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Increasing miR-150 and lowering HMGA2 inhibit proliferation and cycle progression of colon cancer in SW480 cells

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Abstract. – OBJECTIVE: High mobility group protein A2 (HMGA2) is a kind of oncogene that regulates cell proliferation and cycle. HMGA2 up-regulation is related to the occurrence of multiple tumors including colorectal cancer. MiR-150 is found down-regulated in colorectal cancer tissue. Bioinformatics analysis shows the complementary targeted relationship between miR-150 and the 3'-UTR of HMGA2. This study explores the role of microRNA-150 (miR-150) in regulating HMGA2 expression, colorectal cancer cell proliferation, and cycle.

PATIENTS AND METHODS: Colorectal cancer patients were enrolled to collect cancer and para-carcinoma tissues. MiR-150 and HMGA2 expressions were tested in tissue. MiR-150, HMGA2, and Cyclin A levels in colorectal cancer cell line SW480, and normal colorectal epithelial cell line FHC were compared. The targeted relationship between miR-150 and the 3'-UTR of HMGA2 was evaluated by dual luciferase reporter gene assay. SW480 cells were divided into five groups, including miR-control, miR-150 mimic, small interfere normal control (si-NC), si-HMGA2, and miR-150 mimic + si-HMGA2. Cell cycle was determined by using flow cytometry. The cell proliferation was detected by using the cell counting kit 8 (CCK-8) test.

RESULTS: HMGA2 expression was significantly increased, while miR-150 levels were significantly declined in colorectal cancer tissue compared with that in para-carcinoma tissue ($p<0.05$). HMGA2 and Cyclin A levels were higher significantly, whereas miR-150 expression was lower significantly in SW480 cells compared to that in FHC cells ($p<0.05$). MiR-150 targeted band to the 3'-UTR of HMGA. MiR-150 mimic and/or si-HMGA2 significantly reduced HMGA2 and Cyclin A expressions, blocked cell cycle in the G0/G1 phase, and attenuated cell proliferation.

CONCLUSIONS: We observed that miR-150 down-regulated Cyclin A expression to block colorectal cancer cell cycle and inhibit proliferation through targeted inhibiting HMGA2.

Key Words:
MiR-150, HMGA2, Cyclin A, Proliferation, Cell cycle, Colorectal cancer.

Introduction

Colorectal cancer (CRC) is a common malignant tumor in clinic with high morbidity, mortality, and poor prognosis[1]. High mobility group protein A2 (HMGA2) is a member of the HMGA family. It is named because of its high migration speed in polyacrylamide gel electrophoresis (PAGE)[2]. HMGA2 regulates gene transcription and expression by changing the chromatin configuration or interacting with other transcription factors to participate in multiple biological processes, including cell proliferation, cell cycle, and apoptosis[3]. The HMGA2 expression is low in normal tissues except early embryos and immature tissue[4]. A variety of studies revealed that HMGA2 abnormal over-expression was closely related to multiple tumor occurrence, progression, and prognosis, such as breast cancer[5], bladder cancer[6], prostate cancer[7], and ovary cancer[8]. HMGA2 abnormal expression was also found in CRC and might be associated with progression and prognosis[9,10]. MicroRNA (miR) is a type of important epigenetic regulatory factor. It affects cell pro-

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liferation, cycle, and apoptosis by inhibiting mRNA translation or degrading mRNA[11]. Some evidence[12-14] suggested that miRNA abnormal expression and function were critical in CRC. It was showed that miR-150 down-regulation was related to CRC, while its expression may affect CRC progression[15]. Bioinformatics analysis shows the complementary targeted relationship between miR-150 and the 3'-UTR of HMGA2. This study explores the role of miR-150 in regulating HMGA2 expression, colorectal cancer cell proliferation, and cycle.

Patients and Methods

Main Reagents and Materials

Human CRC cell line SW480 and normal colorectal epithelial cell FHC were provided by Jennio Biological Technology (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Biological Industries Co. Ltd. (Israel). Trizol and Lipofectamine® RNA iMAX transfection reagents were purchased from Thermo Scientific Pierce (Rockford, IL, USA). ReverTra Ace quantitative PCR (qPCR), reverse transcription (RT) kit and SYBR Green were purchased from Toyobo Life Science (Osaka, Japan). HMGA2 small interfere RNA (siRNA) was designed and synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). MicroRNA nucleotide fragment was designed and synthesized by Guangzhou RiboBio. Co. Ltd. (Guangzhou, China). Rabbit anti-human HMGA2 antibody was got from Cell Signaling Technology Inc. (Beverly, MA, USA). Mouse anti-Cyclin A antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG (H + L) was purchased from Abcam Biotechnology (Cambridge, MA, USA). Horseradish peroxidase (HRP) labeled anti-mouse and rabbit secondary antibodies were provided by Jackson ImmunoResearch (West Grove, PA, USA). Cell Counting Kit-8 (CCK-8) was got from Boster (Wuhan, China). Cell cycle detection kit was purchased from Beyotime Biotechnology (Shanghai, China). Luciferase reporter gene vector pLUC Luciferase vector was purchased from Ambion Inc. (Austin, Texas, USA). Dual-Luciferase® Reporter Assay System was obtained from Promega (Madison, MI, USA).

Clinical Information

A total of 41 cases of CRC patients received treatment in the First People's Hospital of Wenling, Zhejiang Province between Dec 2015 and Jul 2016 were recruited. The tumor tissue and para-carcinoma tissue were collected. There were 25 males and 16 females with mean age at 61.8 (46-76) years old. No patients received radio or chemotherapy before surgery.

All the subjects signed the informed consent and approved this study. This investigation was approved by the Ethics Committee of First People's Hospital of Wenling, Zhejiang Province, China.

Immunofluorescence Assay

The tissue section was washed with phosphate-buffered saline (PBS) and blocked by serum. Next, it was incubated in rabbit anti-human HMGA2 monoclonal antibody at 4°C for 12-16 h. Then, the section was incubated in IgG Alexa Fluor 594 labeled Goat Anti-Rabbit IgG (H+L) at room temperature for 60 min. At last, the section was stained with 0.1% 4'6-diamidino-2-phenylindole (DAPI) and observed under the microscope.

Cell Culture

SW480 and FHC cells were maintained in RPMI-1640 medium containing 10% FBS, 1% penicillin-streptomycin, and 2 mM L-glutamine. The cells in logarithmic phase were used for the following experiments.

Dual-Luciferase Activity Detection

The PCR product containing the full length fragment of HMGA2 3'-UTR was connected to pLUC plasmid and transformed to DH5α cells. The plasmid was confirmed by sequencing and named as pLUC-HMGA2-3'-UTR-wt and pLUC-HMGA2-3'-UTR-mut, respectively. Lipofectamine RNAiMAX® was used to co-transfect pLUC-HMGA2-3'-UTR-wt or pLUC-HMGA2-3'-mut and miR-150 mimic to HEK293T cells. After cultured for 48 h, the cells were tested by Dual-Luciferase® Reporter Assay System according to the manual.

Cell Grouping and Transfection

SW480 cells in logarithmic phase were divided into five groups, including miR-control, miR-150 mimic, si-NC, si-HMGA2, and miR-150 mimic + si-HMGA2. Si-HMGA2 sense strand, 5′-CAGCCUGAAUAACUUGAACTT-3′. Si-HMGA2 antisense strand, 5′-GUUCAAGUUAUUCAGGCU-

GTT-3′. Si-NC sense strand, 5′-UUCUCCGA-ACGUGUCACGUTT-3′. Si-NC antisense strand, 5′-ACGUGACACGUUCGGAGAATT-3′. The nucleotide fragment and lipofectamine® RNA iMAX were diluted by Opti-MEM, respectively. After 5 min incubation at room temperature, they were mixed and added to the cells. At last, the cells were collected after 48 h and tested.

qRT-PCR

Total RNA was extracted using the TRIzol. ReverTra Ace qPCR RT Kit was applied for qRT-PCR detection. The primers used were as follows. miR-150P_F: 5′-ATAAAGTGCTGACAGTGCAGA-TAGTG-3′, miR-150P_R: 5′-TCAAGTACCCACA-GTGCGGT-3′; U6P_F: 5′-ATTGGAACGATACA-GAGAAGATT-3′, U6P_R: 5′-GGAACGCTTCAC-GAATTTG-3′; HMGA2P_F: 5′-ACCCAGGGGA-AGACCCAAA-3′, HMGA2P_R: 5′-CCTCTTGGC-CGTTTTTCTCCA-3′; CyclinAP_F: 5′-ACATGGA-TGAACTAGAGCAGGG-3′, CyclinAP_R: 5′-GA-GTGTGCCGGTGTCTACTT-3′; β-actinP_F: 5′-GA-ACCCTAAGGCCAAC-3′, β-actinP_R: 5′-TGTCAC-GCACGATTTCC-3′. The qRT-PCR reaction system contained 10.0 μl 2×SYBR Green RT-PCR Mixture, 0.8 μl primers at 10 μm/l, 2 μg complementary DNA (cDNA), and ddH_2O. The PCR reaction composed of 40 cycles of 95°C for 15 s, 60°C for 30 s, and 74°C for 30 s was performed on ABI7500.

Western Blot

The cells were lysed by RIPA and quantified by bicinchoninic acid (BCA). The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 45 V for 200 min. After the protein was transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 60 min, the membrane was blocked at room temperature for 60 min. Next, the membrane was incubated in a primary antibody (HMGA2, Cyclin A, and β-actin at 1:200, 1:200, and 1:500) at 4°C overnight. After washed by Phosphylated-buffered saline and Tween-20 (PBST) for three times, the membrane was further incubated in HRP labeled secondary antibody (1:5000) at room temperature for 60 min. At last, the membrane was treated by enhanced chemiluminescence (ECL) and detected.

Flow Cytometry

The cells were collected by enzyme and transferred to 15 ml centrifuge tube. After centrifuged at 1000 r/min for 5 min, the cells were fixed by 70% ethanol at 4°C overnight. Then, the cells were added with 50 μg/ml propidium iodide (PI) at 37°C avoid of light for 15 min; the sample was tested on the flow cytometry.

CCK-8 Assay

The cells were seeded in 96-well plate at 1×10^4 cells/well. After incubation for 48 h, 10 μl CCK-8 solution was added to each well. The plate was read on a microplate reader at 450 nm after 4 h incubation. Cell viability = (treatment group A450-blank A450)/(control A450 – blank A450) ×100%.

Statistical Analysis

All data analysis was performed on SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was treated as statistical significance.

Results

MiR-150 and HMGA2 Expressions in CRC Tissue

qRT-PCR showed that miR-150 expression was significantly lower in tumor tissue than the para-carcinoma tissue (Figure 1A). Immunofluorescence detection demonstrated that HMGA2 protein level was markedly up-regulated in tumor tissue compared with adjacent tissue (Figure 1B).

MiR-150 Targeting Inhibited HMGA2 Expression

Bioinformatics analysis revealed the complementary targeted relationship between miR-150 and the 3'-UTR of HMGA2 (Figure 2A). Dual luciferase reporter gene assay revealed that miR-150 mimic transfection markedly reduced the relative luciferase activity in HEK293T cells transfected with pLUC-HMGA2-3'-UTR-wt (Figure 2B). On the contrary, it exhibited no significant impact on the relative luciferase activity in HEK293T cells transfected with pLUC-HMGA2-3'-UTR-mut, suggesting that miR-150 can bind to the 3'-UTR of HMGA2 mRNA and inhibit its expression.

MiR-150 Was Down-Regulated, While HMGA2 and Cyclin A Were Enhanced in SW480 Cells

CFSE staining flow cytometry demonstrated that the fluorescence intensity of SW480 was ap-

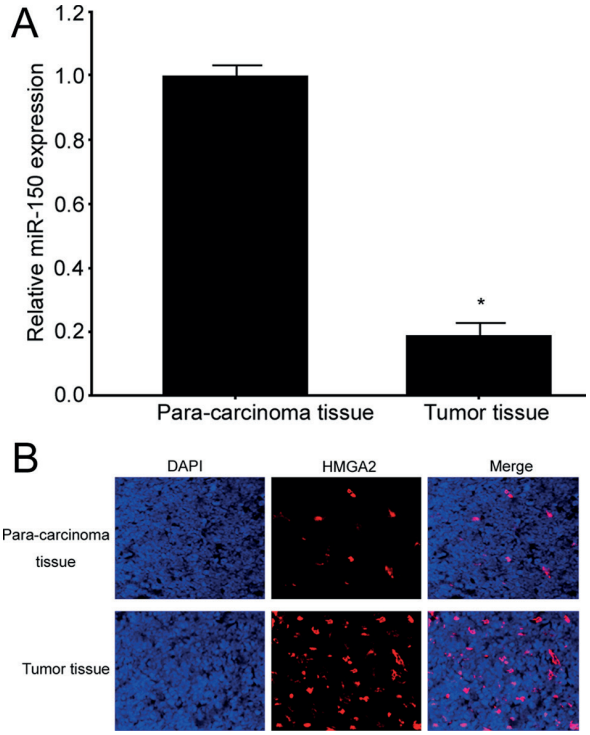

Figure 1. MiR-150 and HMGA2 expressions in CRC tissue. ***A,*** qRT-PCR detection of miR-150 expression. ***B,*** Immunofluorescence detection of HMGA2 protein level. $^{*}p<0.05$, compared with para-carcinoma tissue.

parently lower than FHC (Figure 3A), indicating the proliferative ability of CRC cells was stronger than normal colorectal epithelial cells. Cell cycle detection showed that the cell proportion in S and G2/M phases in SW480 cells was markedly higher, whereas the cell percent in G0/G1 phase was lower than that in FHC cells (Figure 3B). The qRT-PCR revealed that miR-150 expression was significantly lower, while HMGA2 and Cyclin A mRNA levels were markedly higher in SW480 cells compared with FHC cells (Figure 3C). Western blot exhibited that HMG2 and Cyclin A protein expressions markedly elevated in SW480 compared with FHC (Figure 3D). It suggested that miR-150 down-regulation might be the reason of HMGA2 and Cyclin A elevation in CRC cells, thus involving in CRC morbidity.

MiR-150 Over-Expression Blocked SW480 Cell Cycle and Proliferation

MiR-150 mimic and/or si-HMGA2 markedly reduced HMGA2 and Cyclin A expressions (Figure 4A), blocked cell cycle in G0/G1 phase (Figure 4C), and attenuated cell proliferation (Figure 4B).

Discussion

CRC is a common malignant tumor in our country. It is estimated that the incidence of CRC is as high as of 30/100,000, while the mortality at 12/100,000 annually. There are more than 376,000 new cases and 191,000 cases died every year[16]. The prognosis of CRC is poor, resulting in the 3-year survival rate at 60% and 5-year survival rate at lower than 40%[17]. Therefore, searching for the molecular targets in the pathogenesis of CRC is of great significance for early diagnosis and prognosis.

HMGA2 is a kind of nonhistone chromosomal protein without transcriptional activity itself. However, it can regulate gene transcription, replication, and DNA damage impair through changing the spatial structure of chromatin or interacting with protein[18]. Also, Cyclin A plays a critical role in the cell cycle. It can interact with CDK1 and CDK2, thus to promote cells entering S phase and accelerate G2/M phase transition[19]. Tessari et al[20] found that HMGA2 can interact with p120E4F to increase Cyclin A expression, resulting in cell cycle, mitosis, and oncogenesis promotion. It was showed that miR-150 down-regulation was associated with CRC occurrence, which may also affect CRC progression[15]. Bioinformatics analysis shows the complementary targeted relationship between miR-150 and the 3'-UTR of HMGA2. This study explores the role

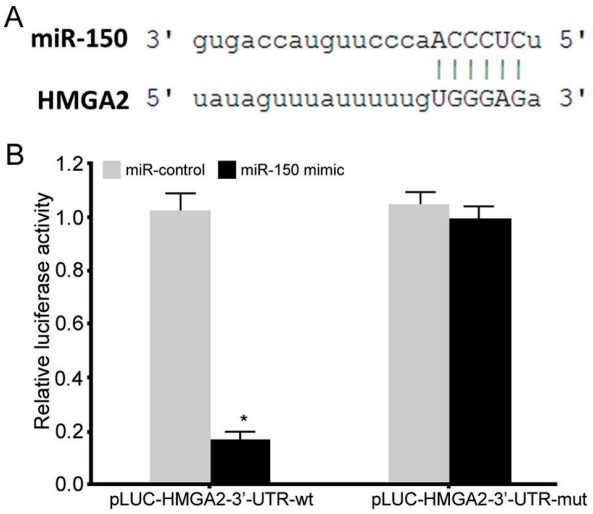

Figure 2. MiR-150 targeted inhibited HMGA2 expression. ***A,*** The potential binding site between miR-150 and the 3'-UTR of HMGA2 mRNA. ***B,*** Dual luciferase reporter gene assay. $^{*}p<0.05$ compared with miR-control.

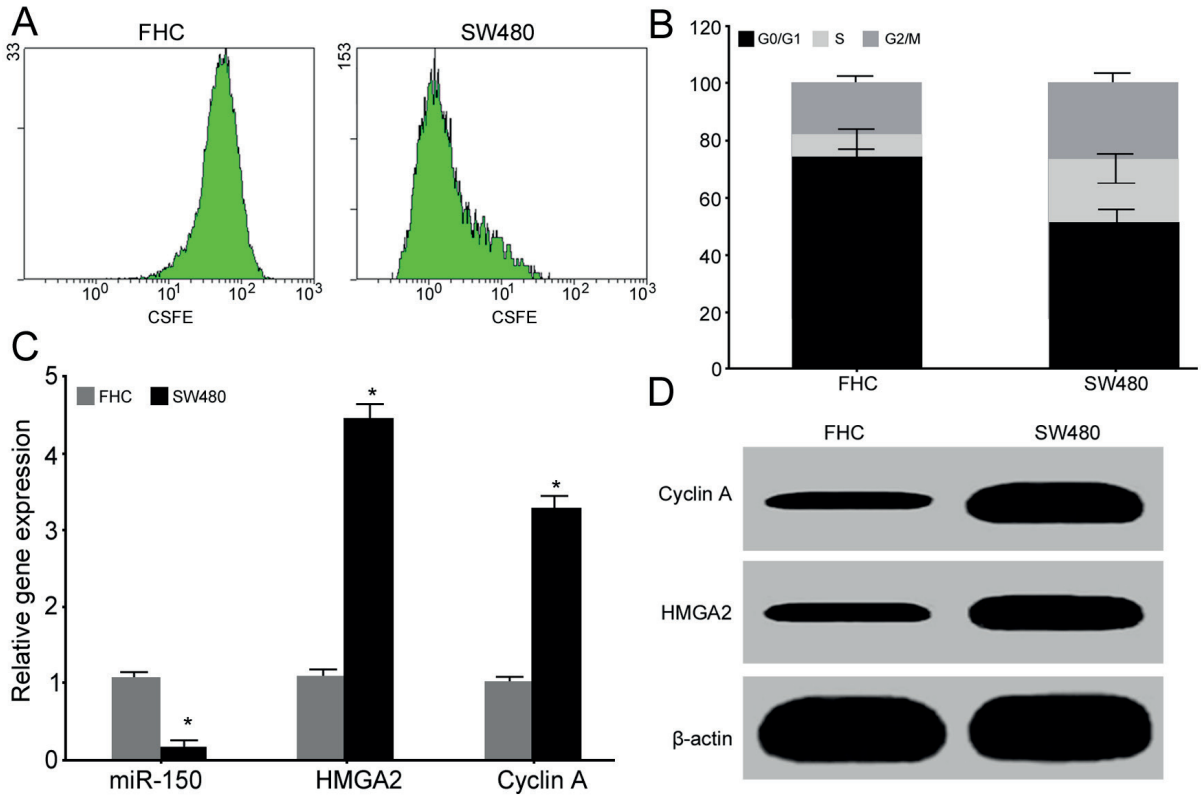

Figure 3. MiR-150 downregulated, while HMGA2 and Cyclin A enhanced in SW480 cells. ***A,*** CFSE detection of cell proliferation. ***B,*** PI detection of cell cycle. ***C,*** qRT-PCR detection of gene expression. ***D,*** Western Blot detection of protein expression. $^{*}p<0.05$, compared with FHC cells.

of miR-150 in regulating HMGA2 expression, colorectal cancer cell proliferation, and cycle.

Our results showed that HMGA2 expression significantly elevated, whereas miR-150 significantly decreased in CRC tissue compared with para-carcinoma tissue. Yu et al[21] revealed that HMGA2 abnormal upregulated in CRC tissue and was correlated with tumor staging and prognosis. Wang et al[10] demonstrated that HMGA2 over-expressed in CRC tissue and its level was associated with distant metastasis and poor prognosis. Razzi et al[22] showed that the HMGA2 elevation in CRC tissue was closely related to blood vessel metastasis, and it could be treated as an indicator to predict tumor progression and clinical outcome. We observed the HMGA2 up-regulation in CRC tissue, which was in accordance with Yu et al[21] and Wang et al[10]. Aherne et al[15] found that miR-150 expression was significantly lower in colon polyps benign lesions and normal colon tissue compared with CRC tissue. Gattolliat et al[23] reported that miR-150 expression was markedly lower in colonic adenoma or CRC tissue than normal colonic mucosa. Ma et al[24] revealed that miR-150 level markedly reduced in CRC tissue compared with adjacent normal tissue. Moreover, patients with miR-150 downregulation exhibited worse survival rate and lower chemotherapy sensitivity. Pizzini et al[25] also observed miR-150 downregulation in CRC tissue compared with normal colonic mucosa. The miR-150 reduction in the peripheral blood of CRC patients was also confirmed by Sarlinova et al[26]. In this research, miR-150 was found declined in CRC tissue, which was similar to the reports of Gattolliat et al[23], Ma et al[24], and Pizzini et al[25]. Compared with the normal colonic epithelial cell FHC, miR-150 expression significantly decreased, whereas HMGA1 and Cyclin A levels up-regulated in CRC cell line SW480. It indicated that miR-150 may play its role in enhancing HMGA2 and Cyclin A, and facilitating CRC cell proliferation and cell cycle. Further analysis demonstrated that miR-150 mimic and/or HMGA2 interference down-regulated HMGA2 and Cyclin A protein levels, blocked cell cycle in the G0/G1 phase, and inhibited cell

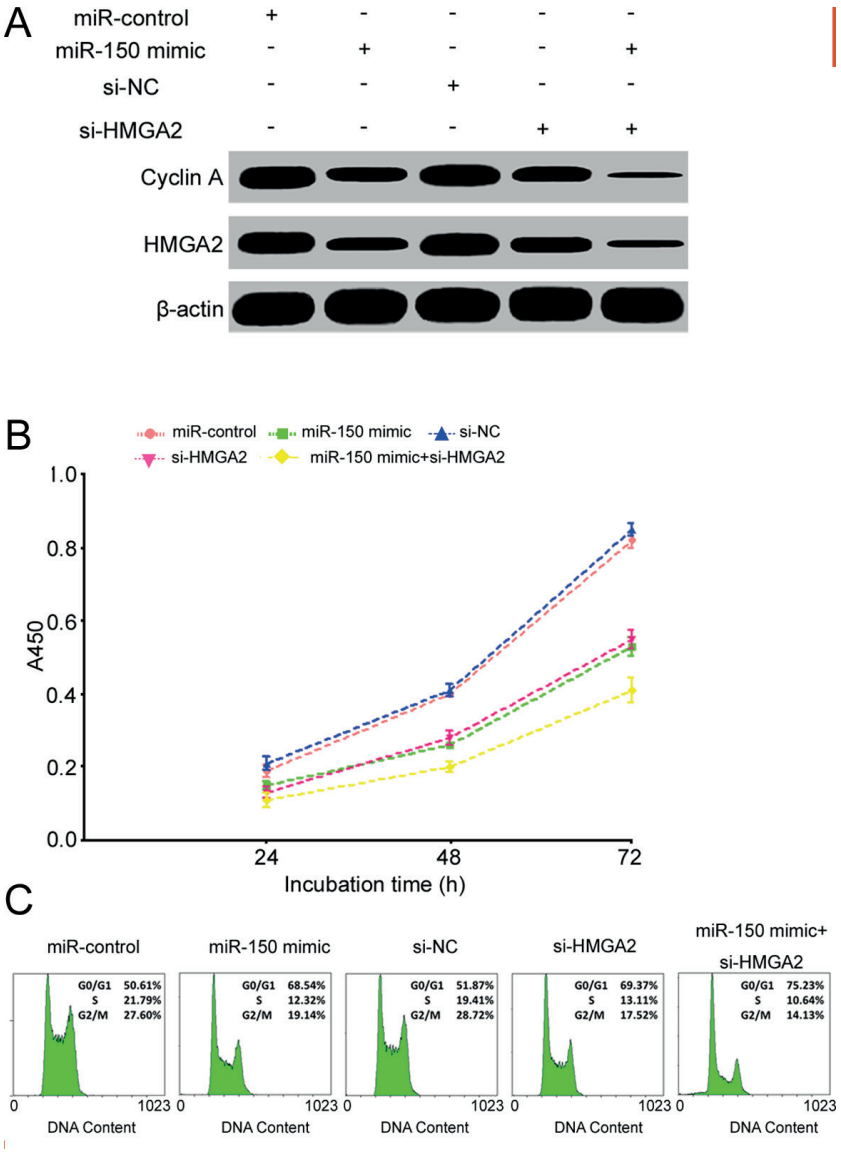

Figure 4. MiR-150 over-expression blocked SW480 cell cycle and proliferation. ***A,*** Western Blot detection of protein expressions. ***B,*** CCK-8 detection of cell viability. ***C,*** PI staining detection of cell cycle.

proliferation in SW480 cells. Wu et al[27] showed that HMGA2 over-expression markedly promoted CRC cell migration and invasion, while HMGA2 reduction exhibited the opposite effect. Wu et al[28] found that HMGA2 siRNA apparently suppressed the cell proliferation of CRC cell line HCT-116 and SW480, while HMGA2 over-expression demonstrated the opposite phenomena. This study showed that HMGA2 down-regulation attenuated the malignant characteristic of CRC cells, which was similar to Wu et al' report[27,28]. Feng et al[29] reported that miR-150 can inhibit oncogene c-Myc expression, suppress cell proliferation and cell cycle, and induce cell apoptosis in CRC LoVo cells. Wang et al[30] detected that miR-150 targeted MUC4 to restrain CRC cell migration and invasion. This study showed that miR-150 induced CRC cell cycle arrest and inhibited cell proliferation by suppressing HMGA2 to down-regulate Cyclin A expression.

Conclusions

We showed that miR-150 arrested CRC cell cycle and inhibited cell proliferation through targeted inhibiting HMGA2 to reduce Cyclin A expression.

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Conflict of Interest
The Authors declare that they have no conflict of interest.

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