

Microcystin-leucine arginine promotes colorectal cancer cell proliferation by activating the PI3K/Akt/Wnt/ β -catenin pathway

YAQI TANG^{1*}, XIAOYU YI^{1*}, XINYU ZHANG^{2*}, BAOJIE LIU¹, YONGZHENG LU¹,
ZHIFANG PAN¹, TAO YU¹ and WEIGUO FENG¹

¹School of Life Science and Technology, Weifang Medical University, Weifang, Shandong 261053;

²Department of Gastrointestinal Surgery, The Second Affiliated Hospital of
Shandong First Medical University, Taian, Shandong 271000, P.R. China

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Abstract. Microcystin-leucine arginine (MC-LR) is an environmental toxin produced by cyanobacteria and is considered to be a potent carcinogen. However, to the best of our knowledge, the effect of MC-LR on colorectal cancer (CRC) cell proliferation has never been studied. The aim of the present study was to investigate the effect of MC-LR on CRC cell proliferation and the underlying mechanisms. Firstly, a Cell Counting Kit-8 (CCK-8) assay was conducted to determine cell viability at different concentrations, and 50 nM MC-LR was chosen for further study. Subsequently, a longer CCK-8 assay and a cell colony formation assay showed that MC-LR promoted SW620 and HT29 cell proliferation. Furthermore, western blotting analysis showed that MC-LR significantly upregulated protein expression of PI3K, p-Akt (Ser473), p-GSK3 β (Ser9), β -catenin, c-myc and cyclin D1, suggesting that MC-LR activated the PI3K/Akt and Wnt/ β -catenin pathways in SW620 and HT29 cells. Finally, the pathway inhibitors LY294002 and ICG001 were used to validate the role of the PI3K/Akt and Wnt/ β -catenin pathways in MC-LR-accelerated cell proliferation. The results revealed that MC-LR activated Wnt/ β -catenin through the PI3K/Akt pathway to promote cell proliferation. Taken together, these data showed that MC-LR promoted CRC cell proliferation by activating the PI3K/Akt/Wnt/ β -catenin pathway. The present study provided a novel insight into the toxicological mechanism of MC-LR.

Introduction

Microcystins (MCs) are a type of toxins synthesized by cyanobacteria (1). Among over 200 MC variants, MC-leucine arginine (MC-LR) is considered to be the most abundant and harmful (2). It has been reported that MC-LR might be transferred to the food chain and accumulate in different organisms (3). For example, Sotton *et al* (4) found that white-fish accumulated MC-LR in the body by ingesting foods containing MC-LR in Lake Hallwil of Switzerland. The World Health Organization recommends 1 μ g/l as a guideline for the maximum tolerated value in drinking water (5). However, an increasing number of studies have reported that the concentration of MC-LR in drinking water is much higher than 1 μ g/l in certain countries, such as Vietnam and China (6,7). Currently, MC-LR is considered to be a potent carcinogen (8,9). In a previous study, MC-LR activated MMP expression and promoted breast cancer cell migration (10). MC-LR also induced hepatocarcinogenesis *in vitro* and in nude mice (11). In addition, MC-LR has been shown to promote prostate epithelial cell proliferation (6). However, the effect of MC-LR on colorectal cancer (CRC) cells is still poorly documented.

CRC is the third leading cause of cancer death worldwide (12,13). Old age, dietary patterns and a bad lifestyle are considered to be risk factors for CRC (13,14). Previous studies have demonstrated that MC-LR is a carcinogen and can be transferred to the food chain (3,8). Therefore, MC-LR may be related to the occurrence and development of CRC. To date, a few studies have shown that MC-LR promotes CRC cell migration and metastasis (14,15). However, to the best of our knowledge, the impact of MC-LR on CRC cell proliferation has never been studied.

Previous studies have demonstrated that the PI3K/Akt and Wnt/ β -catenin signaling pathways are closely associated with the occurrence and development of CRC (16,17). Activation of the PI3K/Akt and Wnt/ β -catenin signaling pathways can promote CRC cell proliferation (18,19). In addition, these two pathways are important downstream targets of MC-LR (6,20). Therefore, we speculated that MC-LR activates the PI3K/Akt and Wnt/ β -catenin pathways to promote CRC cell proliferation. The present study aimed to investigate the effect of

Correspondence to: Professor Weiguo Feng or Professor Zhifang Pan, School of Life Science and Technology, Weifang Medical University, 7166 Baotong Street, Weifang, Shandong 261053, P.R. China
E-mail: fengwg@wfmuc.edu.cn
E-mail: sdwfpzf@126.com

*Contributed equally

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MC-LR on CRC cell proliferation and the underlying mechanisms. For this purpose, SW620 and HT29 cells were treated with 50 nM MC-LR and then examined using Cell Counting Kit-8 (CCK-8) and cell colony formation assays to determine whether MC-LR accelerated cell proliferation. Subsequently, the protein expression of the pathway key genes was detected using western blotting. Finally, the pathway inhibitors LY294002 and ICG001 were used to verify the role of the PI3K/Akt and Wnt/ β -catenin pathways in MC-LR-induced cell proliferation.

Materials and methods

Reagents and antibodies. MC-LR (purity >95%) was purchased from Express Technology, Co., Ltd. Dulbecco's modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. The nuclear and cytoplasmic protein extraction kit (cat. no. P0027), CCK-8 kit (cat. no. C0037), enhanced chemiluminescence (ECL) reaction kit (cat. no. P0018S), LY294002 (a PI3K/Akt pathway inhibitor; cat. no. S1737), ICG001 (a Wnt/ β -catenin pathway inhibitor; cat. no. SF6827), and anti-PI3K (p110; cat. no. AF7749), anti-Akt (cat. no. AA326), anti-GSK3 β (cat. no. AF1543), anti- β -catenin (cat. no. AC106), anti-c-myc (cat. no. AF6513) and anti-cyclin D1 (cat. no. AF1183) antibodies were purchased from Beyotime Institute of Biotechnology. Anti-phosphorylated (p)-Akt (Ser473; cat. no. 4060) and anti-p-GSK3 β (Ser9; cat. no. 9322) were purchased from Cell Signaling Technology, Inc. The HRP-conjugated secondary antibodies (cat. nos. SA00001-1 and SA00001-2), anti-lamin B (cat. no. 12987-1-AP) and anti- β -actin (cat. no. 20536-1-AP) were purchased from Wuhuan Sanying Biotechnology.

Cell culture and treatment. SW620 and HT29 cells were obtained from the Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in DMEM with 10% FBS at 37°C with 5% CO₂. SW620 and HT29 cells were cultured for 6 h before MC-LR (dissolved in DMSO), LY294002 or ICG001 was added. To determine the appropriate concentration of MC-LR, the cells were treated with various concentrations of MC-LR (10, 25, 50, 75, 100 and 200 nM) for 24 h at 37°C, and the relative cell viability was detected by CCK-8 analysis. To study the effect of MC-LR on cell proliferation, cells were treated with 50 nM MC-LR for 48 h or 10 days at 37°C, and then cell proliferation was assessed by CCK-8 and cell colony formation assays, respectively. In addition, the protein expression of the key genes of the PI3K/Akt and Wnt/ β -catenin pathways was assessed by western blotting after treating the cells with or without MC-LR (50 nM), LY294002 (25 μ M) or ICG001 (25 μ M) for 24 h at 37°C. The control group was cultured in media containing the same quantity of DMSO as the treatment group.

CCK-8 and cell colony formation assay. For the CCK-8 assay, after seeding SW620 and HT29 cells separately in 96-well plates (3,000 cells per well) for 48 h, CCK-8 was added and incubated for 2 h to assess relative cell viability, as described in our previous study (5). Light absorbance was examined at 450 nm using a microplate reader, and the optical density values were used directly to analyze the results of the CCK-8

assay as previously reported (21,22). For the cell colony formation assay, SW620 and HT29 cells (400 cells per well) were seeded separately in 6-well plates and incubated for 10 days. Cell colonies were fixed with methanol for 30 min, and stained with 0.1% (w/v) crystal violet for 20 min at 37°C. Cell colony counting was performed under the microscope (>50 cells per cell colony) as previously described (23).

Western blotting. Western blotting was performed as previously reported (24). In brief, the extraction of cell nuclear and cytoplasmic proteins was performed using a nuclear and cytoplasmic protein extraction kit. After quantification by BCA method, the protein was subjected to SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked using 5% skimmed milk in phosphate-buffered saline for 1 h at 37°C, and then incubated with the aforementioned primary antibodies (1:1,000) at 4°C overnight. After incubating with the aforementioned secondary antibodies (1:2,000) for 1 h at room temperature, the protein bands were developed using the ECL kit, and analyzed using ImageJ software (v1.46; National Institutes of Health).

Statistical analysis. Data were analyzed by independent samples t-test or by one-way analysis of variance followed by Tukey's post hoc analysis, using SPSS 19.0 software (IBM Corp.). All experiments were performed in triplicate. Data are presented as the mean \pm SD. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MC-LR promotes cell proliferation in SW620 and HT29 cells. Firstly, the appropriate concentration of MC-LR was screened for by CCK-8 analysis. The result showed that MC-LR affected cell viability, and cell viability of SW620 and HT29 cells was promoted at MC-LR concentrations of 10, 25, 50 and 75 nM, especially at 50 nM (Fig. 1A). Therefore, 50 nM MC-LR was used for the further experiments. Subsequently, CCK-8 and cell colony formation assays were performed to investigate the effect of MC-LR on cell proliferation. The result of both assessments showed that MC-LR promoted cell proliferation significantly in SW620 and HT29 cells compared with that in the control ($P < 0.05$; Fig. 1B-D).

MC-LR activates the PI3K/Akt and Wnt/ β -catenin pathways in SW620 and HT29 cells. As the PI3K/Akt and Wnt/ β -catenin signaling pathways are closely related to cell proliferation (25-28), the present study examined whether MC-LR activated the PI3K/Akt and Wnt/ β -catenin pathways in SW620 and HT29 cells. As shown in Fig. 2, the expression of PI3K and the level of p-Akt (Ser473) increased significantly in the MC-LR treatment group compared with that in the control group ($P < 0.05$). These data demonstrated that MC-LR activated the PI3K/Akt pathway. Meanwhile, the expression levels of p-GSK3 β (Ser9), β -catenin, c-myc and cyclin D1, the downstream targets of the Wnt/ β -catenin pathway, were also significantly upregulated in the MC-LR treatment group compared with those in the control group ($P < 0.05$). These data indicated that MC-LR activated the Wnt/ β -catenin pathway in SW620 and HT29 cells.

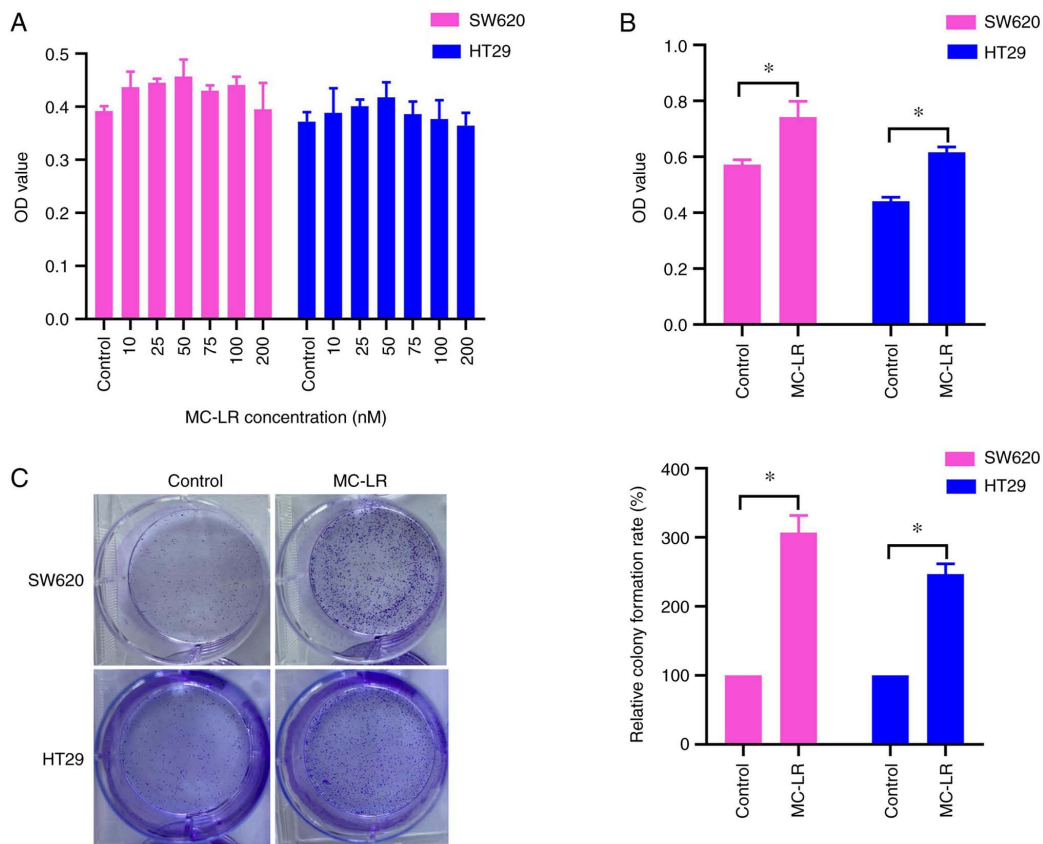


Figure 1. MC-LR promotes cell proliferation in SW620 and HT29 cells. (A) Cells were treated with different concentrations of MC-LR (10, 25, 50, 75, 100 and 200 nM) for 24 h, and the relative cell viability was detected by CCK-8 analysis. (B) Cells were treated with 50 nM MC-LR for 48 h, and cell proliferation was tested by CCK-8 analysis. (C) Cells were treated with 50 nM MC-LR for 10 days, and cell proliferation was assessed by cell colony formation assay. The results are representative of three independent experiments. *P<0.05. Error bars indicate SD. MC-LR, microcystin-leucine arginine; OD, optical density; CCK-8, Cell Counting Kit-8.

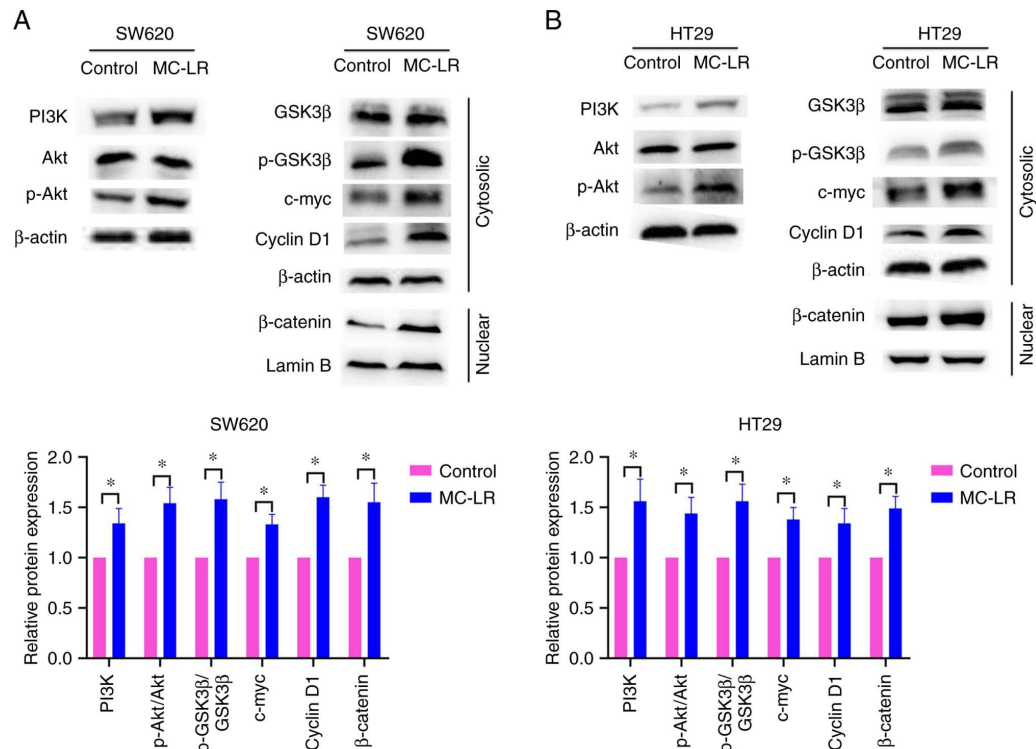


Figure 2. MC-LR activates the PI3K/Akt and Wnt/β-catenin pathways in (A) SW620 and (B) HT29 cells. Cells were treated using 50 nM MC-LR for 24 h, and then the protein expression levels of the key genes of the PI3K/Akt and Wnt/β-catenin pathway were detected by western blotting. The results are representative of three independent experiments. *P<0.05. Error bars indicate SD. MC-LR, microcystin-leucine arginine; p-, phosphorylated.

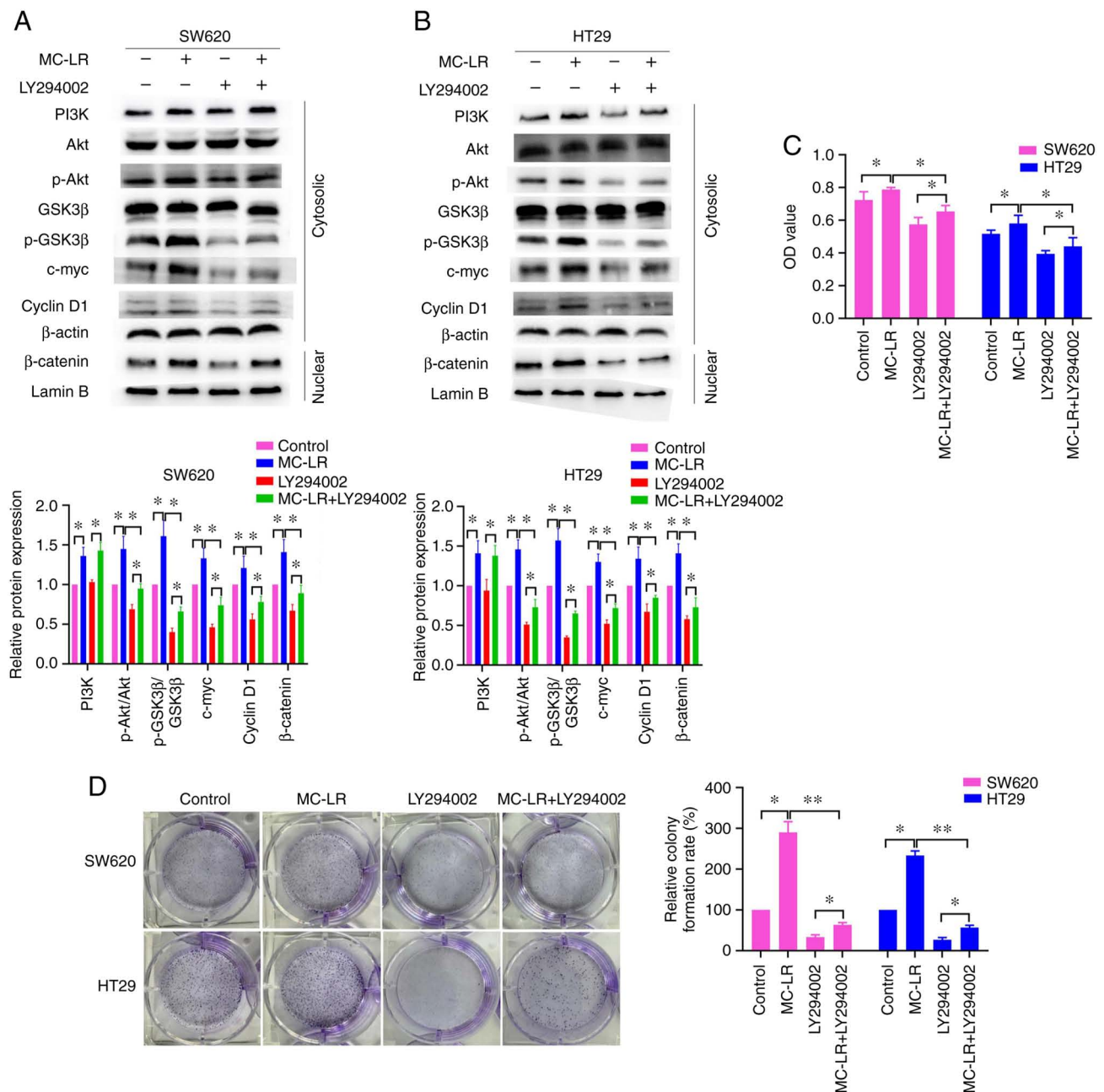


Figure 3. MC-LR promotes SW620 and HT29 cell proliferation by activating the PI3K/Akt/Wnt/β-catenin pathway. (A) SW620 and (B) HT29 cells were treated with or without MC-LR (50 nM) and LY294002 (25 μM) for 24 h, and then the protein expression levels of the pathway key genes was detected by western blotting. (C and D) SW620 and HT29 cells were treated with or without MC-LR (50 nM) and LY294002 (25 μM) for 48 h or 10 days, and then cell proliferation was assessed using (C) Cell Counting Kit-8 or (D) cell colony formation analysis, respectively. The results are representative of three independent experiments. *P<0.05; **P<0.01. Error bars indicate SD. MC-LR, microcystin-leucine arginine; p-, phosphorylated; OD, optical density.

MC-LR promotes SW620 and HT29 cell proliferation by activating the PI3K/Akt/Wnt/β-catenin pathway. To confirm the role of the PI3K/Akt/Wnt/β-catenin pathway in MC-LR-mediated cell proliferation, LY294002, an inhibitor of the PI3K/Akt pathway, was used. The results showed that LY294002 could significantly suppress the overexpression of p-Akt (Ser473), p-GSK3β (Ser9), β-catenin, c-myc and cyclin D1 induced by MC-LR (P<0.05; Fig. 3A and B). This indicated that LY294002 neutralized the activation of the PI3K/Akt/Wnt/β-catenin pathway induced by MC-LR in SW620 and HT29 cells. Additionally, the results of the CCK-8 and colony formation analyses showed that LY294002

significantly inhibited cell proliferation promoted by MC-LR (P<0.05; Fig. 3C and D). These results revealed that MC-LR activated the PI3K/Akt/Wnt/β-catenin pathway to promote SW620 and HT29 cell proliferation.

MC-LR activates Wnt/β-catenin through the PI3K/Akt pathway to promote cell proliferation. In this study, LY294002, as an inhibitor of the PI3K/Akt pathway, could also significantly inhibit the Wnt/β-catenin pathway. Thus, we speculated that MC-LR activated Wnt/β-catenin through the PI3K/Akt pathway in SW620 and HT29 cells. To investigate this, ICG001, an inhibitor of the Wnt/β-catenin pathway, was used. As

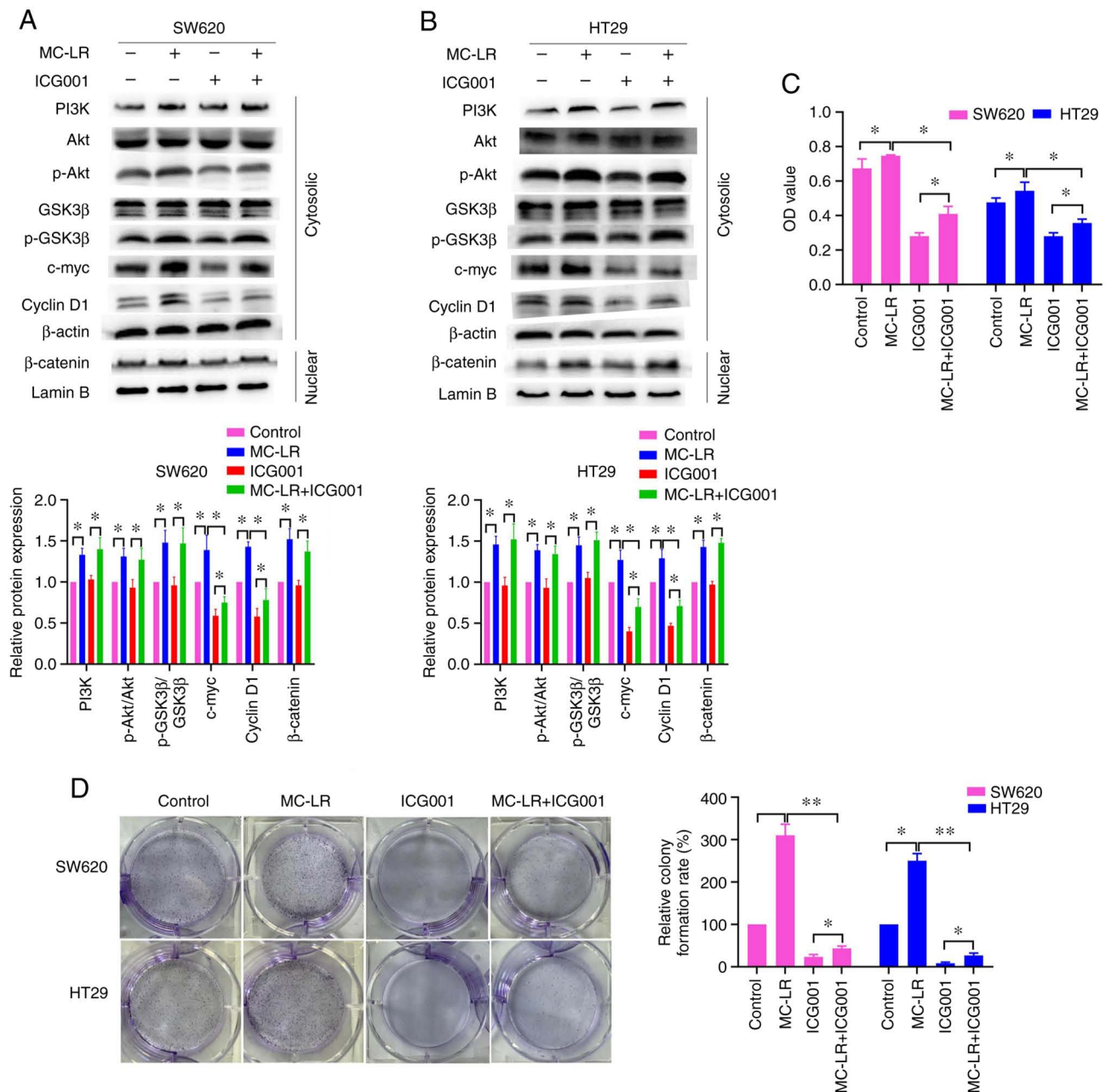


Figure 4. MC-LR activates Wnt/β-catenin through the PI3K/Akt signaling pathway to promote cell proliferation. (A) SW620 and (B) HT29 cells were treated with or without MC-LR (50 nM) and ICG001 (25 μM) for 24 h, and then the protein expression levels of the pathway key genes was detected by western blotting. (C and D) SW620 and HT29 cells were treated with or without MC-LR (50 nM) and ICG001 (25 μM) for 48 h or 10 days, and then the cell proliferation was assessed using (C) Cell Counting Kit-8 or (D) cell colony formation analysis, respectively. The results are representative of three independent experiments. *P<0.05; **P<0.01. Error bars indicate SD. MC-LR, microcystin-leucine arginine; p-, phosphorylated; OD, optical density.

shown in Fig. 4A and B, ICG001 significantly suppressed the overexpression of c-myc and cyclin D1 induced by MC-LR (P<0.05), but not the expression of PI3K-related proteins. These results indicated that ICG001 neutralized the activation of Wnt/β-catenin, rather than the PI3K/Akt pathway, induced by MC-LR, suggesting that MC-LR activated Wnt/β-catenin through the PI3K/Akt pathway in SW620 and HT29 cells. Additionally, the results of the CCK-8 and colony formation analyses showed that ICG001 significantly suppressed cell proliferation promoted by MC-LR (P<0.05; Fig. 4C and D). Together, the aforementioned results suggested that MC-LR activated Wnt/β-catenin through the PI3K/Akt signaling pathway to promote cell proliferation.

Discussion

In the present study, the effect of MC-LR on CRC cell proliferation and the underlying mechanism were investigated. Firstly, an MC-LR concentration of 50 nM was determined to be suitable for the subsequent CCK-8 and cell colony formation assays. Next, the protein expression levels of the key genes of the pathway were measured using western blotting after treating the cells with 50 nM MC-LR. The results revealed that MC-LR activated the PI3K/Akt and Wnt/β-catenin pathways. Finally, LY294002 and ICG001 were used to confirm the role of the PI3K/Akt/Wnt/β-catenin pathway in MC-LR-promoted cell proliferation. The results showed that MC-LR activated

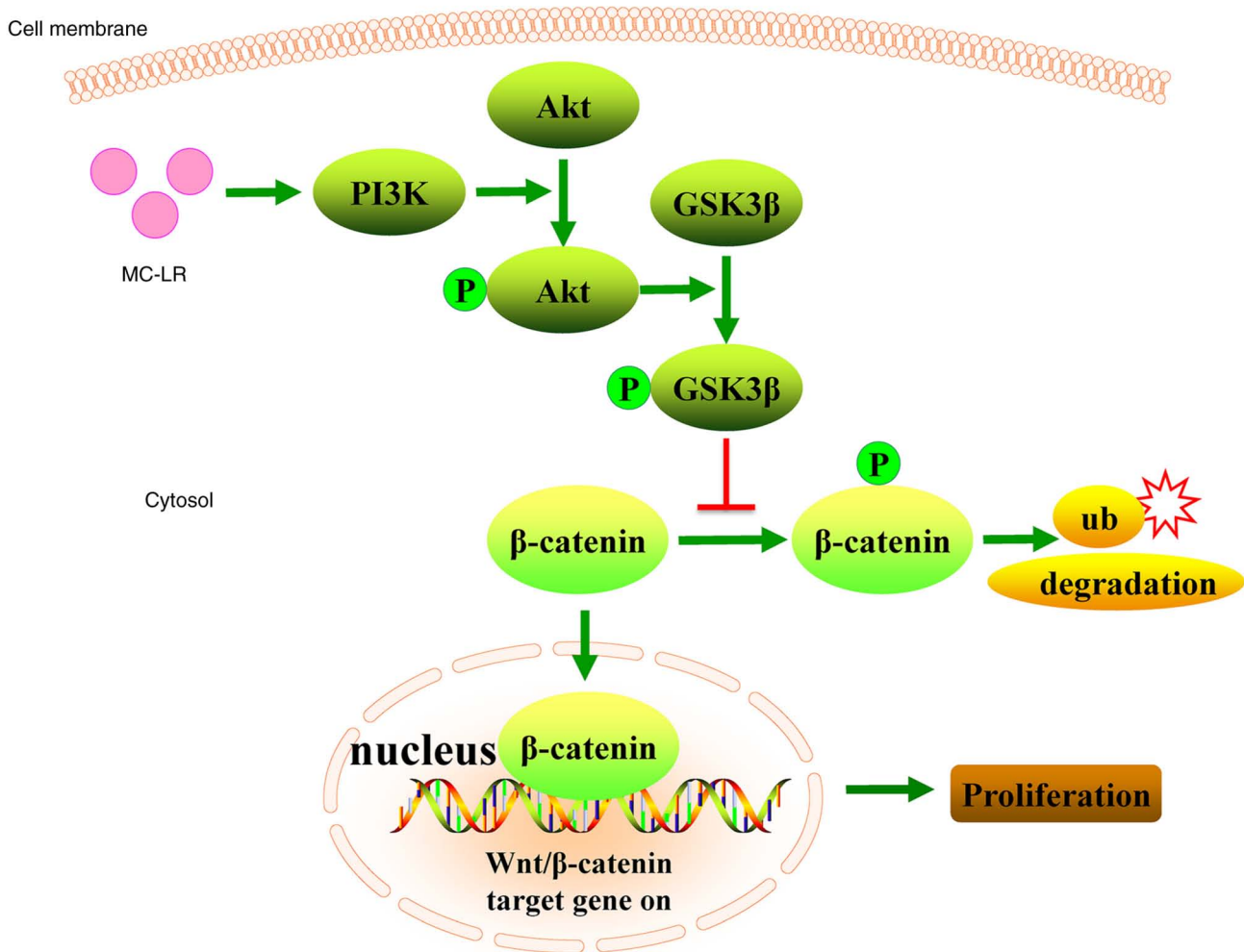


Figure 5. Proposed pathways involved in MC-LR-promoted CRC cell proliferation. MC-LR activates the PI3K/Akt signaling axis, and then induces GSK3β phosphorylation, thereby inhibiting degradation by ubiquitination caused by the phosphorylation of β-catenin. Subsequently, β-catenin is transported to the nucleus leading to increasing expression levels of c-myc and cyclin D1, and consequently promoting CRC cell proliferation. CRC, colorectal cancer; MC-LR, microcystin-leucine arginine; p, phosphate; ub, ubiquitin.

the PI3K/Akt pathway and promoted GSK3β phosphorylation leading to β-catenin accumulation in the cell nucleus, thereby promoting cell proliferation (Fig. 5). Collectively, the results suggested that MC-LR activated Wnt/β-catenin through the PI3K/Akt signaling pathway to promote cell proliferation.

To investigate the impact of MC-LR on CRC cell proliferation, a suitable concentration of MC-LR needs to be selected. Several studies have used 25 or 50 nM MC-LR to treat CRC cells (14,15,29). For example, Miao *et al* (14) treated DLD-1, HT29 and SW480 cells with 12.5, 25 and 50 nM MC-LR. Ren *et al* (15) treated DLD-1 and HT29 cells with 25 and 50 nM MC-LR. Likewise, in the present study, SW620 and HT29 cells were treated using similar concentrations of MC-LR, and the results showed that MC-LR could promote cell proliferation of SW620 and HT29 cells at a concentration of 10, 25, 50 and 75 nM, especially at 50 nM. Therefore, 50 nM MC-LR was determined suitable for use in further experiments.

It has been reported that MC-LR could accelerate CRC cell migration and metastasis (14,15). However, the impact of MC-LR on CRC cell proliferation has never been studied. In the present study, the results of CCK-8 and cell colony formation analyses revealed that MC-LR promoted CRC cell

proliferation significantly. Similarly, previous studies found that MC-LR accelerated cell proliferation in liver, biliary and prostate epithelial cells (6,30-33). It was notable that MC-LR promoted cell proliferation at high doses in these studies. For instance, Liu *et al* (30) found that MC-LR accelerated HL7702 cell proliferation at 5 and 10 μM. Wang *et al* (34) reported that MC-LR increased A549 cell proliferation at 1 and 5 μM. In the present study, the results showed that a low dose of MC-LR (50 nM) significantly increased cell proliferation, highlighting the role of MC-LR in the malignant transformation of CRC. Therefore, the underlying mechanisms need to be investigated.

Previous studies have found that the PI3K/Akt and Wnt/β-catenin signaling pathways play key roles in cell proliferation (25-28). As a result, the key molecules of the PI3K/Akt and Wnt/β-catenin pathways were assessed using western blotting in the present study. The results showed that MC-LR increased the expression of PI3K (p110) and p-Akt (Ser473) significantly. It is well-known that elevated expression of PI3K (p110) and p-Akt (Ser473) is marker of the PI3K/Akt pathway activation (35,36). Therefore, this result suggested that MC-LR activated the PI3K/Akt pathway in CRC cells. In addition, the present data revealed that MC-LR upregulated

the level of p-GSK3 β (Ser9) and the expression of β -catenin, c-myc and cyclin D1. Previous studies have demonstrated that p- β -catenin induced by GSK3 β is degraded by ubiquitination, and that non-p- β -catenin is transferred to and accumulates in the cell nucleus, where it combines with T-cell factor/lymphoid enhancer factor to activate downstream target genes, such as c-myc and cyclin D1 (25,37). Therefore, the present results indicated that MC-LR also activates the Wnt/ β -catenin pathway in CRC cells.

To verify the role of the PI3K/Akt/Wnt/ β -catenin pathway in MC-LR-promoted cell proliferation, LY294002, an inhibitor of the PI3K/Akt pathway (14,38), was used to treat the cells. The results showed that LY294002 neutralized the activation of the PI3K/Akt/Wnt/ β -catenin pathway, leading to the inhibition of cell proliferation promoted by MC-LR in CRC cells. To further study the underlying mechanism, ICG001, an inhibitor of the Wnt/ β -catenin pathway (39), was used to treat the studied cells. The results revealed that ICG001 neutralized the activation of Wnt/ β -catenin rather than the PI3K/Akt pathway, thereby inhibiting cell proliferation promoted by MC-LR in the CRC cells. Based on these results, it was shown that MC-LR activated Wnt/ β -catenin through the PI3K/Akt signaling pathway to promote cell proliferation. In relation to the present study, Liu *et al* (30) reported that MC-LR increased proliferation by activating the Akt pathway in HL7702 cells. The study by Han *et al* showed that MC-LR activated the PI3K/Akt pathway in RM-1 cells (20). Pan *et al* (6) found that MC-LR activated the Wnt/ β -catenin pathway to promote RWPE-1 cell proliferation. To the best of our knowledge, this is the first study to find that MC-LR activated Wnt/ β -catenin through the PI3K/Akt pathway, leading to the promotion of CRC cell proliferation.

In conclusion, the present study demonstrated that MC-LR increased CRC cell proliferation by activating the PI3K/Akt/Wnt/ β -catenin pathway. The present study provides a novel insight into the toxicological mechanism of MC-LR.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WF, XZ and ZP designed the experiments and wrote the manuscript. YT, XY and TY performed the experiments. BL and YL analyzed the data. All authors read and approved the final manuscript. YT and XY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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