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ORIGINAL RESEARCH

LncRNA MCM3AP-AS1 Upregulates CDK4 by Sponging miR-545 to Suppress G1 Arrest in Colorectal Cancer

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Xudong Ma[1]
Jing Luo[1]
Yi Zhang[2]
Dali Sun[1]
Yueying Lin[1]

[1]Gastrointestinal Surgery, The Second Affiliated Hospital of Kunming Medical University, Kunming City, Yunnan Province 650031, People's Republic of China; [2]The General Surgery Department, The Teng-Chong People's Hospital, Tengchong City, Yunan Province, 679100, People's Republic of China

Introduction: This study aimed to investigate the role of lncRNA MCM3AP-AS1 in colorectal cancer (CRC).

Methods: Paired tumor and non-tumor tissues were collected from 60 CRC patients. Expression of MCM3AP-AS1 was determined by RT-qPCR. Overexpression of MCM3AP-AS1, miR-545, and CDK4 in CRC cells was achieved to explore the interactions between them. Cell cycle assay was performed to analyze the roles of MCM3AP-AS1, miR-545, and CDK4 in regulating the cell cycle progression of CRC cells.

Results: We found that MCM3AP-AS1 was upregulated in CRC and its high expression levels predicted poor survival of CRC patients. MCM3AP-AS1 is predicted to interact with miR-545. In CRC cells, overexpression of MCM3AP-AS1 and miR-545 was achieved, while their overexpression did not affect the expression of each other. Instead, overexpression of MCM3AP-AS1 led to the increased expression levels of CDK4, which is a downstream target of miR-545. Cell cycle analysis showed that overexpression of MCM3AP-AS1 and CDK4 suppressed G1 arrest induced by miR-545. In addition, overexpression of MCM3AP-AS1 reduced the enhancing effects of overexpressing miR-545 on cell cycle progression.

Conclusion: MCM3AP-AS1 may upregulate CDK4 by sponging miR-545 to induce G1 arrest in CRC cells.

Keywords: MCM3AP-AS1, colorectal cancer, miR-545, CDK4, G1 arrest

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in many countries, such as China and America.[1–3] In 2018, CRC affects a total of 1,096,601 new cases, which were 6.1% of all new cancer cases and caused 551,269 deaths, which were 5.8% of all cancer deaths.[3] Despite the development of diagnostic and treatment approaches, the survival of CRC patients is still poor.[4] Especially for patients with metastatic tumors, only 15% patients live longer than 5 years after the initial diagnosis.[4,5] Therefore, novel therapeutic approaches are still needed.

Previous studies on the molecular pathogenesis of CRC have identified a considerable number of molecular pathways involved in the development and progression of this cancer, such as the PTEN signaling and NF-κB signaling.[6,7] In effect, understanding the functions of these molecular factors (such as PTEN) involved in CRC provides novel insights into the development of anti-cancer approaches, such as targeted therapies.[8,9] Extensive studies have revealed that non-coding RNAs (ncRNAs), such as miRNAs and long (> 200 nt) ncRNAs (lncRNAs),

Correspondence: Yueying Lin
Email us5010@163.com

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encode no proteins but regulate cancer-related gene expression to participate in cancer biology.[10] Therefore, regulating the expression of certain crucial ncRNA players may indirectly suppress the development of cancers. However, the functions of most ncRNAs in CRC remain hardly known. MCM3AP-AS1 is a recently characterized oncogenic lncRNA in several types of cancer, such as hepatocellular carcinoma, glioblastoma, and papillary thyroid cancer.[11–13] However, the involvement of MCM3AP-AS1 in CRC remains unknown. Our preliminary bioinformatics analysis showed that MCM3AP-AS1 may interact with miR-545, which can target CDK4 to suppress cancer development.[14] This study was therefore carried out to investigate the interactions among MCM3AP-AS1, miR-545, and CDK4 in CRC.

Materials and Methods

CRC Patients

Research subjects of the present study included 60 patients (32 males and 28 females, 41– 67 years old, mean age 53.2 ± 6.6 years old) diagnosed as CRC between March 2012 and March 2014 at the Second Affiliated Hospital of Kunming Medical University. This study was approved by the Ethics Committee of this hospital. All 60 patients were diagnosed for the first time and patients with other clinical disorders were excluded. No therapy was initiated before admission. All CRC patients were diagnosed by histopathological biopsy (fine needle aspiration). During biopsy, paired CRC and non-tumor tissues were collected from each patient. All fresh tissues were stored in liquid nitrogen before use. All patients signed the informed consent.

Treatment and Follow-Up

The 60 patients included 10, 12, 18, and 20 cases at clinical stage I, II, III, and IV, respectively. Based on patient's health condition and clinical stages, different treatment approaches, such as surgical resection, chemotherapy, radiotherapy, and targeted therapy were applied. Patients were followed up for 5 years after admission to record patients' survival.

CRC Cells and Transfections

Human CRC cell line CR4 (Sigma-Aldrich, USA) was used as the cell model. Cells were cultivated in a medium composed of 90% EMEM medium and 10% FBS. Cell culture conditions were 37°C, 5% CO_2, and 95% humidity. Expression vectors of MCM3AP-AS1 and CDK4 were constructed using pcDNA 3.1 vector as a backbone. Negative control (NC) miRNA and miR-545 mimic were purchased from Sigma-Aldrich. CR4 cells were transfected with 10 nM expression vector or 40 nM miRNA using lipofectamine 2000 (Invitrogen). Untransfected cells were used as the control (C) cells. Cells transfected with empty vector or NC miRNA were NC cells.

Nude Mice Experiments

CR4 cells with successful transfection of C (approximately 1×10^6 cells/mouse) and MCM3AP-AS1 were subcutaneously injected into the armpit of 6-week-old BALB/c athymic nude mice (five of each group, male:female: 3:2). Tumor volume and weight were measured at 5, 10, 15, 20, 25, and 30 days. The mice were euthanized 30 days after injection, and photographs of excised tumors were obtained. The excised tumors were then used for further analyses.

Fish

FISH was used to detect the localization of MCM3AP-AS1 in CRC cells. MCM3AP-AS1 probes were prepared using Ribo™ lncRNA FISH Probe Mix (Red) (Guangzhou RiboBio, Guangzhou, China). In brief, PCa cells were inoculated into a six-well culture plate, fixed with 4% polyformaldehyde, and treated with protease K (2 mg/mL), glycine, and ethylphthalate reagent. After pre-hybridization, 250 Ml hybridization solution containing probes (300 ng/mL) was added to the plate, followed by overnight incubation at 42°C. DAPI diluted by Tween 20 (PBST) solution (1:800) was added to stain the nuclei. Five randomly selected visual fields were observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan), and photographed.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates (2×10^4 cells/well) and cultured for 24, 48, and 72 hours, respectively. Then 10 μL CCK-8 solution (BD Biosciences, San Jose, CA, USA) was added into each well. After incubated at 37°C for 2 hours, the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA)

Colony Formation Assay

DLD-1 cells were firstly transfected for 24 hours, and then cultured in six-well plates (1×10^3 cells/well) for 2 weeks.

Colonies were fixed with 4% of paraformaldehyde for 10 minutes, stained with 1% crystal violet, and washed twice with PBS. Finally, cell colonies were counted and images were taken.

Dual Luciferase Activity Assay

MCM3AP-AS1 luciferase vector was constructed using pGL3 vector (Promega Corporation) as backbone. CR4 cells were co-transfected with either MCM3AP-AS1 vector + miR-545 mimic (miR-545 group) or MCM3AP-AS1 vector + NC miRNA (NC group) using the aforementioned method. Luciferase activity was measured at 48 hours post-transfection.

RNA Preparations and qPCR

Total RNAs from cells and paired tissue samples were extracted using RNAzol reagent (Sigma-Aldrich). To harvest miRNAs, 85% ethanol was used to precipitate and wash RNA samples. DNA removal was performed using a gDNA eraser (Takara). NanoDrop 2000 Spectrophotometer (Thermo Scientific) was performed to measure RNA concentrations. A reverse transcription system (Promega Corporation) was used to perform reverse transcription using RNA samples as a template to synthesize cDNA samples. With cDNA samples as the template, SYBR Premix Ex Taq II kit (Takara Bio) was used to perform all qPCR reactions. The expression levels of MCM3AP-AS1 were determined with GAPDH as internal control. All-in-One™ miRNA qRT-PCR Reagent Kit (Genecopoeia) was used to measure the expression levels of mature miR-545. All qPCR reactions reached an about 100% amplification rate, some of the PCR products were randomly selected for sequencing to confirm correct PCR products were obtained. All reactions were repeated three times and the method of $2^{-\Delta\Delta cq}$ was used to analyze Ct values to calculate relative gene expression levels.

Western Blot

Isolation of total proteins from CR4 cells using RIPA solution (Invitrogen) was performed. Protein concentrations were measured using a BCA protein detection kit (Takara bio). Following protein denaturation in boiling water for 8 minutes, protein separation was performed using 10% SDS-PAGE. The separated proteins were transferred to PVDF membranes, and membranes were blocked in PBS containing 5% non-fat milk at room temperature for 2 hours. Membranes were first incubated with GAPDH (ab9845, Abcam) and CDK4 (ab137675, Abcam) primary antibodies at 4°C for 12 hours. Following that, membranes were further incubated with HRP Goat Anti-Rabbit (IgG) (ab6721, Abcam) at room temperature for a further 2

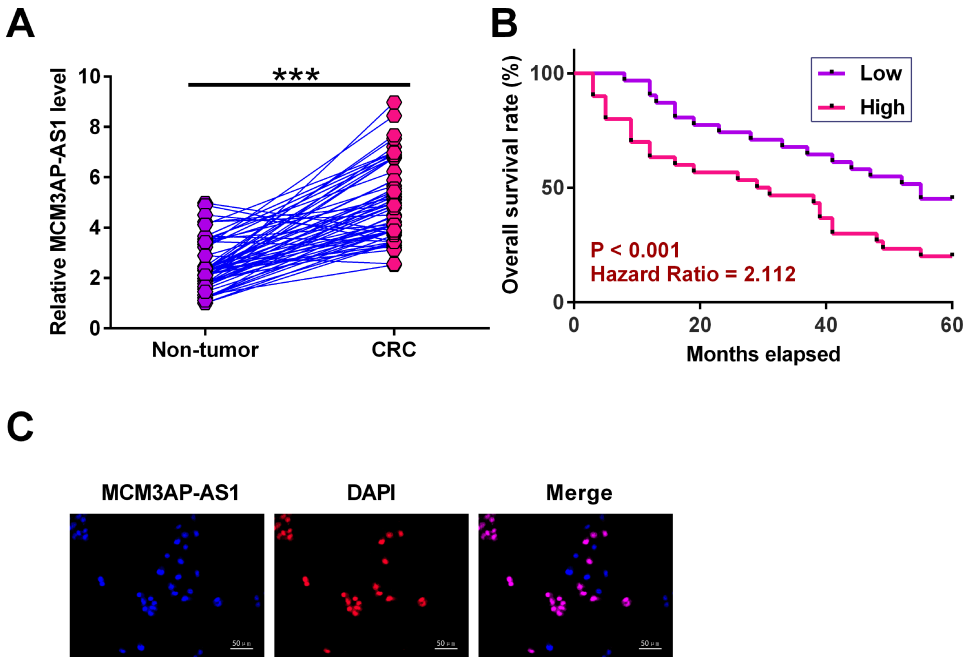

Figure 1 MCM3AP-AS1 was upregulated in CRC and predicted poor survival. The expression levels of MCM3AP-AS1 in paired tissues were measured by performing RT-qPCR. PCR reactions were repeated three times and mean values were presented and compared (**A**). ***P<0.001. The 60 patients were divided into high and low MCM3AP-AS1 level groups (n=30) with the median level of MCM3AP-AS1 in CRC tissues a cutoff value. Survival curves were plotted for both groups and compared by Log rank test (**B**). Subcellular localization of MCM3AP-AS1 in PCa cells as detected by FISH (**C**). ***P<0.001.

Table 1 Correlations Between the Expression Levels of MCM3AP-AS1 and Clinical Characteristics Data of Patients

Items	Groups	Cases	High	Low	χ^2	*P*-value
Age	>55 (years old) ≤55 (years old)	34 26	16 14	18 12	0.27	0.60
Gender	Male Female	32 28	14 16	18 12	1.07	0.30
Tumor stage	I II III IV	10 12 18 20	4 6 10 10	6 6 8 10	0.62	0.88
Tumor grade	1 and 2 3 and 4	23 37	10 20	13 17	0.63	0.43

hours. Enhanced Chemiluminescence (ECL, Sigma-Aldrich) was used for signal production. Quantity One software was used to normalize signals.

Cell Cycle Analysis

Following trypsinization, cold PBS was used to wash CR4 cells. After that, cells were incubated with 75% ethanol at 4°C for 3 hours, followed by washing with cold PBS. The washed cells were then stained with BD Pharmingen™ PI/RNase at room temperature for 30 minutes. Finally, cells were separated using a flow cytometer. A total of 10^5 events were included in each experiment.

Immunohistochemistry (IHC)

Xenograft tumors were immunostained for H&E and Ki-67. Signals were amplified and visualized with 3′-diaminobenzidine chromogen, and the tumors were subsequently counterstained with hematoxylin. Expression was considered positive when 60% or more of the tumor cells were stained. The IHC staining results were independently scored by the author and a pathologist to minimize subjectivity and then compared, and final comprehensive results were obtained. AntiKi-67 (1:100) antibodies were purchased from R&D.

Statistical Analyses

Data of three biological replicates were expressed as mean ±SD values. Paired *t*-test was used to compare paired tissues. Unpaired *t*-test was used to compare two independent groups. ANOVA Tukey's test was used to compare multiple groups. The 60 patients were divided into high and low MCM3AP-AS1 level groups (n=30) with the median level of MCM3AP-AS1 in CRC tissues a cutoff value. Survival curves were plotted for both groups and compared by Log rank test. Chi-squared test was

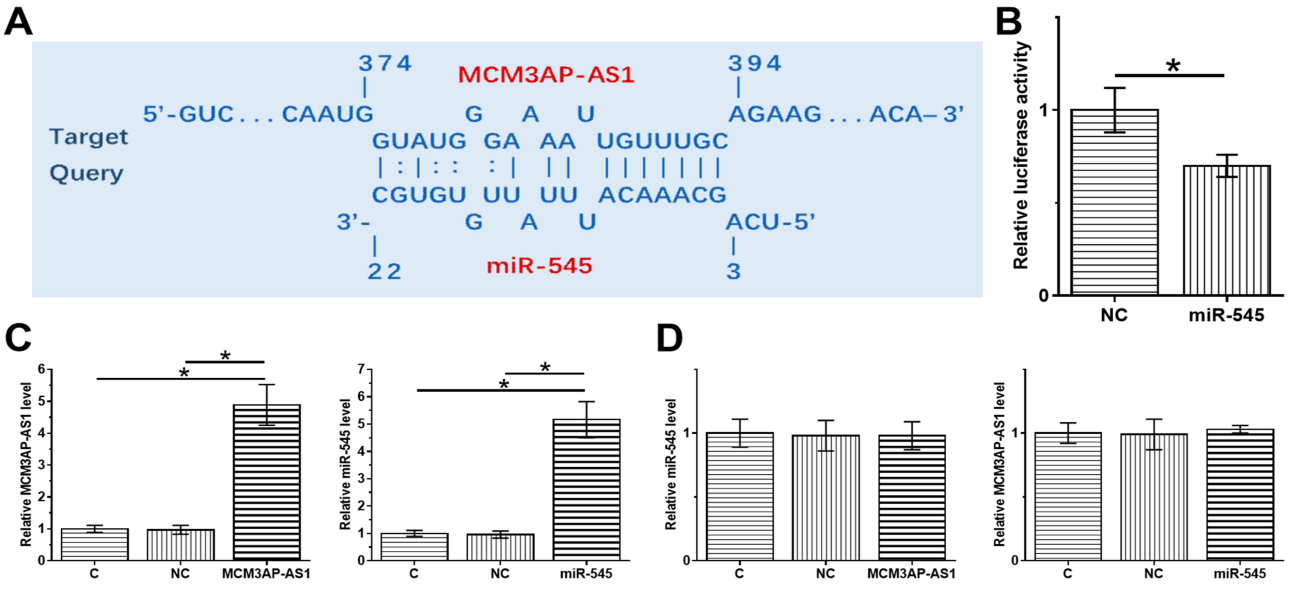

Figure 2 MCM3AP-AS1 and miR-545 interacted with each other but did not regulate the expression of each other. Potential base pairs can be formed by MCM3AP-AS1 and miR-545 were predicted by IntaRNA 2.0 (**A**). Dual luciferase activity assay was performed by co-transfecting CR4 cells with either MCM3AP-AS1 vector + miR-545 mimic (miR-545 group) or MCM3AP-AS1 vector + NC miRNA (NC group). Luciferase activity was measured and compared at 48 hours post-transfection. Luciferase activity was set to "1", and the miR-545 group was normalized to the NC group (**B**). To further characterize the interaction between them, CR4 cells were transfected with either MCM3AP-AS1 expression vector or miR-545 mimic, and the overexpression of MCM3AP-AS1 and miR-545 was confirmed by RT-qPCR at 48 hours post-transfection (**C**). Effects of overexpression of MCM3AP-AS1 and miR-545 on the expression of each other were analyzed by RT-qPCR (**D**). All experiments were repeated three times and data were expressed as mean±SD values. **P*<0.05.

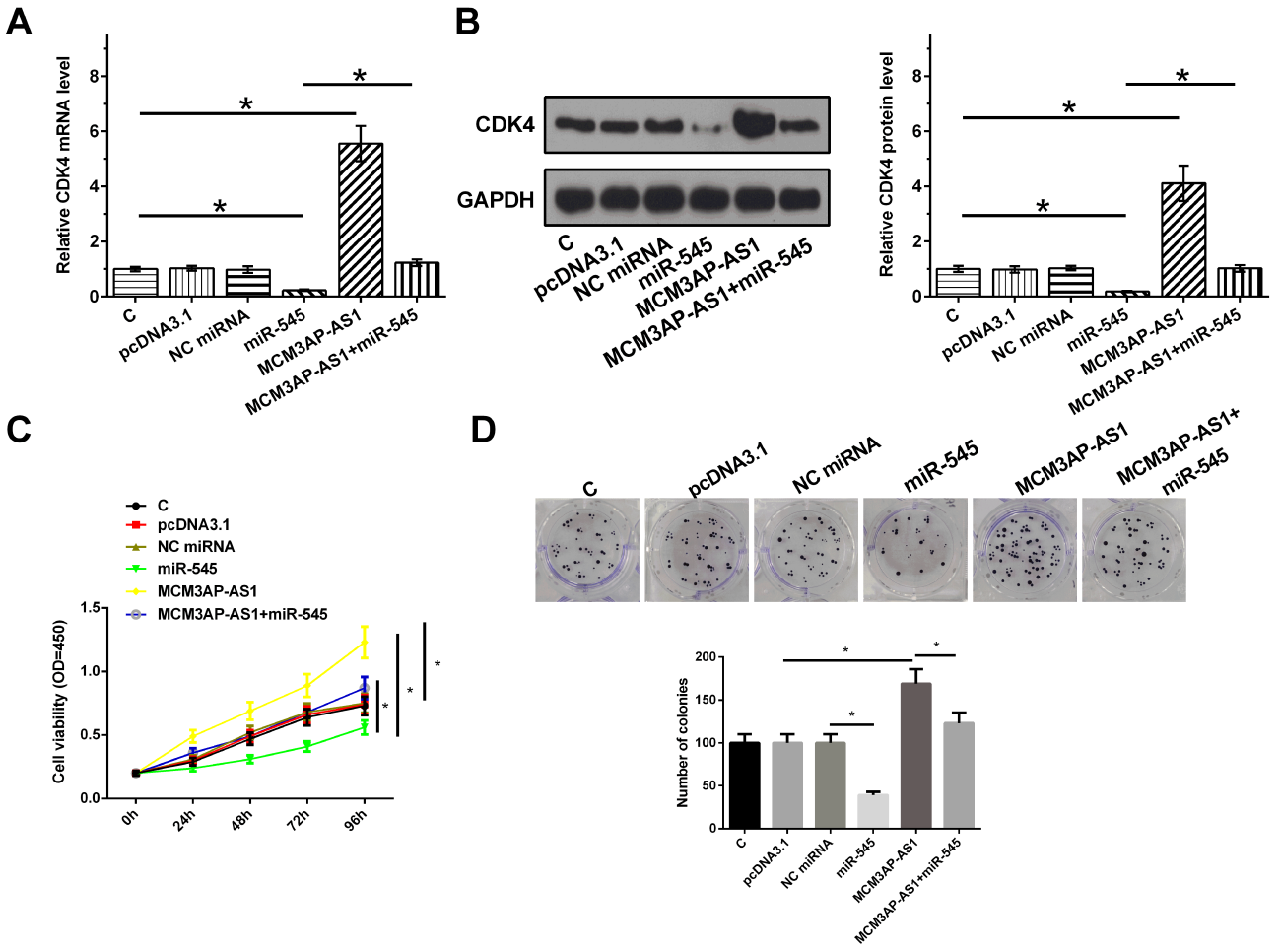

Figure 3 Overexpression of MCM3AP-AS1 led to the increased expression levels of CDK4. CDK4 is a downstream target of miR-545. To test whether MCM3AP-AS1 can sponge miR-545, the effects of overexpression of MCM3AP-AS1 and miR-545 on the expression of CD4 were analyzed by RT-qPCR (**A**) and Western blot (**B**). CCK-8 and colony formation assays were used to measure proliferation of CRC cells (**C** and **D**). All experiments were repeated three times and data were expressed as mean±SD values. C, control cells without transfections; *P<0.05.

performed to analyze the correlations between the expression levels of MCM3AP-AS1 and clinicopathological data of patients. P<0.05 was statistically significant.

Results

MCM3AP-AS1 Was Upregulated in CRC and Predicted Poor Survival

Expression levels of MCM3AP-AS1 in paired tissues were measured by performing RT-qPCR. Compared to non-tumor tissues, the expression levels of MCM3AP-AS1 were significantly higher in CRC tissues (Figure 1A, P<0.001). Analysis of survival curves were plotted for both high and low MCM3AP-AS1 level groups. Compared to patients in the low MCM3AP-AS1 level group, patients in the high MCM3AP-AS1 level group experienced a significantly lower overall survival rate (Figure 1B). Fluorescence in situ hybridization (FISH) showed that MCM3AP-AS1 was mainly localized in the nucleus (Figure 1C). Chi-squared test showed that the expression levels of MCM3AP-AS1 in CRC tissues were not significantly correlated with patients' age, gender, clinical stage, and tumor grades. The detailed clinical characteristics, including age, gender, tumor stage, and tumor grade are shown in Table 1.

MCM3AP-AS1 and miR-545 Interacted with Each Other but Did Not Regulate the Expression of Each Other

The interaction between MCM3AP-AS1 and miR-545 was predicted by IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp). It is predicted that MCM3AP-AS1 and miR-545 may form multiple base pairs (Figure 2A). To further confirm the interaction,

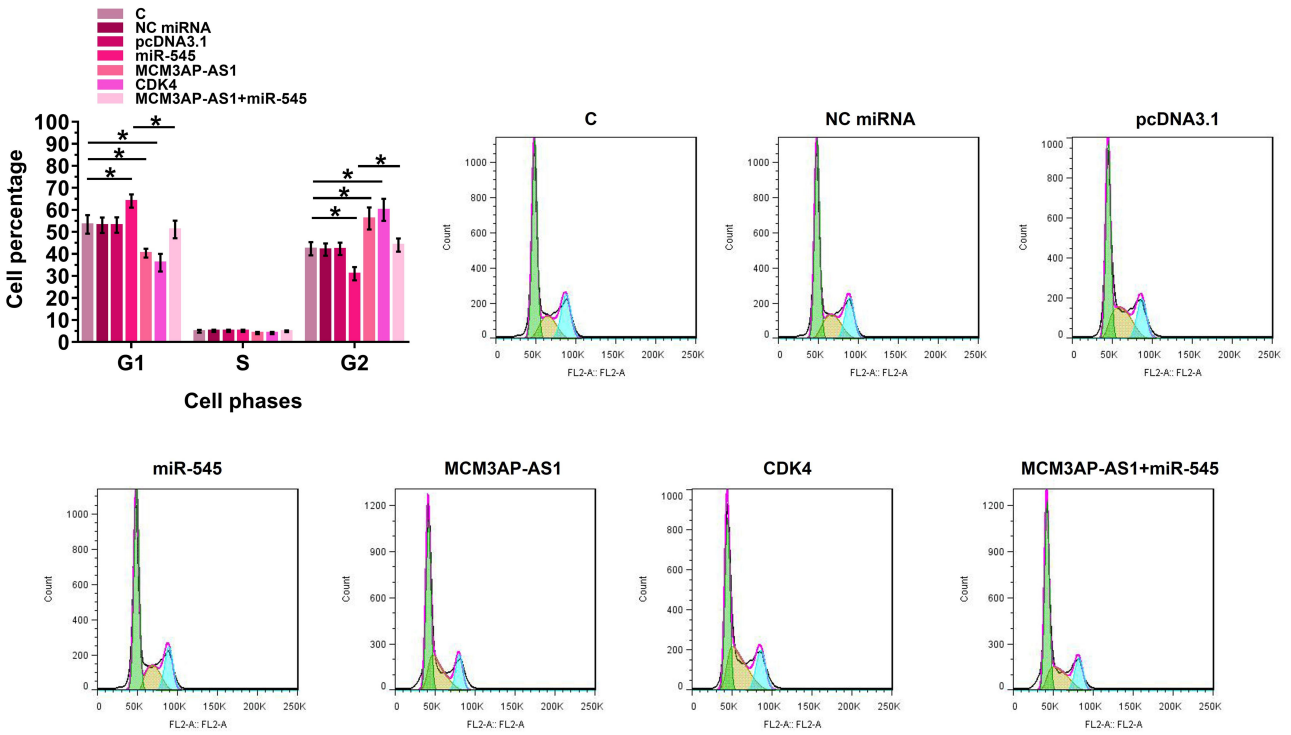

Figure 4 MCM3AP-AS1 suppressed G1 arrest through miR-545/CDK4 axis. Cell cycle analysis was performed to evaluate the effects of overexpression of MCM3AP-AS1, miR-545, and CDK4 on the cell cycle progression of CR4 cell. All experiments were repeated three times and data were expressed as mean±SD values. C, control cells without transfections; *P<0.05.

dual luciferase activity assay was performed by co-transfecting CR4 cells with either MCM3AP-AS1 vector + miR-545 mimic (miR-545 group) or MCM3AP-AS1 vector + NC miRNA (NC group). Compared to NC group, relative luciferase activity was significantly lower in the miR-545 group (Figure 2B, P<0.05). To further characterize the interaction, CR4 cells were transfected with either MCM3AP-AS1 expression vector or miR-545 mimic, and overexpression of MCM3AP-AS1 and miR-545 were confirmed by RT-qPCR (Figure 2C, P<0.05). Compared to the C and NC groups, overexpression of MCM3AP-AS1 and miR-545 did not significantly affect the expression of each other (Figure 2D).

Overexpression of MCM3AP-AS1 Led to Increased Expression Levels of CDK4 and Promotes Cell Proliferation of CRC Cells

CDK4 is a downstream target of miR-545. To test whether MCM3AP-AS1 can sponge miR-545, the effects of overexpression of MCM3AP-AS1 and miR-545 on the expression of CDK4 were analyzed by RT-qPCR (Figure 3A) and Western blot (Figure 3B). It was observed that overexpression of miR-545 led to the downregulation of CDK4, while overexpression of MCM3AP-AS1 played an opposite role and attenuated the effects of overexpressing miR-545. In addition, CCK-8 and colony formation assays indicated that MCM3AP-AS1 significantly promoted cell proliferation, but the transfection of miR-545 significantly reversed the promoting effects of MCM3AP-AS1 (P<0.05) (Figure 3C and D).

MCM3AP-AS1 Suppressed G1 Arrest Through miR-545/CDK4 Axis

Cell cycle analysis was performed to evaluate the effects of overexpression of MCM3AP-AS1, miR-545, and CDK4 on the cell cycle progression of CR4 cells. It was observed that overexpression of miR-545 led to an increased percentage of cells at G1 stage and a decreased percentage of cells at G2 stage (Figure 4, P<0.05). Overexpression of MCM3AP-AS1 and CDK4 played an opposite role. In addition, overexpression of MCM3AP-AS1 reduced the enhancing effects of overexpressing miR-545 on cell cycle progression (P<0.05).

Overexpression of MCM3AP-AS1 Promoted Tumor Growth in Nude Mice

Within 30 days after the xenograft model establishment, the average tumor volume and tumor weight in CR4 cells

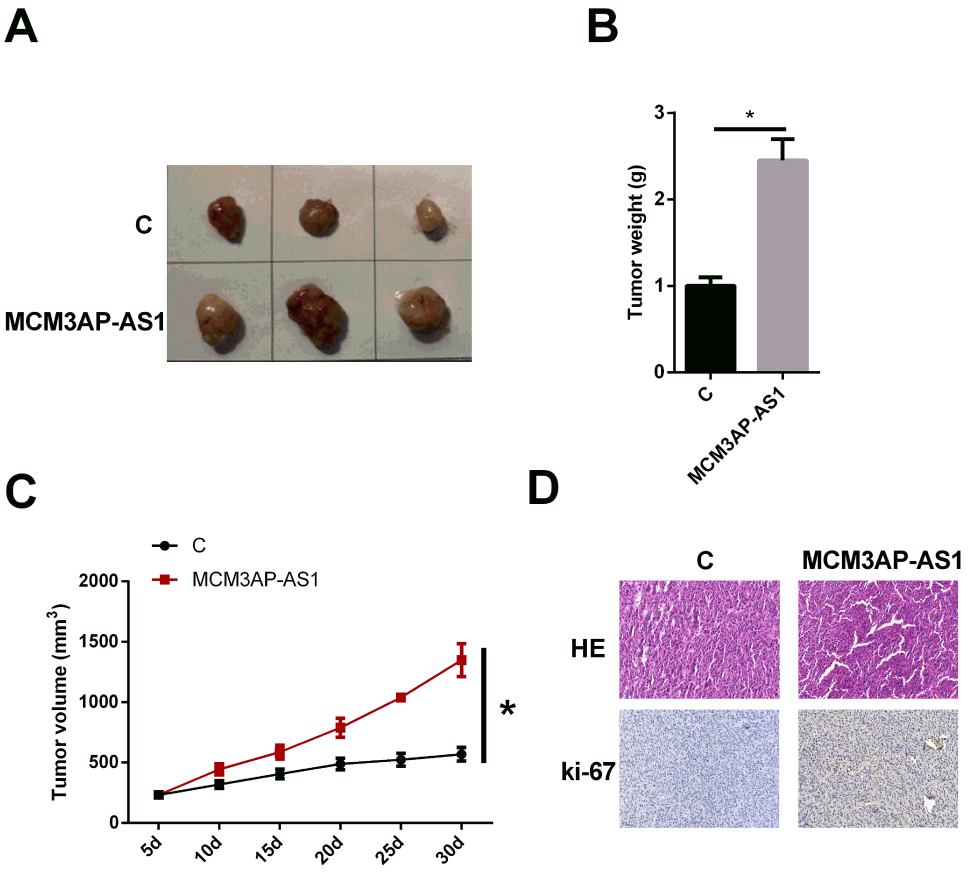

Figure 5 Overexpression of MCM3AP-AS1 promoted tumor growth in nude mice. Overexpression of MCM3AP-AS1 promoted tumor growth in nude mice. (**A**–**C**) The in vivo tumor formation assay suggested that overexpression of MCM3AP-AS1 dramatically reduced tumor size and weight in five samples of each group. (**D**) Immunohistochemistry showed that overexpression of MCM3AP-AS1 increased the proliferation index Ki67 and HE. *P<0.05.

with overexpression of MCM3AP-AS1 was increased compared with that in mock cells (Figure 5AC, P<0.05). Immunohistochemical staining of tumor tissues for Ki-67 and HE detection was then conducted, and it showed that the proliferation was increased in tumor tissues (P<0.01, Figure 5D).

Discussion

In this study we mainly investigated the interactions among MCM3AP-AS1, miR-545, and CDK4 in CRC. We found that MCM3AP-AS1 was upregulated in CRC and may regulate the miR-545/CDK4 axis to regulate cell cycle progression of CRC cells.

Previous studies have characterized the functionality of MCM3AP-AS1 in several types of cancer, such as hepatocellular carcinoma, glioblastoma, and papillary thyroid cancer.[11–13] MCM3AP-AS1 is overexpressed in hepatocellular carcinoma and may regulate the axis of miR-194-5p/FOXA1 to promote tumor growth.[11] In glioblastoma, overexpression of MCM3AP-AS1 promotes angiogenesis by regulating the axis of miR-211/KLF5/AGGF1.[12] Another study reported that the overexpression of MCM3AP-AS1 in papillary thyroid cancer led to increased cancer cell invasion and proliferation rates.[13] This study is the first to report the upregulation of MCM3AP-AS1 in CRC. In addition, the overexpression of MCM3AP-AS1 promoted cancer cell cycle progression. Therefore, MCM3AP-AS1 may play an oncogenic role in CRC.

Due to the lack of classic symptoms at early stages, the early diagnostic rate of CRC is still low.[15] This situation is unlikely to be significantly changed in the near future due to the lack of sensitive diagnostic markers and approaches.[15] Our study demonstrated that the high expression levels of MCM3AP-AS1 in CRC tissues were closely correlated with the poor survival of CRC patients. Therefore, measuring the expression levels of MCM3AP-AS1 before treatment may guide the determination of treatment approaches, thereby improving the survival of CRC patients.

MiR-545 plays different roles in different types of cancer.[14,16] For instance, miR-545 targets RIG-I to promote cell proliferation in hepatocellular carcinoma, indicating its oncogenic role in this disease.[16] In contrast, miR-454 targets CDK4 in lung cancer to suppress cancer cell proliferation.[14] In our study we showed that miR-454 may also target CKD4 to induce G1 arrest in CRC, suggesting its tumor suppressive role in this disease. The key finding of the present study is that MCM3AP-AS1 may sponge miR-545 to upregulate CDK4 to participate in CRC. However, other mechanisms may also exist and further studies are still needed.

In conclusion, MCM3AP-AS1 is upregulated in CRC and may sponge miR-545 to upregulate CDK4, thereby promoting cell cycle progression of CRC cells.

Funding

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Disclosure

The authors report no conflicts of interest for this work.

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