European Review for Medical and Pharmacological Sciences 2020; 24: 1794-1806

Metabolites of intestinal microflora upregulate miR-192-5p to suppress proliferation of colon cancer cells *via* RhoA-ROCK-LIMK2 pathway

Y.-L. HUANG[1], X.-H. LI[2], H. MA[1], H.-Y. YUE[1], X.-Y. HU[1]

[1]Department of Gastroenterology, Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia, P.R. China
[2]Department of Pediatrics, Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia, P.R. China

Abstract. – OBJECTIVE: The application of intestinal microflora is involved in various cancers; however, researches reporting the potential of metabolites of intestinal microflora (MIM) on biological activities of colon cancer (CC) cells are unavailable. This study was designed to testify the functions of MIM on CC cells and its mechanism.

MATERIALS AND METHODS: qRT-PCR/Western blot were applied to test the expression levels of miR-192-5p and BMPR2 in human colonic epithelial cells and CC cells (HCT116, SW480). The effects of MIM, mimics-miR-192-5p or inhibitors-miR-192-5p on mRNA and protein expressions of miR-192-5p and BMPR2 were verified by qRT-PCR and Western blot. MTT assay for CC cell viability, flow cytometry for CC cells apoptosis rate, and cell scratch and cell chamber served for the analysis of invasion and migration ability of CC cells. The relationship between miR-192-5p and BMPR2 was validated employing Luciferase reporter gene assay.

RESULTS: Compared with human normal colonic epithelial cells, HCT116 and SW480 cells had lower expression of miR-192-5p and higher expression of BMPR2 ($p < 0.01$). MIM and mimics-miR-192-5p could enhance cell apoptosis and suppress the migration and proliferation of CC cells. MIM were also found to up-regulate miR-192-5p and down-regulate the expression levels of BMPR2 and p-LIMK2 ($p < 0.01$). Transfection of inhibitors-miR-192-5p reversed the inhibitory effect of MIM on CC cells.

CONCLUSIONS: MIM could up-regulate miR-192-5p to inhibit CC cell growth *via* down-regulating BMPR2 and inhibiting the activity of RhoA-ROCK-LIMK2 pathway.

Key Words:
MiR-192, BMPR2, Colon Cancer, RhoA/ROCK/LIMK2 signaling pathway.

Introduction

Colorectal cancer, covering rectal cancer and colon cancer (CC), is characterized by high cancer recurrence rate and poor prognosis, and represents one of the most prevalent vicious neoplasms[1,2]. It remains a dominating cause of cancer-related death worldwide, which accounts for more than 600,000 mortality each year[3]. In addition to lifestyle and environmental influence, genetic factors are important risk factors for CC. Furthermore, a major finding indicated that the dominating cause of cancerization lies in gene expression dysregulation[4].

MicroRNAs (miRNAs) could restrain the expression of target proteins through binding to 3'-untrans-lated region (3'-UTR) of target mRNAs to induce translational repression or mRNA degradation[5,6]. There was increasing evidence supporting the diagnostic and therapeutic biomarker value of miRNAs in CC cells. miRNAs can regulate proliferation, invasion, and apoptosis of CC cells. Of note, LINC00261 represses human CC cell progression through regulating miR-324-3p and the Wnt/β-catenin pathway[7]. Another work stated that miR-32 plays a blocking role in the growth of CC cells through arresting cell cycle[8]. Numerous researches demonstrated that miR-192-5p has gained increasing attention for its implication in various diseases. In osteosarcoma, miR-192-5p negatively regulates tumor cell initiation and progression by targeting USP1[9]. Besides, miR-192-5p mediates hypoxia/reoxygenation-induced cardiomyocyte apoptosis *via* targeting FABP3[6]. However, the expression pattern and the specific function of miR-192-5p in CC still need to be identified.

Corresponding Authors: Li Xiaohua, MD; e-mail: bananaywsw@163.com
Huang Yinglong, MD; e-mail: bananaywsw@163.com

A diverse microbial community exists in the intestinal environment that modifies and forms chemical signals and composition of metabolites within the gut[10]. The human intestinal microflora, comprising 500-1,000 species and millions of genes, contributes to the regulation of many host physiological processes, such as energy consumption, nutritional homeostasis, and immunity[11,12]. Microbiota presents itself as a major player involving in pathology of bowel disease and digestive diseases, and the complex breakdown of communication between the host and its microbiota can lead to CC[13]. MiR-21 is proved to cause intestinal dysbacteriosis and thus induce intestinal inflammation, indicating the possible link of microRNA and intestinal microflora[14]. However, the function of metabolites of intestinal microflora (MIM) in digestive diseases, especially in CC remains unclear.

In this research, we found up-regulated expression of miR-192-5p in MIM, therefore we gave an assumption that MIM may regulate biological activities of CC cells *via* mediating miR-192-5p. Hence, we designed this study with the purpose of seeking out the effect of MIM on CC cells and clarify its specific mechanism. We obtained that MIM could up-regulate miR-192-5p to inhibit CC cell proliferation through the RhoA-ROCK-LIMK2 pathway.

Materials and Methods

Ethical Statement

The experimental scheme was authorized by the Committee of Experimental Animals of Affiliated Hospital of Inner Mongolia Medical University. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals.

Culture of Intestinal Microflora and Collection of MIM in Mice

Sixty healthy male balb/c mice (aged 8 weeks, weighing 200-250 g, and purchased from Changsha, Hunan SJA Laboratory Animal Co., Ltd.) were the subjects of this experiment. All animals were raised in specific pathogen free (SPF) conditions with free access to food on 12 h light and 12 h dark condition. Intestinal microflora suspension was prepared by means of mixing 0.25 g of fresh intestinal contents of mice with 1 mL of 0.9% NaCl in a sterile tube. The suspension was then cultured in 9 mL anaerobic medium in culture bags. The culture bag was sealed after the addition of an anaerobic bag and an oxygen indicator. The anaerobic culture shall be conducted at 36° C for 48 h, during which the culture bag shall be oscillated at 100 r/min for the first 24 h and maintained for the later 24 h. After that, MIM were collected and centrifuged for twice at 10,000 g for 15 min. The supernatant was preserved and filtered through a 0.22 mm membrane under sterile conditions.

Cell Culture and Cell Transfection

CC cells (HCT116 and SW480, purchased from American Type Culture Collection, Manassas, VA, USA) were reserved and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose medium containing 100 U/mL penicillin, 10% inactivated fetal bovine serum (FBS), and 100 μg/mL streptomycin at 37°C in 5% CO_2 saturated humidity atmosphere.

Cells in logarithmic growth phase were subjected to corresponding treatments and grouped into MIM group (treated with MIM), mimics-miR-192-5p group (transfected with miR-192-5p mimics), inhibitors-miR-192-5p group (transfected with miR-192-5p inhibitors), and MIM + inhibitors-miR-192-5p group (treated with MIM and miR-192-5p inhibitors). The dosage of MIM in MIM group and MIM + inhibitors-miR-192-5p group shall account for 30% of culture medium. The final concentration of mimics-miR-192-5p and inhibitors-miR-192-5p in culture medium was 50 nm. All mimics and inhibitors were synthesized by Gene Pharma (Shanghai, China). Transfection was accomplished with the lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA). The transfected cells were made into cell suspension with DMEM (10% FBS), and then, seeded in 24-well plates at the density of 1×10^5 cells/well for incubation at 37° C with 5% CO_2 and 95% humidity.

MTT Assay

After each group was cultured for 24 h, 48 h, 72 h, and 96 h, respectively, 20 μL of MTT solution (5 mg/mL, Merck KGaA, Darmstadt, Germany) was added to each well for incubation at 37° C with 5% CO_2 for 4 h. The culture was terminated, and the culture solution was discarded. MTT derivative was dissolved with 150 μL of dimethylsulfoxide (DMSO) in each well and the crystallization was dissolved by gently shaking. The absorbance values (OD) of each well were measured at the wavelength of 495 nm on en-

zyme-linked immunosorbent assay (ELISA). Set the absorbance value as the ordinate and the time as the abscissa, and then draw the MTT curve. The OD of each group was measured three times and averaged.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from CC cells using TRIzol (Invitrogen, Carlsbad, CA, USA) based on the protocol. Total RNA samples of 5 μL were taken and diluted 20 times with RNAase-free ultra-pure water, and the OD values at 260 nm and 280 nm in ultraviolet spectrophotometer were obtained to determine the purity and concentration of RNA. The ratio of OD260/OD280 between 1.7 and 2.1 indicated the high purity of RNA. The cDNA template was synthesized by reverse transcription reaction in a PCR amplification instrument, and the real-time quantitative RT-PCR experiment was carried out with CFX96 quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). The reaction conditions were 95° C predenaturation for 10 min, followed by 40 cycles of 95° C denaturation for 10 s, 60° C annealing for 20 s, and 72° C extension for 34 s. The threshold value was selected manually at the lowest point of parallel rise of each logarithmic amplification curve, and the Ct value (Threshold cycle) of each reaction tube was obtained. The ratio of the expression of the target gene was calculated employing $2^{-\Delta\Delta Ct}$ method. The formula is as follows: $\Delta\Delta Ct = [Ct_{(target\ gene)}-Ct_{(reference\ gene)}]_{experimental\ group}-[Ct_{(target\ gene)}-Ct_{(reference\ gene)}]_{control\ group}$. Ct refers to the cycle number when the real-time fluorescence intensity of the reaction reaches the set threshold. The amplification was performed in cell in logarithmic stage. The internal reference of miRNA was U6, internal reference of mRNA was GAPDH, and the test was repeated three times. The amplified primer sequences of each gene and its primers are in Table I.

Flow Cytometry

HCT116 and SW480 cells in each group were washed twice with phosphate buffer solution (PBS), and then digested with trypsin. Cells were centrifuged at 1,000 rpm for 5 min to prepare and discard the supernatant of cells. Then cells were re-suspended in PBS before centrifugation at 1,000 rpm for 5 min and removal of cell supernatant. After that, cells were re-suspended in 490 μL pre-cooled 1 × binding buffer with a concentration of 10^5-10^6 cells/mL. Then, 5 μL of Annexin V-FITC and 5 μL of PI (Roche, Basel, Switzerland) were added to cell suspension and incubated on ice for 10 min in the dark. Flow cytometry was employed to evaluate the apoptosis.

Western Blotting

After washed twice with PBS, HCT116 and SW480 cells were treated with RIPA lysate (Beyotime, Beijing, China) containing phenylmethanesulfonyl fluoride (PMSF), and lysed on ice for 30 min. The supernatant was centrifuged at 12,000 rpm at 4°C for 10 min and then stored at -80°C. The protein concentration was analyzed by BCA method. The protein was separated by electrophoresis with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to polyvinylidene difluoride (PVDF) membranes employing wet electric transfer method for 2 h, which was conducted in a cold chamber at 4°C. PVDF membranes were removed and blocked in 5% non-fat dry milk-TBST for 1-2 h at room temperature. After blocking, the PVDF membranes were placed in an incubator overnight

Table I. Primer information for reverse transcription polymerase chain reaction.

Name of primer	Sequences
miR-192-F	CATGATCAGCTGGGCCAAGATGTCAATTCATA
miR-192-R	CTGACCTATGAATTG
U6-F	CTCGCTTCGGCAGCACATATACT
U6-R	ACGCTTCACGAATTTGCGTGTC
BMPR2-F	GTGATACGGGCAGGATCAGTC
BMPR2-R	GGGGACGCATGGCGAAG
GAPDH-F	GTCGATGGCTAGTCGTAGCATCGAT
GAPDH-R	TGCTAGCTGGCATGCCCGATCGATC

Note: F, forward; R, reverse.

at 4° C with the primary antibodies of rabbit anti-human BMPR2 (ab78422, 1:1000, Abcam, Cambridge, MA, USA), p-LIMK2 (ab38499, 1:1000, Abcam, Cambridge, MA, USA), or LIMK2 (ab97766), 1:1000, Abcam, Cambridge, MA, USA). After 3 × 10 min wash in TBST, the PVDF membranes were incubated at room temperature for 1 h with goat anti-rabbit IgG (1:5000, Beijing ComWin Biotech Co., Ltd. Beijing, China) labeled with horseradish peroxidase. Membranes were then washed in TBST for 3 × 10 min before ECL kit was applied for chemiluminescence.

Cell Scratch Assay

HCT116 and SW480 cells were seeded on six-well plates. About 24 h later, cells were scratched using a 10 μL pipette tip perpendicular to cells as possible during the scratch process. PBS was used to wash the cells for 3 times to remove the scratched cells, and then cells were cultured with serum-free DMEM in an incubator at 37° C with 5% CO_2. Photographs were taken under a microscope at time 0 and 24 h post-cell scratch.

Transwell Migration Assay

Cell suspension (100 μL) was added to transwell chamber (Coring, NY, USA) to incubate for 24 h before cell counting. The culture medium in transwell chamber was removed, and then chamber was subjected to PBS wash and formaldehyde fixation for 30 min. The chamber was air-dried naturally and stained with 0.1% crystal violet for 20 min. Cotton swab was utilized to remove the cells on the upper surface of chamber. The chamber was washed with PBS for 3 times to remove unattached cells before cell counting under a microscope. Five high power fields (× 200) were randomly selected to count cells.

Luciferase Reporter Assay

Online prediction software Target Scan predicted the binding site of BMPR2 and miR-192-5p. The mutated type sequences (MT) and wild type sequences (WT) in the binding sites of BMPR2 and miR-192-5p were determined in accordance with the predicted results. MT and the WT fragments were cloned and bound to the Promega vector and then co-transfected with miR-192-5p mimics or miR-192-5p negative control into HEK293 cells respectively, correspondingly renamed as MT + mimics group, MT + NC group, WT + mimics group and WT + NC group. Forty-eight h after transfection, a Luciferase kit (Promega, Madison, WI, USA) was used to test the fluorescence activity intensity of cells in each group.

Statistical Analysis

Data were analyzed utilizing SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Data were displayed as mean ± standard deviation (SD). *t*-test was applied for comparison between two groups, whereas comparisons among multiple groups were analyzed utilizing one-way analysis of variance (ANOVA) and pairwise comparisons were validated using post-hoc Tukey test. Significance was set at $p<0.05$.

Results

Downregulated Expression of miR-192-5p and Upregulated Expression of BMPR2 in CC Cells

Compared with human colon epithelial cells HCoEpiC, the expression of miR-192-5p in HCT116 and SW480 cells was down-regulated ($p<0.01$) (Figure 1A), and the mRNA ($p<0.01$) and protein levels ($p < 0.05$) of BMPR2 were up-regulated (Figure 1B-C).

Effects of Overexpression or Silencing of miR-192-5p on Regulating miR-192-5p, BMPR2 and RhoA-ROCK-LIMK2 Pathway

To investigate the function of miR-192-5p on biological activities of CC cells, mimics-miR-192-5p or inhibitors-miR-192-5p were transfected into CC cells. qRT-PCR results presented that mimics-miR-192-5p group had increased expression level of miR-192-5p and inhibitors-miR-192-5p group had suppressed expression level of miR-192-5p (Figure 2A) compared with mimics-NC group, indicating for satisfactory transfection efficiency. Comparison between mimics-NC group and mimics-miR-192-5p group found that up-regulation of miR-192-5p could lower the mRNA and protein expression levels of BMPR2 (Figure 2B-C) and refrain cell proliferation (Figure 2D), migration (Figure 2F) and invasion ability (Figure 2G) in addition to improving cell apoptotic rate (Figure 2E) of CC cells. MiR-192-5p could also inhibit the phosphorylation of LIMK2 (p-LIMK2) (Figure 2H). Totally reversed expression patterns were found in inhibitors-miR-192-5p group.

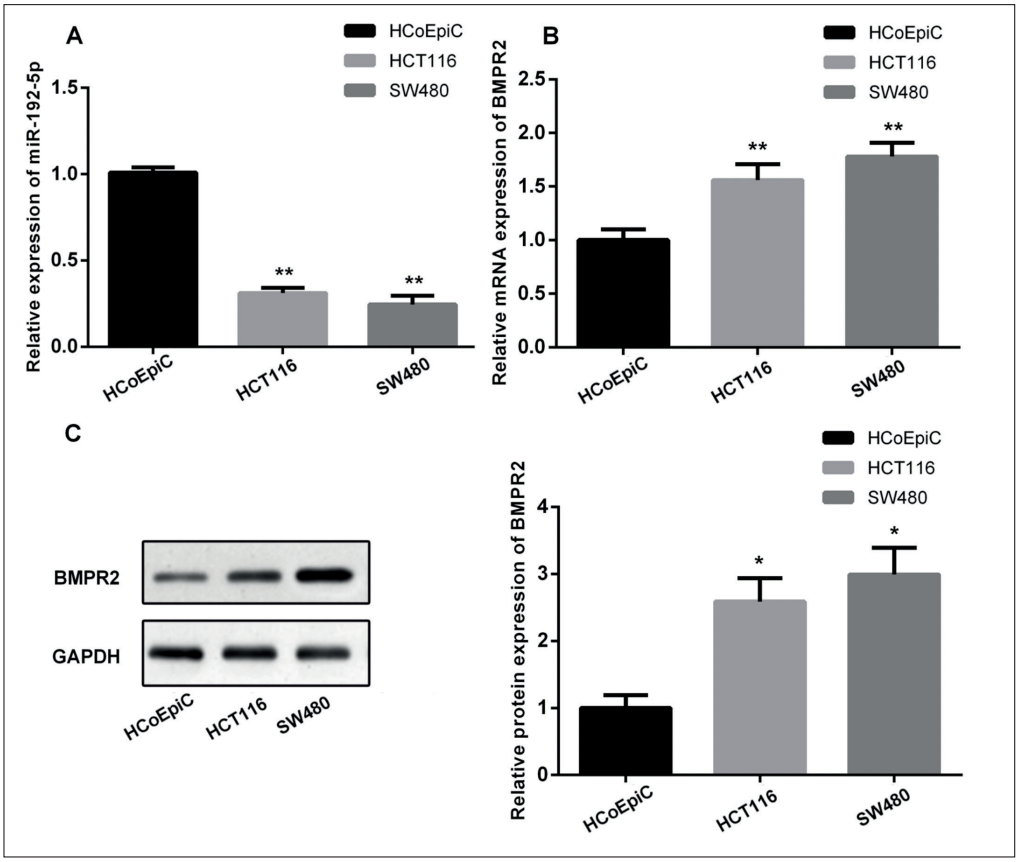

Figure 1. miR-192-5p was lowly expressed while BMPR2 was highly expressed in CC cells. **A,** Expression levels of miR-192-5p in human colon epithelial cells and CC cells were inspected by qRT-PCR; **B-C,** qRT-PCR and Western blot assay examined the mRNA and protein expressions of BMPR2 in human colon epithelial cells and CC cells. *p<0.05, **p<0.01, compared to HCoEpiC group; CC, colon cancer.

Effect of MIM on Regulating Expression of miR-192-5p, BMPR2, as well as Downstream Signaling Pathway in CC Cells

The detection on MIM incubated CC cells employing qRT-PCR showed that compared with Control group, cells subjected to MIM treatment had increased expression level of miR-192-5p (Figure 3A), and decreased mRNA and protein expression level of BMPR2 (Figure 3B-C) and phosphorylation level of LIMK2 (Figure 3H). MIM were also found to enhance cell apoptosis (Figure 3E), and suppress cell proliferation (Figure 3D), migration ability (Figure 3F) and invasive ability (Figure 3G).

Transfection of Inhibitors-miR-192-5p Reverses the Suppression of MIM on CC Cells

A rescue experiment was designed to further validate the impact of miR-192-5p on CC cells. MIM treatment in CC cells increased the expression of miR-192-5p and decreased the mRNA and protein expressions of BMPR2. Transfection of inhibitors-miR-192-5p in MIM treated CC cells could increase the mRNA and protein expressions of BMPR2 and reduce the expression of miR-192-5p, compared with cells with MIM treatment (Figure 4A-C). Results in this study proved that MIM could inhibit cell proliferation, invasion and migration, while enhance cell apoptosis of CC cells. Transfection on MIM treated CC cells supported that inhibitors-miR-192-5p could reverse the suppression of MIM on CC cell progression (Figure 4D-G). Inhibitors-miR-192-5p could also restore the down-regulated expression of p-LIMK2 (p<0.05) (Figure 4H).

BMPR2 is a Target Gene of miR-192-5p

The prediction results of StarBase V3.0 revealed that the binding site of BMPR2 and miR-

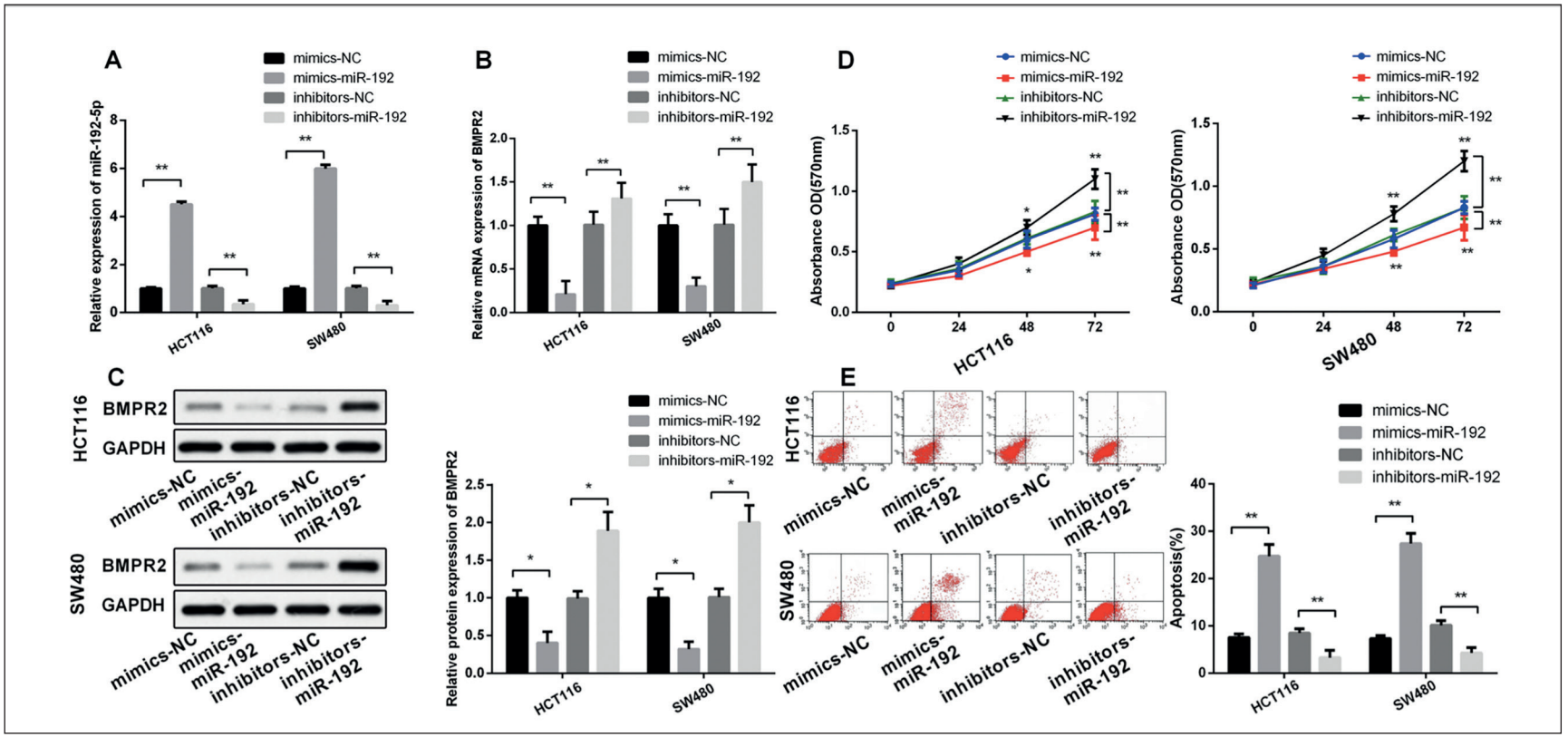

Figure 2. Transfection of mimics-miR-192-5p or inhibitors-miR-192-5p influences expression levels of BMPR2 and miR-192-5p, and activation of RhoA-ROCK-LIMK2 pathway. **A,** Transfection of mimics-miR-192-5p or inhibitors-miR-192-5p regulates miR-192-5p expression in CC cells as detected by qRT-PCR. **B-C,** Regulatory effect of miR-192-5p on mRNA and protein expressions of BMPR2 was determined by qRT-PCR and Western blot. **D,** Cell proliferation was tested by MTT assay. **E,** Cell apoptosis after transfection was detected by flow cytometry.

Figure Continued

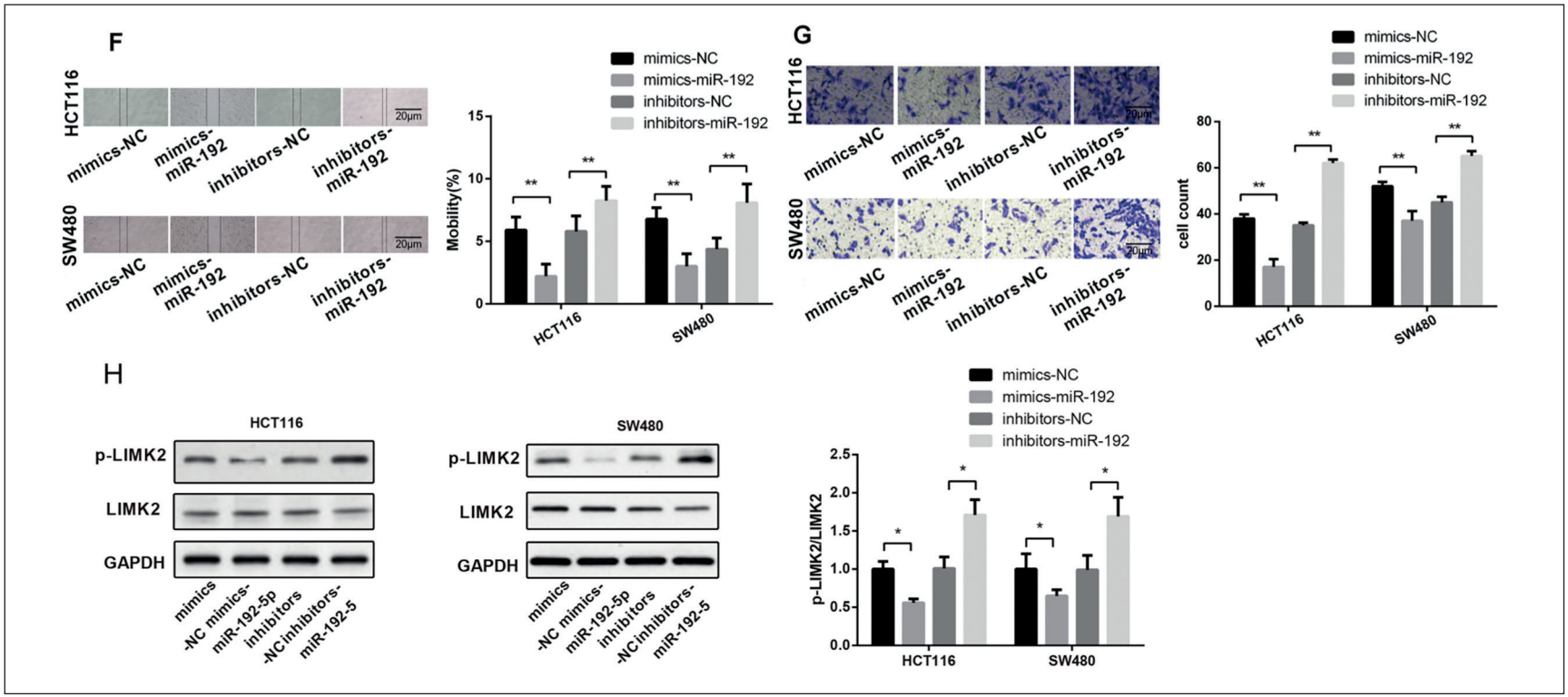

Figure 2 *(Continued).* **F-G,** Cell scratch assay and transwell assay was applied to verify the effect of miR-192-5p on cell invasion and migration ability (magnification of ×16 in F, magnification of ×200 in G). **H,** The phosphorylation and total protein levels of LIMK2 were detected using Western blot; *$p<0.05$, **$p<0.01$, compared to mimics-NC group or inhibitors-NC group; CC, colon cancer; NC, negative control.

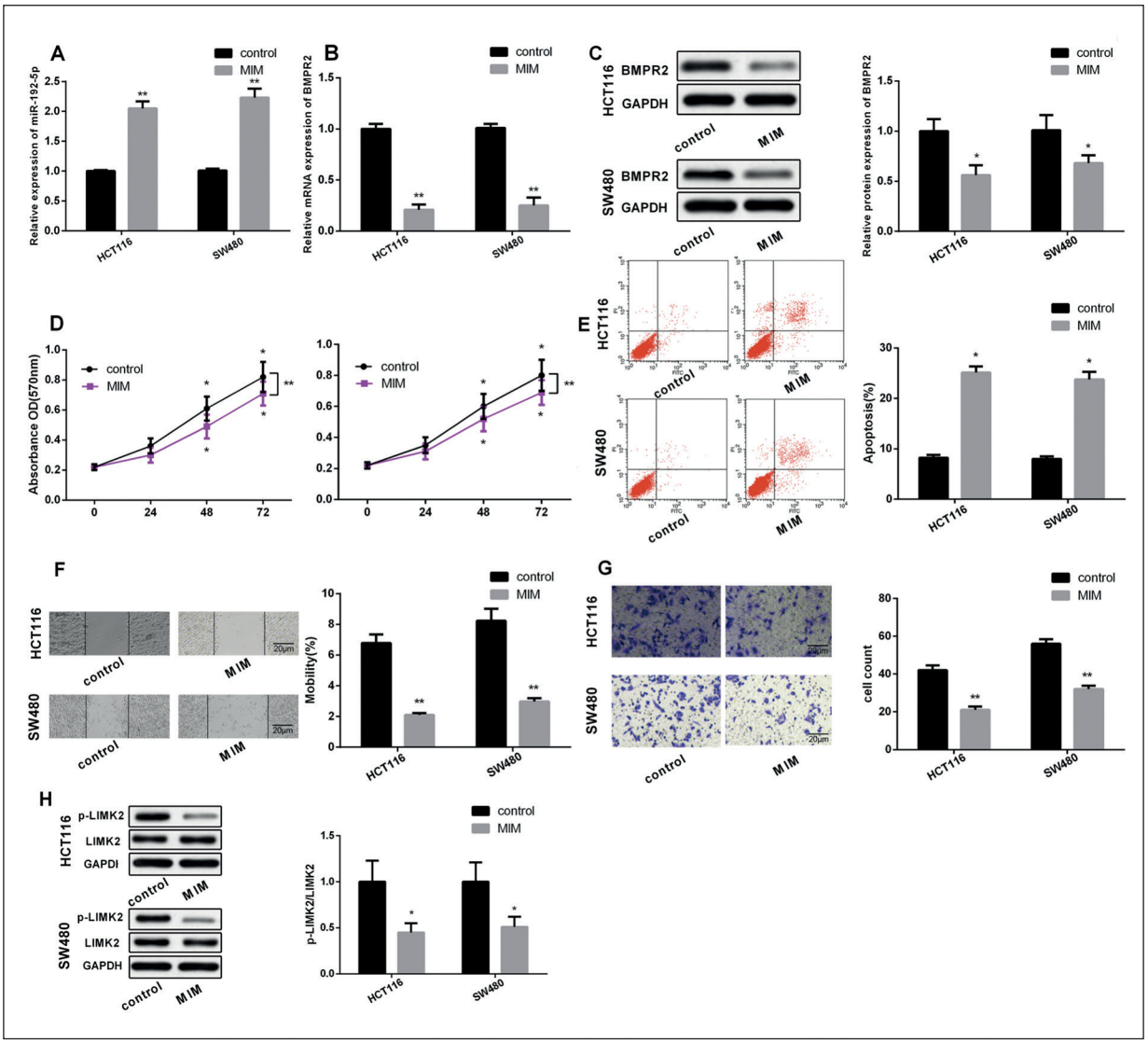

Figure 3. MIM up-regulate the expression level of miR-192-5p and down-regulate BMPR2 as well as regulating downstream signaling pathway in CC cells. CC cells were incubated with MIM of mice to verify the regulatory role of MIM on expression of miR-192-5p and BMPR2, and to identify the effect of MIM on biological activities of CC cells. **A,** Expression of miR-192-5p was determined by qRT-PCR. **B-C,** mRNA and protein expressions of BMPR2 in CC cells treated with MIM were examined by qRT-PCR and Western blot. **D,** The regulatory role of MIM on cell viability of CC cells was determined employing MTT. **E,** Apoptosis rate of CC cells treated with MIM was tested by flow cytometry. **F-G,** Detection of migration and invasion ability of CC cells treated with MIM by cell scratch assay and transwell (magnification of ×16 in F, magnification of ×200 in G). **H,** The phosphorylation and total protein levels of LIMK2 were detected using Western blot; **p*<0.05, ***p*<0.01, compared to Control group; MIM, metabolites of intestinal microflora; CC, colon cancer.

192-5p was on the 3'-UTR region (Figure 5A). The results of Luciferase reporter gene assay displayed that the Luciferase activity in the WT + mimics group was markedly lower than that in the WT + NC group, while there was no visible difference in the Luciferase activity between the MT + mimics group and the MT + NC group (p>0.01) (Figure 5B).

Discussion

The development of cancer was the consequence of the complex implication of many regulatory factors, which can regulate the expression of oncogenes or tumor suppressor genes, thus playing a role in tumor promotion or inhibition[15]. We hypothesized that miR-192-5p might exert a

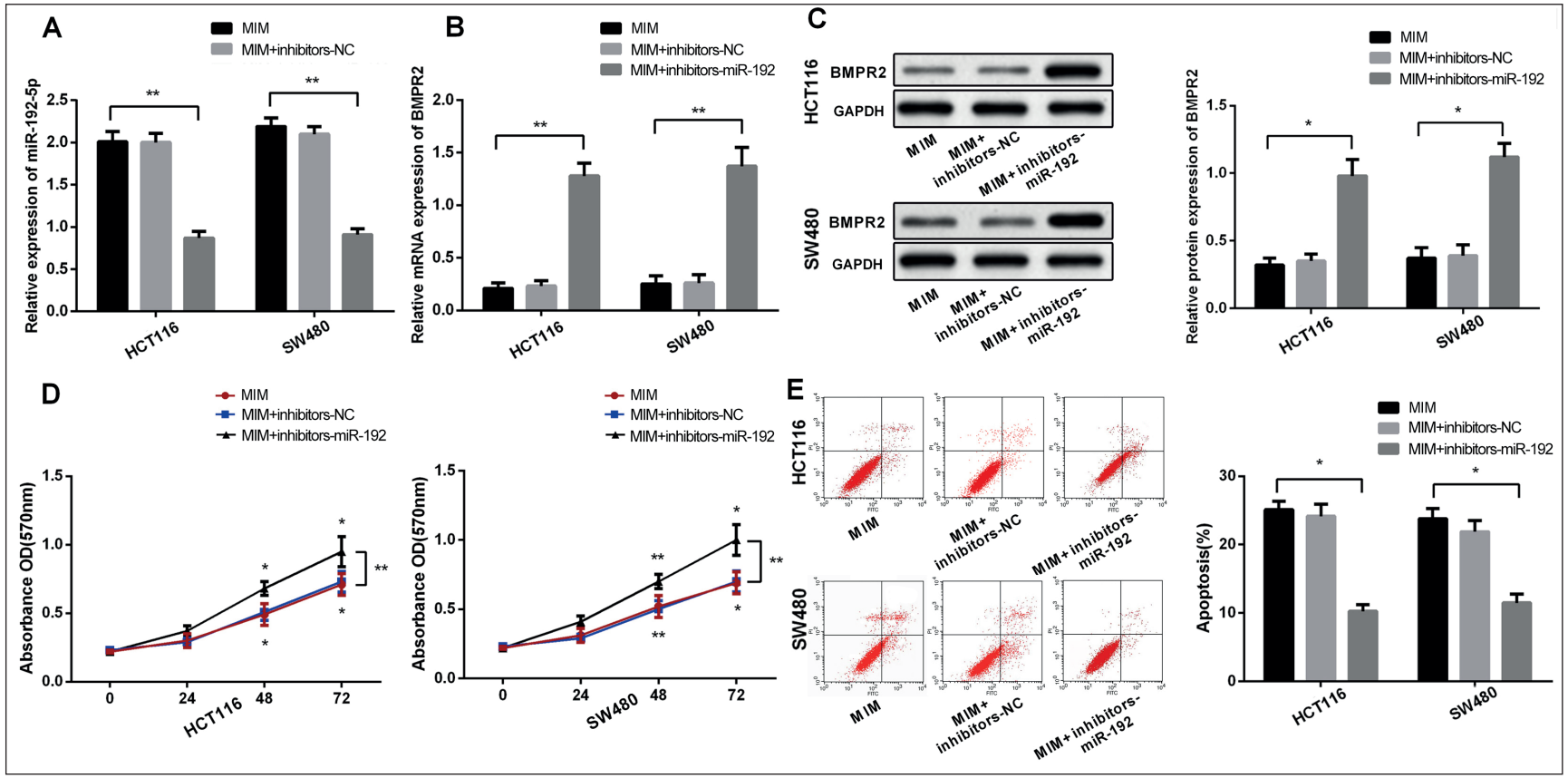

Figure 4. MIM suppress the biological activities of CC cells while inhibitors-miR-192-5p could reverse the protective effect of MIM in CC progression. To further understand the effect of miR-192-5p on CC cells, we designed a rescue experiment. **A-C,** Expression of miR-192-5p and BMPR2 in MIM treated CC cells or in cells with MIM + inhibitors-miR-192-5p treatment. **D-G,** Comparison on cell apoptosis, proliferation, invasion (× 16), and migration (× 16) in MIM treated CC cells or in cells with MIM + inhibitors-miR-192-5p treatment.

Figure Continued

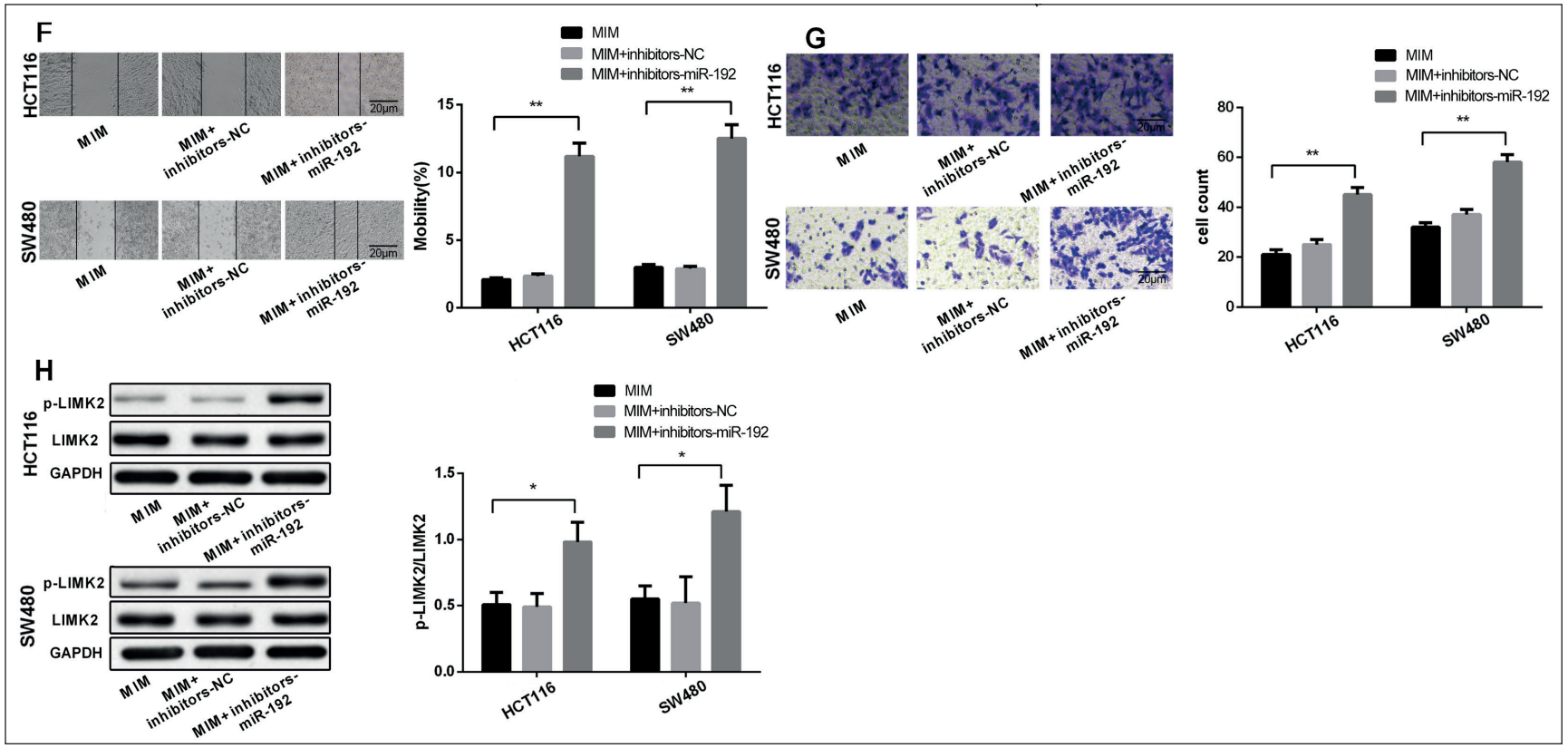

Figure 4 ***(Contiued).*** **H,** effect of MIM and MIM + inhibitors-miR-192-5p on phosphorylation level of LIMK2; *p<0.05, **p<0.01, compared to MIM group; magnifications of × 16 in F-G; MIM, metabolites of intestinal microflora; CC, colon cancer.

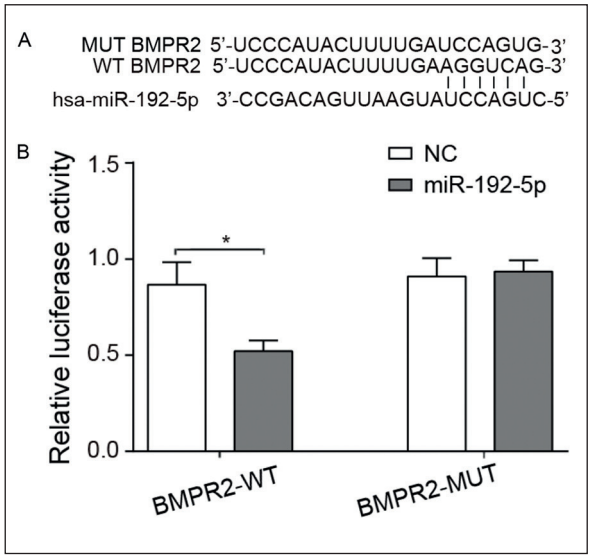

Figure 5. BMPR2 is a target gene of miR-192-5p. **A,** The binding site of BMPR2 and miR-192-5p predicted by StarBase. **B,** Analysis of Dual-Luciferase reporter assay on relative Luciferase activity of WT + mimics group; *$p<0.01$ when compared to WT + NC group; WT, wild type; NC, negative control.

significant effect in CC cells, and MIM may have certain relationship with CC cell growth. Hence, in our study, HCT116 and SW480 cells were treated with MIM to testify the significance and mechanism of MIM on biological activities of CC cells. The results supported that MIM could up-regulate miR-192-5p to inhibit BMPR2 and the RhoA-Rock-LIMK2 pathway, and thus suppress cell growth and development of CC cells.

A vital contributor of intestinal microflora to human health is also mentioned in previous studies. Intestinal microflora exerts an irreplaceable function in promoting or preventing atherosclerotic cardiovascular disease[16]. The importance of intestinal microflora in the development of autism and mood disorders also has been found[17]. However, the significance of miRNAs in mediating the host and intestinal microflora has only recently been elucidated[18]. The inspection of cell invasion, proliferation, as well as migration in this research recommended that MIM could enhance the inhibitory impact of miR-192-5p on CC cell growth.

MiR-192-5p is originally identified as the gene of rat, while it has recently been confirmed in humans[19]. In view of recent reports on the use of miR-192-5p in the diagnosis of other cancers, further researches need to verify the diagnostic potential of miR-192-5p in CC. Kim et al[20] suggested that miR-195 can promote the acquisition of drug resistance on CC to 5-FU. Yan-Chun et al[21] explained that miR-192-5p affects the biological activity of hepatocellular carcinoma cells by directly targeting SEMA3A. Besides, another previous research also highlighted that miR-192-5p may directly regulate NOTCH2 expression to inhibit colorectal cancer EMT and M2-like TAM polarization[22]. Those data verified the tumor suppression role of miR-192-5p on cancer cells. Consistently, we found decreased expression of miR-192-5p in HCT116 and SW480 cells and overexpression of miR-192-5p could inhibit migration, invasion, and proliferation of CC cells. Unlike previous studies, we further showed that BMPR2 was a direct target of miR-192-5p. Through prediction methods of bioinformatics or literature, we found that miR-195 could target BMPR2 gene thus inhibiting the transcription and translation of BMPR2 gene. Growing evidence for clinically-relevant functions of BMPR2 has been found in the remodeling of the postnatal skeleton and pulmonary vascular homeostasis[23]. Besides, BMPR2 is involved in the mediation of miR-191 in human pulmonary artery endothelial cell proliferation[24]. The physiological importance of BMPR2 has been expounded that the genetic alteration of the BMPR2 gene is relevant to various clinical circumstances, covering metabolic diseases, typical pulmonary hypertension, and cancer[25]. Therefore, we speculated that miR-192-5p may implicate in CC cell development through regulating BMPR2.

While the accurate mechanism by which MIM up-regulated miR-192-5p to mediate CC cell activities in CC was ambiguous. Hence, our research further investigated the functional mechanism herein. RhoA is a member of the GTPase family and covers cell-cycle progression, focal adhesion complex assembly, gene transcription, and cell polarity[26]. As the downstream molecules of RhoA, Rho-associated protein kinase (ROCK) and LIM kinases (LIMKs) are closely related to multiple cytobiology functions such as cell motility, proliferation and protein expression, cytoskeleton reorganization, and smooth muscle contraction[26,27]. The result of current study supported that MIM could down-regulate the phosphorylation level of LIMK2. LIMK 1 and LIMK 2, activated by Rho-ROCK kinase and the corresponding phosphatase Slingshot, can regulate cofilin activity by phosphorylation and dephosphorylation reactions[28]. Activation of LIMK 1 results in increasing phosphorylation of

ADF/cofilin, reducing depolymerization of ADF/cofilin and actin cytoskeletal reorganization, thereby promoting tumor cell filopodia formation and metastasis[29]. Consistent with our observation, MIM could decrease the phosphorylation of LIMK2, which suggesting that MIM can inhibit Rho-ROCK kinase and phosphorylation of ADF/cofilin and therefore attenuate cell development of CC cells. Previous research advised that the silencing of BMPR2 inhibits metastasis and invasion of osteosarcoma cells through RhoA/ROCK/LIMK pathway[30]. Similar to that in osteosarcoma cells, present study revealed that miR-192-5p could inhibit the RhoA/ROCK/LIMK signaling pathway *via* silencing BMPR2 in CC.

Conclusions

All the experimental results supported our hypothesis that MIM could up-regulate miR-192-5p in CC cells to inhibit cell migration, proliferation, and invasion *via* the RhoA/ROCK/LIMK signaling pathway. Therefore, these discoveries suggested that MIM have an inhibitory role in CC and may likely apply for clinical CC therapy. More studies are required to verify the results of the current study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We thank for all the supports and contributions of participators.

Funding

This work was supported by the Natural Science Foundation of Inner Mongolia (2018MS08028).

References

1) Wei R, Yang Q, Han B, Li Y, Yao K, Yang X, Chen Z, Yang S, Zhou J, Li M, Yu H, Yu M, Cui Q. MicroRNA-375 inhibits colorectal cancer cells proliferation by downregulating JAK2/STAT3 and MAP3K8/ERK signaling pathways. Oncotarget 2017; 8: 16633-16641.
2) Zhu K, Wang Y, Liu L, Li S, Yu W. Long non-coding RNA MBNL1-AS1 regulates proliferation, migration, and invasion of cancer stem cells in colon cancer by interacting with MYL9 via sponging microRNA-412-3p. Clin Res Hepatol Gastroenterol 2019. pii: S2210-7401(19)30098-1. doi: 10.1016/j.clinre.2019.05.001. [Epub ahead of print].
3) Vishnubalaji R, Yue S, Alfayez M, Kassem M, Liu FF, Aldahmash A, Alajez NM. Bone morphogenetic protein 2 (BMP2) induces growth suppression and enhances chemosensitivity of human colon cancer cells. Cancer Cell Int 2016; 16: 77.
4) Ji D, Jiang L, Li Y. MiR-192-5p suppresses the growth of bladder cancer cells via targeting Yin Yang 1. Hum Cell 2018; 31: 210-219.
5) Jin H, Qiao F, Wang Y, Xu Y, Shang Y. Curcumin inhibits cell proliferation and induces apoptosis of human non-small cell lung cancer cells through the upregulation of miR-192-5p and suppression of PI3K/Akt signaling pathway. Oncol Rep 2015; 34: 2782-2789.
6) Zhang Y, Huang R, Zhou W, Zhao Q, Lu Z. MiR-192-5p mediates hypoxia/reoxygenation-induced apoptosis in H9c2 cardiomyocytes via targeting of FABP3. J Biochem Mol Toxicol 2017; 31. doi: 10.1002/jbt.21873. Epub 2016 Oct 25.
7) Yan D, Liu W, Liu Y, Luo M. LINC00261 suppresses human colon cancer progression via sponging miR-324-3p and inactivating the Wnt/beta-catenin pathway. J Cell Physiol 2019; 234: 22648-22656.
8) Yang F, Chen L, Wang ZJ. MicroRNA-32 inhibits the proliferation, migration and invasion of human colon cancer cell lines by targeting E2F transcription factor 5. Eur Rev Med Pharmacol Sci 2019; 23: 4156-4163.
9) Zhou S, Xiong M, Dai G, Yu L, Zhang Z, Chen J, Guo W. MicroRNA-192-5p suppresses the initiation and progression of osteosarcoma by targeting USP1. Oncol Lett 2018; 15: 6947-6956.
10) Lustri BC, Sperandio V, Moreira CG. Bacterial chat: intestinal metabolites and signals in host-microbiota-pathogen interactions. Infect Immun 2017; 85. pii: e00476-17.
11) Tuddenham S, Sears CL. The intestinal microbiome and health. Curr Opin Infect Dis 2015; 28: 464-470.
12) Wang G, Huang S, Wang Y, Cai S, Yu H, Liu H, Zeng X, Zhang G, Qiao S. Bridging intestinal immunity and gut microbiota by metabolites. Cell Mol Life Sci 2019; 76: 3917-3937.
13) Yang Y, Jobin C. Novel insights into microbiome in colitis and colorectal cancer. Curr Opin Gastroenterol 2017; 33: 422-427.*
14) Johnston DGW, Williams MA, Thaiss CA, Cabrera-Rubio R, Raverdeau M, McEntee C, Cotter PD, Elinav E, O'Neill LAJ, Corr SC. Loss of microRNA-21 influences the gut microbiota, causing reduced susceptibility in a murine model of colitis. J Crohns Colitis 2018; 12: 835-848.
15) Zeng M, Zhu L, Li L, Kang C. MiR-378 suppresses the proliferation, migration and invasion of colon cancer cells by inhibiting SDAD1. Cell Mol Biol Lett 2017; 22: 12.

16) Jie Z, Xia H, Zhong SL, Feng Q, Li S, Liang S, Zhong H, Liu Z, Gao Y, Zhao H, Zhang D, Su Z, Fang Z, Lan Z, Li J, Xiao L, Li J, Li R, Li X, Li F, Ren H, Huang Y, Peng Y, Li G, Wen B, Dong B, Chen JY, Geng QS, Zhang ZW, Yang H, Wang J, Wang J, Zhang X, Madsen L, Brix S, Ning G, Xu X, Liu X, Hou Y, Jia H, He K, Kristiansen K. The gut microbiome in atherosclerotic cardiovascular disease. Nat Commun 2017; 8: 845.

17) Mangiola F, Ianiro G, Franceschi F, Fagiuoli S, Gasbarrini G, Gasbarrini A. Gut microbiota in autism and mood disorders. World J Gastroenterol 2016; 22: 361-368.

18) Riaz Rajoka MS, Jin M, Haobin Z, Li Q, Shao D, Huang Q, Shi J. Impact of dietary compounds on cancer-related gut microbiota and microRNA. Appl Microbiol Biotechnol 2018; 102: 4291-4303.

19) Yang B, Tan Z, Song Y. Study on the molecular regulatory mechanism of microRNA-195 in the invasion and metastasis of colorectal carcinoma. Int J Clin Exp Med 2015; 8: 3793-800.

20) Kim C, Hong Y, Lee H, Kang H, Lee EK. MicroRNA-195 desensitizes HCT116 human colon cancer cells to 5-fluorouracil. Cancer Lett 2018; 412: 264-271.

21) Yan-Chun L, Hong-Mei Y, Zhi-Hong C, Qing H, Yan-Hong Z, Ji-Fang W. MicroRNA-192-5p promote the proliferation and metastasis of hepatocellular carcinoma cell by targeting SEMA3A. Appl Immunohistochem Mol Morphol 2017; 25: 251-260.

22) Lin X, Wang S, Sun M, Zhang C, Wei C, Yang C, Dou R, Liu Q, Xiong B. MiR-195-5p/NOTCH2-mediated EMT modulates IL-4 secretion in colorectal cancer to affect M2-like TAM polarization. J Hematol Oncol 2019; 12: 20.

23) Gorrell RE, Totten MH, Schoerning LJ, Newby JB, Geyman LJ, Lawless WG, Hum JM, Lowery JW. Identification of a bone morphogenetic protein type 2 receptor neutralizing antibody. BMC Res Notes 2019; 12: 331.

24) Zhang C, Wang P, Mohammed A, Zhou Z, Zhang S, Ni S, Tang Z. Function of adipose-derived mesenchymal stem cells in monocrotaline-induced pulmonary arterial hypertension through miR-191 via regulation of BMPR2. Biomed Res Int 2019; 2019: 2858750.

25) Kim MJ, Park SY, Chang HR, Jung EY, Munkhjargal A, Lim JS, Lee MS, Kim Y. Clinical significance linked to functional defects in bone morphogenetic protein type 2 receptor, BMPR2. BMB Rep 2017; 50: 308-317.

26) Yao J, Gao P, Xu Y, Li Z. Alpha-TEA inhibits the growth and motility of human colon cancer cells via targeting RhoA/ROCK signaling. Mol Med Rep 2016; 14: 2534-2540.

27) Ren T, Zheng B, Huang Y, Wang S, Bao X, Liu K, Guo W. Osteosarcoma cell intrinsic PD-L2 signals promote invasion and metastasis via the RhoA-ROCK-LIMK2 and autophagy pathways. Cell Death Dis 2019; 10: 261.

28) Nowak D, Mazur AJ, Popow-Wozniak A, Radwanska A, Mannherz HG, Malicka-Blaszkiewicz M. Subcellular distribution and expression of cofilin and ezrin in human colon adenocarcinoma cell lines with different metastatic potential. Eur J Histochem 2010; 54: e14.

29) Su J, Zhou Y, Pan Z, Shi L, Yang J, Liao A, Liao Q, Su Q. Downregulation of LIMK1-ADF/cofilin by DADS inhibits the migration and invasion of colon cancer. Sci Rep 2017; 7: 45624.

30) Wang S, Ren T, Jiao G, Huang Y, Bao X, Zhang F, Liu K, Zheng B, Sun K, Guo W. BMPR2 promotes invasion and metastasis via the RhoA-ROCK-LIMK2 pathway in human osteosarcoma cells. Oncotarget 2017; 8: 58625-58641.