(a) and (b)). Consistent with the flow cytometry results, the expression of apoptosis-related proteins (Bax and p53 protein) was significantly upregulated, while the anti-apoptosis-related protein BCL-2 was dramatically inhibited (Figure 3(c)). We also detected the effect of circHMGCS1 on metastasis abilities in LoVo CRC cells. Interestingly, the results indicated no significant differences in circHMGCS1 downregulated groups and the control group (Supplemental Fig. S1), meaning that circHMGCS1 did not significantly change the metastasis abilities, reinforcing the importance of circHMGCS1 for proliferation.

**MiR-503-5p is a direct target of circHMGCS1 in CRC**

From publicly available bioinformatic algorithms (StarBase), we predicted that miR-503-5p might be a downstream target for circHMGCS1, and the potential binding site between them is shown in Figure 4(a). We further evaluated miR-503-5p’s expression pattern in CRC samples. The results displayed that miR-503-5p is dramatically downregulated (Figure 4(b)). Meanwhile, the low expression of miR-503-5p accounted for 76% (76/100) of CRC specimens (Figure 4(c)), and miR-503-3p was negatively correlated with the expression of circHMGCS1 in CRC (Figure 4(d)). To further explore their association, the luciferase reporter assay was employed, which indicates that the luciferase activity was significantly lower in
Downregulation of miR-503-5p rescue cell proliferation of CRC cells induced by depletion of circHMGCS1

We further detected the content of miR-503-5p in LoVo and SW480 cells, while si-circHMGCS1-1 group shows higher expression of miR-503-5p, compared to si-Control and si-circHMGCS1-2 groups (Figure 5(a)). In order to explore the function of miR-503-5p, we decreased miR-503-5p in si-circHMGCS1-1 LoVo and SW480 cells, using miR-503-5p-specific inhibitors (Figure 5(b)). From the results of CCK-8, miR-503-5p inhibitors could rescue the cell growth in circHMGCS1-knockdown LoVo and SW480 cells (Figure 5(c) and (d)). Furthermore, the downregulation of miR-503-5p reduced cell apoptosis compared with that in miR-503-5p NC group (Figure 6(a) and (b)). Consistent with the flow cytometry results, the expression of apoptosis-related proteins (Bax and p53 protein) was significantly downregulated, while the anti-apoptosis-related protein BCL-2 was dramatically increased (Figure 6(c)). Consequently, miR-503-5p’s
downregulation could reverse anti-cell proliferation induced by depletion of circHMGCS1.

**Discussion**

Despite the rapid development of therapeutic approaches, including endoscopic, laparotomy, and laparoscopic removal of tumor; neoadjuvant and adjuvant chemotherapy or radiotherapy; as well as the immunotherapy, the prognosis of patients with CRC remains unsatisfactory, which indicates that more precise diagnostic methods and effective treatments are required. Therefore, the underlying mechanisms of CRC progression need being further elucidated.

Accumulating evidences have clarified that circRNAs play a vital role during the cancer generation. Wang et al.\(^{19}\) indicated that circ_0027599 suppresses the gastric cancer progression by sponging miR-101-3p.\(^{1}\) Zhen et al.\(^{15}\) reported that circHMGCS1 could regulate cell growth of liver cancer. In our study, we first validated that the circHMGCS1 was upregulated in the CRC. Then, we focused on the relationship between the circHMGCS1 expression and the clinical parameters in CRC, and the results consistently indicated that the CRC patients with higher expression level of circHMGCS1 were more likely to have poor oncological outcomes. Consistent with the previous results, the CCK-8 results showed that the circHMGCS1 downregulation could significantly inhibit the CRC cells’ proliferative activities.

Cell apoptosis is an important aspect during the growth of cancer cells.\(^{20,21}\) Our study demonstrated that downregulation of circHMGCS1 could significantly increase the CRC cells’ apoptosis.
rates. However, its related mechanism remained unclear to us. P53 is a tumor suppressor gene, which can regulate the Bcl-2/Bax pathway in apoptotic pathway to mediate apoptosis and control the initiation of cell cycle. Bcl-2 can inhibit apoptosis, while Bax can inactivate it to promote apoptosis by forming heterodimers with Bcl-2 protein through its encoded products. P53 promotes the expression of Bax and inhibits the expression of Bcl-2 protein. According to our study, the expression of p53 and Bax increased while Bcl-2 decreased in circHMGCS1-downregulated CRC cells. We suggest that circHMGCS1 may downregulate the expression of p53, thereby reducing the Bax/Bcl-2 ratio and prolonging cell life.

Emerging evidence established that circRNAs could act as molecular sponges for miRNA and then suppress the function of miRNA. We predicted that miR-503-5p might be the downstream of circHMGCS1. To validate that, we further demonstrated that miR-503-5p was downregulated in the CRC tissues. And the results of the correlation analysis revealed that the circHMGCS1 expression was negatively related to the miR-503-5p, which was also confirmed by the dual-luciferase reporter assays. Interestingly, we found that the decrease in miR-503-5p could rescue the cell proliferation inhibition induced by circHMGCS1. Consistent with others’ researches, miR-503-5p was reported to regulate the progress of neuroblastoma and colorectal carcinoma. Therefore, we concluded that circHMGCS1 might promote proliferation of CRC cells by sponging miR-503-5p.

In conclusion, we have demonstrated the overexpression of circHMGCS1 in CRC, which was negatively correlated with miR-503-5p expression. Furthermore, circHMGCS1 promotes proliferation of cancer cells by sponging miR-503-5p in CRC. However, there are also some limitations in our study. As only dual-luciferase reporter assays were utilized to validate that circHMGCS1 could sponge with miR-503, pull-down assay would be used to

Figure 6. Downregulation of miR-503-5p increases anti-apoptotic abilities of CRC cells. (a) Cell apoptosis images of circHMGCS1-downregulated LoVo cells with or without depletion of miR-503-5p. (b) Flow cytometry statistics analysis of circHMGCS1-downregulated LoVo cells with or without depletion of miR-503-5p. (c) The expression of apoptosis-related proteins (Bax, Bcl-2, and p53) with or without depletion of miR-503-5p, as detected by western blot. All experiments were repeated at least three times (**P < 0.01).
prove their relationship in our further research. Furthermore, gain-functional studies or mechanisms of research will also be applied in our next study.

Declaration of conflicting interests
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