THE EFFECT OF LNCRNA ON THE PROLIFERATION AND APOPTOSIS OF COLORECTAL CANCER CELLS BY REGULATING THE MIRNAARPP19 AXIS

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ABSTRACT

Objective: *To explore the influence of long non-coding RNA (lncRNA) on the proliferation and apoptosis of colorectal cancer cells (CRC) by regulating the Phosphoprotein 19 (ARPP19) axis regulated by cAMP, a microRNA.*

Method: *CRC cell lines-Colo 320 and normal colorectal cell lines (NCM460, 293T) were obtained from the American Type Culture Collection. All cell lines were in a humid environment at 37°C and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The experiment had a control group (Colo 320 for experimental control), MCM3AP-AS1 silent group (infecting Colo 320 cells with sh-MCM3AP-AS1 for silence expression), and ARPP19 transfection group (ARPP19 overexpression in this group of cells). The mRNA expressions of MCM3AP-AS1, miR-599, and ARPP19 in normal rectal system (NCM460, 293T) and rectal cancer cell line Colo 320 were analyzed by PCR in real time. CCK-8 and the 24h, 48h, and 96h OD values of the cells were used to reflect their proliferation ability. Cell apoptosis was compared by detecting cell caspase activity. Wound healing and Transwell analysis were used to detect cell migration and invasion ability. The Epithelial-mesenchymal transition (EMT) process and the expressions of N-cadherin and vimentin were analyzed by Western blot. The dual luciferase reporter gene assay was adopted to verify the MCM3AP-AS1-miR-599-ARPP19 interaction relationship.*

Results: *Compared with (NCM460, 293T), the mRNA expressions of MCM3AP-AS1 and RPP19 in Colo 320 increased, and the expression of miR-599 mRNA in Colo 320 decreased (P<0.05). Compared with the control group and ARPP19 transfection group, the cell proliferation ability of the MCM3AP-AS1 silenced group was lower (P<0.05), and the cell proliferation of the ARPP19 transfection group was higher than that of the MCM3AP-AS1 silenced group (P<0.05). At 48h, the Caspase-3 activity of the MCM3AP-AS1 silenced group was higher than that of the control group and the ARPP19 transfection group (P<0.05), and there was no difference in the Caspase-3 activity of the control group and the ARPP19 transfection group (P>0.05). At 96h, the Caspase-3 activity of the MCM3AP-AS1 silenced group was higher than that of the control group and ARPP19 transfection group (P<0.05), and the Caspase-3 activity of the ARPP19 transfection group was lower than that of the control group (P<0.05). Compared with the control group and ARPP19 transfection group, the number of cell migration and invasion in the MCM3AP-AS1 silenced group was lower (P<0.05), and the number of cell migration and invasion in the ARPP19 transfection group was higher than that in the control group (P<0.05). Compared with the control group and the ARPP19 transfection group, the expression of E-cadherin in the MCM3AP-AS1 silenced group increased, and the expression of N-cadherin and vimentin decreased (P<0.05). Compared with the MCM3AP-AS1 silenced group, the expression of E-cadherin in the ARPP19 transfection group decreased, and the expression of N-cadherin and vimentin increased (P<0.05). In the co-culture of the ARPP19-WT and miR-599 transfection group, the enzyme activity decreased (P<0.05), and the ARPP19-Mut enzyme activity did not change (P>0.05). When miR-599 and MCM3AP-AS1 were co-transfected and overexpressed, the luciferase activity of co-cultured ARPP19-WT was higher than that of miR-599 transfected and co-cultured alone (P<0.05).*

Conclusion: *lncRNA MCM3AP-AS1 promoted the proliferation and apoptosis of CRC cells by regulating the miR-599/ARPP19 axis, and therefore might be an effective therapeutic target for CRC.*

Keywords: *CRC, lMCM3AP-AS1, miR-599, cell proliferation and apoptosis.*

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Introduction

CRC is the second leading cause of cancer-related death in the United States. Rectal cancer accounts for about one-third of newly diagnosed cases, which is considered a challenging disease due to its anatomical location and the risk of local recurrence[(1)]. In addition, the incidence of rectal cancer has been increasing among young patients. In the past decades, the incidence of CRC has been greatly reduced, and the overall survival time and disease-free survival rate are still very poor, which is due to late diagnosis and inherent or acquired drug resistance of CRC[(2)]. Therefore, it is still vitally

important to develop new and reliable molecular markers for CRC treatment. MiRNA is a group of small non-coding RNA, which is capable of binding to the 3'- untranslated region of RNA transcript to induce mRNA degradation or inhibiting translation[3]. MiRNA is a promising biomarker in cancer and can be easily detected in serum. Disordered miRNA often participates in different types of human cancers as carcinogens and tumor suppressor[4]. Overexpression of miR-187, for example, inhibits cell proliferation and glioma metastasis by down-regulating SMAD1 expression. According to reports, miR-599 plays an inhibitory role in several types of cancers. For example, miR-599 inhibits glioma progression by targeting RAB27B[5]. In addition, ARPP19 is also reported as the direct target of miR-599. However, the regulatory mechanism of miR-599 in CRC is still unclear. LncRNA is an RNA molecule with a length of more than 200 nucleotides, which involves different types of cell processes, such as proliferation, migration, and differentiation[63]. Previous studies have shown that lncRNA is an important regulatory factor involved in cell proliferation, invasion, and metastasis[7]. Lots of evidences shows that lncRNA has specific biological functions. For example, lncRNA RBM5-AS1 promotes the invasion of oral squamous cell carcinoma by regulating miR-1285-3p/YAP1 axis[8]. Previous studies have also shown that lncRNAs can regulate the progression of different types of cancer by sponging miRNA[9]. For instance, lncRNA RBMS3-AS3 acts as a sponge for miR-4534, and inhibits the progression of prostate cancer by up-regulating VASH1 expression[10].

According to reports, MCM3AP-AS1 is associated with several types of cancers. MCM3AP-AS1/miR-211/KLF5/AGGF1 axis regulates angiogenesis of glioblastoma[11]. Other studies have reported that overexpression of lncRNA MCM3AP-AS1 promotes the progression of lung cancer through miR. However, the molecular mechanism of MCM3AP-AS1 in CRC is still unknown. In this study, we investigated the effect of lncRNA MCM3AP-AS1miRNA-ARPP19 axis on the proliferation and apoptosis of CRC cells and its mechanism.

Material and methods

Experimental materials

Experimental cells

CRC cell line -Colo 320 and normal colorectal cell lines (NCM460, 293T) were obtained from American Type Culture Collection. All the cell lines were kept at 37°C in a humid environment containing 5% carbon dioxide and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.).

Cell transfection

Short hairpin (SH) RNA targeting MCM3AP-AS1 (SH-MCM3AP-AS1; 10 nM; 5'-GCGCCUUCCCUCUAACCUUAA-3') and non-targeted scrambled negative control (sh-NC; 10 nM; 5'-GGCAAGAUGAACGUCUGAAAU-3') were all purchased from Shanghai Gene Pharmaceutical Co., Ltd. The full-length MCM3AP-AS1 and ARPP19 gene sequences were amplified and sub-cloned into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to synthesize pcDNA3.1/MCM3AP-AS1 and pcDNA3.1/ARPP19. According to the manufacturer's instructions, Lipofectamine®2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for all transfections. And incubation was carried out at 37°C for 48 hours. All functional experiments were performed 48h after transfection.

Experimental grouping

The experiment had a control group (Colo 320 for experimental control), MCM3AP-AS1 silent group (infecting Colo 320 cells with sh-MCM3AP-AS1 for silence expression), and ARPP19 transfection group (ARPP19 overexpression in this group of cells).

Experimental method

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from cells, and the extracted total RNA was reversely transcribed into cDNA by RT kit (cat). According to the manufacturer's agreement, the number was RR047A (Takara Biotechnology Co., Ltd.).

According to the manufacturer's regulations, SYBR-Green PCR Master Mix kit (catalog number DRR041A; Takara Biotechnology Co, ltd.) was used for qPCR under the following thermal cycling conditions: pre-denaturation at 95°C for 15 seconds, denaturation at 94°C for 30 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 40 seconds for 40 cycles. GAPDH was used as the internal control of MCM3AP-AS1 and ARPP19,

while U6 was used as the internal control of miR-599. The relative mRNA expression level was quantified by $2^{-\Delta\Delta Ct}$ method.

Assay of cell proliferation

The proliferation ability of cells was evaluated by cell counting kit-8(CCK-8) kit (Dojindo). The cells were inoculated in a 96-well plate. After transfection, the cells were cultured for 0h, 24h, 48h or 96h, and 10μL CCK-8(5 mg/mL) was added to the culture medium. The absorbance was measured at 450 nm using an Exl 800 microplate reader (Bio-tek). Cell proliferation was also analyzed using colony formation assay. In brief, equal numbers of cells from different treatment groups were inoculated into the 6-well plate and cultured in RPMI-1640 medium supplemented with 10%FBS for 10 days. Cells were fixed with paraformaldehyde and stained with 0.1% crystal violet to calculate the number of stained colonies.

Caspase-3 activity

According to the manufacturer's instructions, caspase-3 activity in transfected cells was tested by a caspase activity assay kit (Beyotime, Haimen, China). Samples were monitored by microplate reader (Tecan Group, Nedov, Germany).

Assay of cell invasion

The migration efficiency of CRC cell line was evaluated by Transwell assay. Transfected cells were inoculated into serum-free DMEM in the upper culture chamber at a density of 2×10^5 cells per well.

DMEM filled with 10%FBS was added to the bottom chamber. After incubation at 37°C for 48h, the migrated cells were stained with 0.1% crystal violet for 20 min at room temperature and counted under an optical microscope (magnification: 200 times, Olympus Corporation).

Measurement of wound healing

After transfection, SGC-7901 or BGC-823 cells were inoculated into a six-well plate and cultured until fusion. The single wound was scratched with a sterile pipette tip (yellow). The floating cells were washed away with PBS, and the adherent cells were cultured in DMEM medium containing 1%FBS. The wound closure at 0h and 48h was monitored by an inverted optical microscope (Olympus).

Western blot analysis of protein

Cells were lysed in RIPA buffer containing 1 mM PMSF. The concentration of total protein was quantified by the BCA protein test kit (Thermo). Protein was separated in 10% gel by SDS-PAGE, and then transferred to PVDF membrane (Millipore). The membrane was incubated with anti-E- cadherin (Abcam), anti-N- cadherin, anti-vimentin and anti-β-actin (Abcam) antibodies overnight at 4°C, and incubated with HRP-labeled IgG secondary antibody (Abcam) at room temperature for 1h. The signals were visualized by chemiluminescence detection system, and GAPDH was selected as the internal control.

Luciferase test

Luciferase reporter vector pmirGLO (Promega, Madison, Wisconsin) was used to transfect MCM3AP-AS1-Wt or MCM3AP-AS1-Mut vector into cells with NC mimetic or miR-599 mimetic, respectively. The vector pmirGLO-ARPP19-Wt or pmirGLO-ARPP19-Mut was also co-transfected into Colo 320 cells with miR-599 mimetic or NC mimetic. Transfection was performed using Lipofectamine 2000. After 48h, luciferase reporter analysis system (Promega) checked the relative signal of luciferase.

Statistical analysis

GraphPad Prism 7.0 software was used for statistical analysis, and the data were expressed as mean±standard deviation. One-way ANOVA was used for statistical comparison, and $P<0.05$ indicated a statistically significant difference.

Results

RT-PCR analysis

The mRNA expressions of MCM3AP-AS1, miR-599, and ARPP19 in Colo 320 of normal colorectal cell lines (NCM460, 293T) and colonic cancer cell lines were analyzed by PCR in real time. The mRNA expressions of MCM3AP-AS1 and RPP19 in Colo 320 were higher than those in NCM460, 293T, while the mRNA expression of mir-599 was lower ($P<0.05$) (Table 1).

Detection of cell proliferation

The proliferation ability was reflected by CCK-8 and OD values at 24h, 48h, and 96h. The proliferation ability of MCM3AP-AS1 silenced group was lower than that of the control group and ARPP19 transfection group ($P<0.05$), while the proliferation of ARPP19 transfection group was higher than that of MCM3AP-AS1 silenced group ($P<0.05$). The data showed that the high expression of ARPP19 reversed

the inhibitory effect of MCM3AP-AS1 silencing on cell proliferation. Figure 1 shows the cell colony formation test after 48 hours of culture. The proliferation ability of MCM3AP-AS1 silencing was lower than that of control group cells, and the high expression of ARPP19 promoted the proliferation of cells. (Figure 1, Table 2).

Group	MCM3AP-AS1	miR-599	ARPP19
NCM460	1.13±0.11	1.79±0.16	1.14±0.11
293T	1.24±0.12	1.83±0.18	1.21±0.11
Colo 320	1.96±0.24	1.15±0.09	2.13±0.25
F value	11.304	9.415	13.628
P value	0.021	0.015	0.037

Table 1: RT-PCR analysis of target genes ($\bar{x}\pm s$).

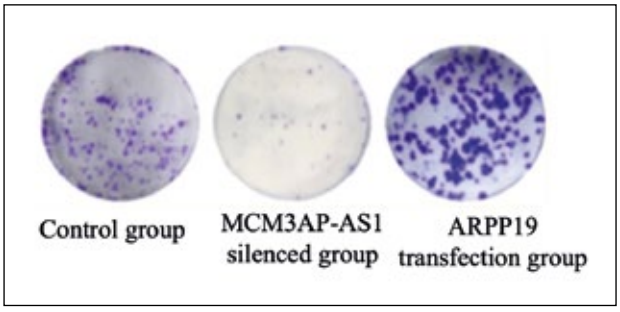

Figure 1: Cell colony formation.

Group	24h	48h	96h
Control group	0.52±0.09	0.85±0.12	1.34±0.15
MCM3AP-AS1 silenced group	0.28±0.02	0.47±0.08	0.79±0.12
ARPP19 transfection group	0.74±0.11	1.36±0.16	1.75±0.18
F value	9.377	13.196	10.423
P value	0.012	0.024	0.035

Table 2: OD value analysis of cell proliferation ($\bar{x}\pm s$).

Evaluation of apoptosis by Caspase-3 activity assay

Caspase-3 activity of cells was detected using a caspase activity assay kit. At 48h, the Caspase-3 activity of MCM3AP-AS1 silenced group was higher than those of the control group and ARPP19 transformation group ($P<0.05$), but there was no difference between the control group and ARPP19 transformation group ($P>0.05$).

At 96h, the Caspase-3 activity of MCM3AP-AS1 silenced group was higher than those of the control group and ARPP19 transformation group ($P<0.05$), while that of ARPP19 transformation group was lower than that of the control group ($P<0.05$). (Table 3).

Group	48h	96h
Control group	1.24±0.08	1.92±0.18
MCM3AP-AS1 silenced group	1.85±0.17	3.24±0.46
ARPP19 transfection group	1.03±0.05	1.33±0.12
F value	11.814	9.238
P value	0.013	0.036

Table 3: OD value analysis of cell proliferation ($\bar{x}\pm s$).

Wound healing and Transwell test

The ability of migration and invasion of cells was detected by wound healing and Transwell analysis. Compared with the control group and ARPP19 transformation group, the number of migration and invasion of cells in MCM3AP-AS1 silenced group decreased ($P<0.05$), while that in ARPP19 transformation group increased ($P<0.05$). The migration and invasion ability of cells was analyzed by wound healing and Transwell. ARPP19 transfected SGC-7901 or BGC-823 cells. As shown in Figure 2, the silencing of MCM3AP-AS1 inhibited cell migration and cell invasion, while the overexpression of ARPP19 showed the opposite effect. (Figure 2, Table 4).

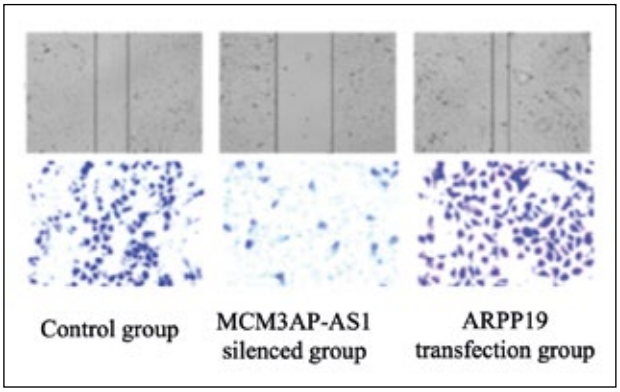

Figure 2: Cell migration and invasion ($\bar{x}\pm s$).

Group	Cell migration	Cell invasion
Control group	104.32±18.52	86.19±11.46
MCM3AP-AS1 silenced group	62.74±9.33	45.02±5.27
ARPP19 transfection group	149.58±24.68	127.36±20.14
F value	9.442	13.826
P value	0.013	0.026

Table 4: Detection of cell migration and invasion ($\bar{x}\pm s$).

Western blot analysis of EMT related protein expression

The EMT process and the expression of N-cadherin and vimentin were analyzed by western blot. Compared with the control group and ARPP19

transformation group, MCM3AP-AS1 silenced group showed higher expression of E-cadherin and lower expression of N-cadherin and vimentin (P<0.05). Compared with MCM3AP-AS1 silenced group, the expression of E-cadherin in ARPP19 transfection group decreased, while the expression of N-cadherin and vimentin increased (P<0.05). (Table 5).

Group	E-cadherin	N-cadherin	Vimentin
Control group	1.68±0.15	1.64±0.15	1.82±0.18
MCM3AP-AS1 silenced group	2.23±0.22	1.12±0.09	1.05±0.04
ARPP19 transfection group	1.01±0.07	2.16±0.24	2.24±0.25
F value	11.528	9.364	13.426
P value	0.015	0.025	0.033

Table 5: Western blot analysis of EMT related protein expression ($\bar{x}\pm s$).

MCM3AP-AS1 interacted with miR-599 and regulated ARPP19

To verify the interaction between MCM3AP-AS1-miR-599-ARPP19, a dual luciferase reporter gene was determined. The enzyme activity of ARPP19-WT co-cultured with miR-599 transfection group decreased (P<0.05), while the enzyme activity of ARPP19-Mut did not change (P>0.05). When miR-599 and MCM3AP-AS1 were co-transfected and overexpressed, the enzyme activity of ARPP19-WT co-cultured was higher than that of miR-599 co-cultured alone (P<0.05), which indicated that MCM3AP-AS1 interacted with miR-599 to regulate ARPP19 in CRC. (Table 6).

Group	ARPP19-WT	ARPP19-Mut
miR-599 control group	1.93±0.18	1.83±0.14
miR-599 transfection group	1.04±0.05	1.88±0.15
Co-transfection group	1.85±0.16	1.92±0.17
F value	11.476	13.225
P value	0.025	0.014

Table 6: Assay of luciferase activity ($\bar{x}\pm s$).

Discussion

LncRNA is a kind of long transcript (>200 nucleotides) which cannot be translated into protein. According to reports, several lncRNA are involved in various biological processes, especially in regulating cell proliferation, migration, invasion, and apoptosis in tumorigenesis[12]. LncRNA can be used as oncogene or tumor suppressor gene in CRC. It is reported that although a high level of lncRNA GHET1 can lead to multidrug resistance in CRC cells, silencing GHET1 can inhibit the activation of CRC cells[13]. Other lncRNA, such as LEIGC, play a role in tumor suppression in CRC. MiRNA is a group of small non-coding RNAs, which has been identified as GC diagnosis and biomarker. Increasing evidence shows that miRNA may have an anti-tumor effect in different types of tumors. It has been proved that miR-192 can inhibit the progression of bone metastasis of lung cancer by regulating TRIM44[14]. The miR-506-3p inhibits the tumorigenesis of thyroid papillary carcinoma cells by targeting YAP1. MiR-296-5p inhibits the occurrence of esophageal squamous cell carcinoma by inhibiting STAT3 signaling[15]. Our study showed that miR-599 was involved in CRC, which decreased its expression in CRC cell lines. In addition, the overexpression of miR-599 inhibited cell proliferation and migration in CRC. Interestingly, increasing evidence shows that lncRNA may play the role of competitive endogenous RNA (ceRNA) by interacting with miRNA. The activity of ceRNA is regulated by many factors, such as abundance and subcellular affinity for locating ceRNA, binding affinity with miRNA, and RNA secondary structure. A study proved that lncRNA HOTAIR can be used as ceRNA function, and regulate HER2 expression by sponging miR-331-3p in GC[16]. In this study, MCM3AP-AS1 was proved to negatively regulate miR-599 through direct interaction. It is worth noting that MCM3AP-AS1 is reported to be involved in the progression of different types of cancers. A study has proved that MCM3AP-AS1 can promote the proliferation and invasion of papillary thyroid carcinoma by regulating miR-211-5p/SPARC axis[17].

There is also evidence that MCM3AP-AS1 can promote the progression of liver cancer by regulating miR-194-5p/FOXA1 axis. However, the molecular mechanism of MCM3AP-AS1 in CRC is still unclear. The results of this study showed that MCM3AP-AS1 sponged miR-599, which accelerated the progress of CRC. In addition, MCM3AP-AS1 mRNA expression increased in CRC cell lines. MCM3AP-AS1-knockdown decreased the proliferation and migration of CRC cells. Overall, the results of this study showed that MCM3AP-AS1 accelerated the progress of CRC by combining with miR-599. RT-PCR confirmed that the expression of MCM3AP-AS1 in CRC cells was higher than that in normal colorectal cells. Knocking down MCM3AP-AS1 can reduce the proliferation, migration, and EMT process of Colo 320 cells, and increase

their apoptosis. Previous studies have reported that miRNA participates in tumor progression by targeting mRNA. For example, miR-26a inhibits the proliferation of thyroid cancer cells by targeting ARPP19[18]. In addition, miR-9-5p targets GOT1 to inhibit the proliferation, invasion, and metabolism of pancreatic cancer cells.

MiRNA is closely related to proliferation and differentiation, and it has been found that it can regulate specific steps in the metastasis pathway and the process of transforming tumor cells into metastatic malignant tumors. It is found that MiR-320b is up-regulated in CRC with liver metastasis and positively regulated the expression of metastasis promoting genes. The MiR-320d is highly expressed in the hyperplasia area of the colon crypt of colon mucosa with normal CRC. In addition, a study found that serum miR-320a in IVR CRC patients is up-regulated compared with that in stage I-II[19]. MiR-599 family also promotes metastatic colonization by regulating the secretory group of tumor cells in breast cancer, thus playing a role in migration. Overexpression of these miRNA can increase the lung colonization ability of poorly metastatic cancer cells and reduce the process of tumor cells entering the circulation from mouse primary tumors, which may be achieved by inhibiting EMT. In addition to miRNA, there are several groups studying whether other ncRNA can be used as biomarkers of cancer. As many gene expression changes are reported to be related to epigenetic changes, any transcript may be affected[20]. In this study, a dual luciferase reporter gene assay confirmed that miR-599 is directly bound to ARPP19. In addition, MCM3AP-AS1-knockdown could reduce ARPP19 mRNA expression by promoting miR-599 expression.

The results also showed that knocking down ARPP19 could weaken the proliferation and migration of CRC cells. Furthermore, the overexpression of MCM3AP-AS1 increased the expression of ARPP19 mRNA, and its effect was reversed after transfection with miR-599 mimetic. It is worth noting that MCM3AP-AS1 promoted CRC progress through miR-599/ARPP19 axis. To sum up, the results of this study showed that lncRNA MCM3AP-AS1 played its biological function by regulating miR-599/ARPP19 axis, and therefore might be an effective therapeutic target for CRC.

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Acknowledgment

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