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MiR-195 alleviates ulcerative colitis in rats *via* MAPK signaling pathway

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Abstract. – OBJECTIVE: To study the effect of micro ribonucleic acid (miR)-195 on the inflammatory response of ulcerative colitis (UC) model rats and to explore its regulatory mechanism, thus providing a new scheme for the clinical treatment of UC.

MATERIALS AND METHODS: A rat model of UC was prepared by 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)/ethanol assay, and the rats were randomly divided into Control group, Model group, and miR-195 mimic (miR-195 agomir) group. The disease activity index (DAI) in each group was observed. Hematoxylin and eosin (H&E) staining was utilized to detect the pathological changes in the rat colon tissues in each group. The levels of interleukin-6 (IL-6) and IL-1β in the colon tissues of the rats in each group were detected by enzyme-linked immunosorbent assay (ELISA). In addition, the messenger RNA (mRNA) and protein levels of p38 mitogen-activated protein kinase (p38 MAPK) and tumor necrosis factor-alpha (TNF-α) in the colon tissues of each group of rats were examined *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting, respectively.

RESULTS: Compared with those in Control group, the rats in Model group had an increased DAI score, severely pathologically damaged colon tissues, raised levels of IL-6 and IL-1β in the colon tissues and significantly elevated mRNA and protein levels of p38 MAPK and TNF-α. In comparison with those in Model group, the DAI score was decreased, the pathological damage to the rat colon tissues was improved, the levels of IL-6 and IL-1β in the rat colon tissues were reduced, and the mRNA and protein levels of p38 MAPK and TNF-α were notably lowered in miR-195 agomir group.

CONCLUSIONS: MiR-195 mimics can alleviate the pathological damage to the colon and inflammatory responses in UC model rats, and its mechanism may be related to the inhibition on the p38 MAPK signaling pathway.

Key Words:

Ulcerative colitis, MiRNA, Inflammatory response, P38 MAPK.

Introduction

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease, the former of which is a chronic non-specific disease involving colorectal mucosa and mucosa[1-3]. As people's diet and lifestyle change, the morbidity rate of UC shows an increasing trend year by year, causing great physical pain and mental burden to patients. The disease can occur in people at any age, mostly in those aged 20-40 years old. The canceration rates of UC in people aged 20-30 years old are about 7.0% and 16.0%, respectively[4,5].

As molecular biology continuously advances, researchers have made some progress in the etiology of UC. Its pathogenesis primarily involves genetic factors, environmental factors, and immune factors. Cytokines released by abnormal inflammatory responses play a pivotal role in the pathogenesis of UC[6-9]. The p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway exerts crucial effects during the development of UC[10]. It is one of the crucial MAPK signaling pathways discovered so far and plays important regulatory roles in inflammation and cell proliferation, differentiation, and apoptosis. Recent research results manifested that p38 MAPK signaling pathway is activated in the development of UC, which elevates the expression of tumor necrosis factor-alpha (TNF-α), a downstream target. This provides an idea for researchers that blocking the transmission of this signaling pathway can suppress abnormal inflammation responses. Therefore, it is expected to become a new approach for the treatment of UC[11,13].

Recent studies have confirmed that miR-195 has a close correlation with the pathogenesis of UC. Finding miRNAs that can adjust the differential expression of the p38 MAPK signaling pathway will further provide an experimental basis

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for revealing the pathological and physiological processes of UC.

This study, therefore, plans to explore the regulatory effect of miR-195 on the inflammatory response of UC rats and its regulatory mechanism by preparing a rat model of UC using 2,4,6-trinitrobenzenesulfonic acid (TNBS)/ethanol assay.

Materials and Methods

Reagents

MiR-195 mimics were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China; miR0017149-4-5); TNBS from Sigma-Aldrich (St. Louis, MO, USA), interleukin-6 (IL-6), and IL-1β enzyme-linked immunosorbent assay (ELISA) kits from R&D System (Minneapolis, MN, USA); the first strand complementary deoxyribonucleic acid (cDNA) synthesis kit and p38 MAPK and TNF-α primers from Invitrogen (Carlsbad, CA, USA); rabbit anti-p38 MAPK and TNF-α primary antibodies from Beijing ZSGB-BIO Co., Ltd. (Beijing, China), and horseradish peroxidase (HRP)-labeled secondary antibody from Beijing Bioss Co., Ltd (Beijing, China).

Instruments

A microplate reader was bought from Bio-Tek (Biotek Winooski, VT, USA), electrophoresis apparatus and semi-dry film transfer apparatus from Bio-Rad (Hercules, CA, USA), a thermostatic water bath pot from Shanghai Yiheng Scientific Instrument Co., Ltd (Shanghai, China), and an analytical balance from Sartorius-Mechatronics Co., Ltd (Beijing, China).

Animals

Thirty Sprague Dawley (SD) rats weighing (220 ± 10) g were purchased from Jinan Jinfeng Laboratory Animal Co., Ltd. [License No.: SCXK (Shandong, China) 2014-0006]. This study was approved by the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine Animal Center.

Preparation of a Rat model of UC

A rat model of UC was prepared using the TNBS/ethanol assay. The rats were fasted for 12 h before the experiment, anesthetized, and fixed. Then, a hose coated with Vaseline was inserted into about 8 cm of the anus intestinal tract of the rats, TNBS/ethanol solution was injected, and the anus was clamped. Thereafter, the rats were hung upside down for about 1 min and then put into a cage for routine feeding. Finally, the disease activity index (DAI) of the rats was scored and counted.

Detection of the Pathological Damage to the Colon Tissues of the Rats in Each Group Via Hematoxylin and Eosin (H&E) Staining

The colon tissues of the rats in each group were embedded with paraffin and cut into sections with a thickness of about 8 μm. Then, the sections were soaked in xylene for deparaffinization for 5 min, put into 100%, 95%, 85%, and 70% ethanol for 1 min each, and washed with deionized water. Subsequently, the hematoxylin solution and eosin solution were added dropwise for staining for 5 min, respectively. After dehydration, the sections were soaked in xylene solution for transparentization for 10 min, followed by mounting with neutral resin and staining observation.

Measurement of the Levels of IL-6 and IL-1β in the Colon Tissues of Each Group of Rats by ELISA

After the last intervention with miR-195 agomir, the rats were anesthetized with 10% chloral hydrate. Then, blood was taken from the abdominal aorta, let stand at room temperature, and centrifuged at 5000 rpm for 15 min after coagulation. After that, the upper serum was taken, added into a new Eppendorf (EP) tube, and marked. According to the instruction of the ELISA kit, the absorbance of IL-6 and IL-1β in the rat colon tissues in each group was detected and statistically analyzed.

Examination of the MRNA levels o f p38 MAPK and TNF-α in the Rat Colon Tissues in Each Group Via reverse transcription-polymerase chain reaction (RT-PCR)

The total RNAs in the colon tissues of the rats in each group were extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) lysis assay and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) according to the instructions of the first-strand cDNA kit (TaKaRa, Otsu, Shiga, Japan). Subsequently, PCR amplification was carried out on a PCR instrument. The sequences of the primers added are shown in Table I. Reaction system: annealing at 65°C and extension at 72°C for 30 cycles. The reaction product was subjected to gel electrophoresis, and the optical density value was analyzed under a gel instrument.

Table I. P38 MAPK and TNF-α primer sequences.

Gene	Sequences
P38 MAPK	GTCCTGAGCACCTGGTTTCT GAGATGACAGTTCCCATCGGC
TNF-α	CCTCTCTCTAATCAGCCCTCTG GAGGACCTGGGAGTAGATGAG
β-actin	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT

Determination of the protein levels of p38 MAPK and TNF-α in the rat colon tissues in each group via Western blotting

The rat colon tissues in each group were collected and lysed by protein lysate, and the supernatant was collected. The protein concentration in each group was determined *via* Bradford assay, and loading dye was then added to boil and denature the proteins. Thereafter, 8% gel was prepared, and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) under 25 V for 2 h after electrophoresis and blocked for 1 h. After that, rabbit antibodies against p38 MAPK and TNF-α were added for incubation overnight. On the next day, incubation was conducted again with the HRP-labeled secondary antibody, the color was developed using diaminobenzidine (DAB) assay (Solarbio, Beijing, China), and the optical density of the bands was tested.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA) software was adopted for data analysis. The measurement data were expressed as mean ± standard deviation. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-195 Mimics Could Reduce the DAI Score of UC Rats

The calculation based on body weight changes, stool characteristics and bloody stool scores showed that the rats in Control group had a normal diet, no diarrhea, no bloody stool, and no abnormal condition, while anorexia, weight loss, and fecal occult blood or hematochezia occurred in the rats in Model group. After treatment with miR-195 mimics, the weight of the rats recovered, and the hematochezia was relieved. Compared with Control group, Model group had a notably increased DAI score ($^*p<0.05$). In comparison with that in Model group, the DAI score of the rats in miR-195 agomir group was decreased significantly ($^\#p<0.05$) (Figure 1).

MiR-195 Mimics Could Improve Colon Pathological Injury in UC Rats

H&E staining results (Figure 2) revealed that the rats in Control group had intact colonic mucosa epithelium, a clear cell structure, more goblet cells, better crypt morphology, and no inflammatory cell infiltration, whereas those in Model group suffered from edema and hyperemia in the colonic mucosa, disordered cells, and mucosal tissue hyperplasia in some cases. Compared with that in Model group, the pathological damage to the colon tissues in miR-195 agomir group was remarkably improved.

MiR-195 Mimics Was Able to Reduce IL-6 and IL-1β Levels in the Colon Tissues of UC Rats

According to the results of ELISA kit (Figure 3-4), statistical analysis manifested that in comparison with those in Control group, the levels of IL-6 and IL-1β in the colon tissues of the rats in Model group were raised ($^*p<0.05$). Besides, compared with those in Model group, these levels in the colon tissues of the rats in miR-195 agomir group were reduced ($^\#p<0.05$).

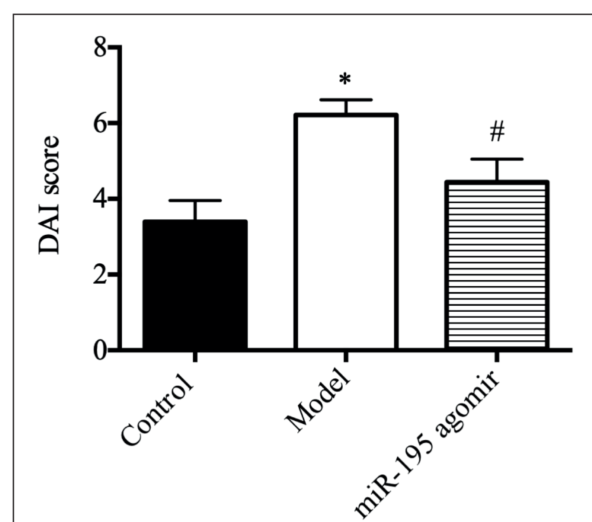

Figure 1. Comparison of the DAI score of the rats in each group (*$p<0.05$: Control group vs. Model group, $^\#p<0.05$: Model group vs. miR-195 agomir group).

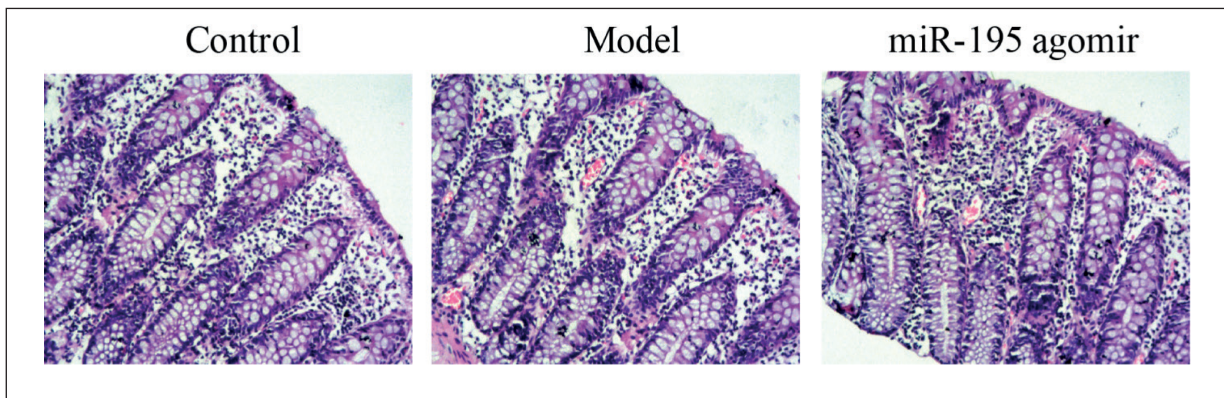

Figure 2. Pathological damage to the colon tissues of the rats in each group (20×).

MiR-195 Mimics Were Capable of Lowering the mRNA levels of p38 MAPK and TNF-α in the Colon Tissues of UC Rats

It was found from RT-PCR bands (Figure 5) that the rats in Model group had elevated mRNA levels of p38 MAPK and TNF-α in the colon tissues compared with those in Control group ($^{*}p<0.05$), while the rats in miR-195 agomir group exhibited decreased mRNA levels of p38 MAPK and TNF-α in the colon tissues in comparison with those in Model group ($^{\#}p<0.05$) (Figure 6).

MiR-195 Mimics Could Decrease the Protein Levels of p38 MAPK and TNF-α in the Colon Tissue of UC Rats

Western blotting bands (Figure 7) illustrated that the rats in Model group had raised protein levels of p38 MAPK and TNF-α in the colon tissues compared with those in Control group ($^{*}p<0.05$), while the rats in miR-195 agomir group had decreased protein levels of p38 MAPK and TNF-α in the colon tissues in comparison with those in Model group ($^{\#}p<0.05$) (Figure 8).

Discussion

As people's diet structure and lifestyle has changed in recent years, the incidence rate of UC in China shows a year-by-year uptrend. UC is mainly clinically manifested as abdominal pain, diarrhea, hematochezia, tenesmus, and joint pain, and primarily occurs in the rectum and the colon mucosa and submucosa[14]. In severe

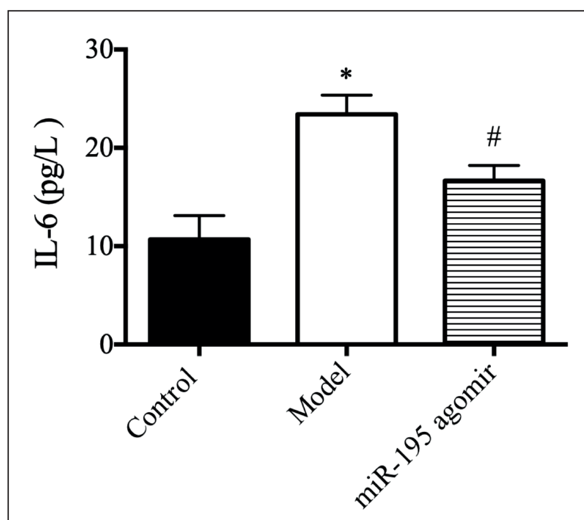

Figure 3. Comparison of the IL-6 level ($*p<0.05$: Control group vs. Model group, $^{\#}p<0.05$: Model group vs. miR-195 agomir group).

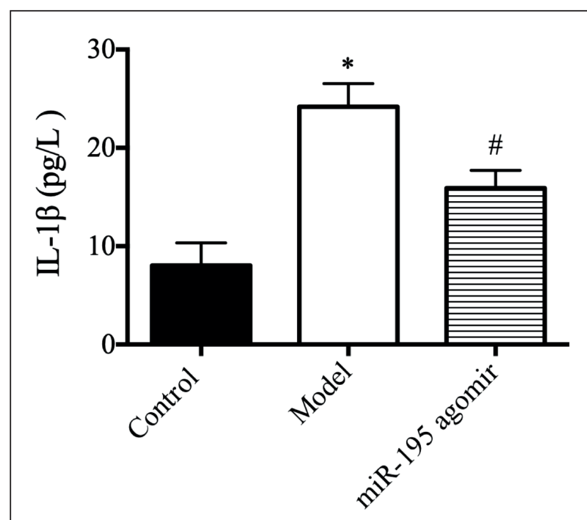

Figure 4. Comparison of the IL-1β level ($*p<0.05$: Control group vs. Model group, $^{\#}p<0.05$: Model group vs. miR-195 agomir group).

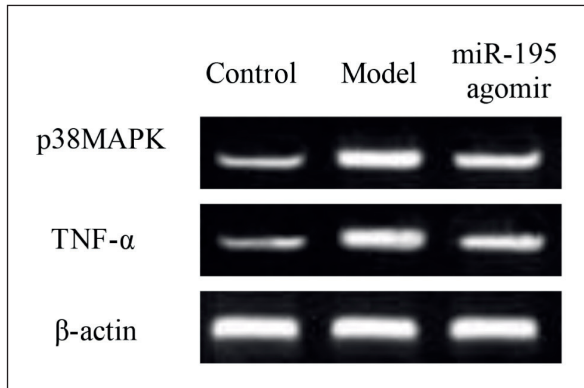

Figure 5. RT-PCR bands.

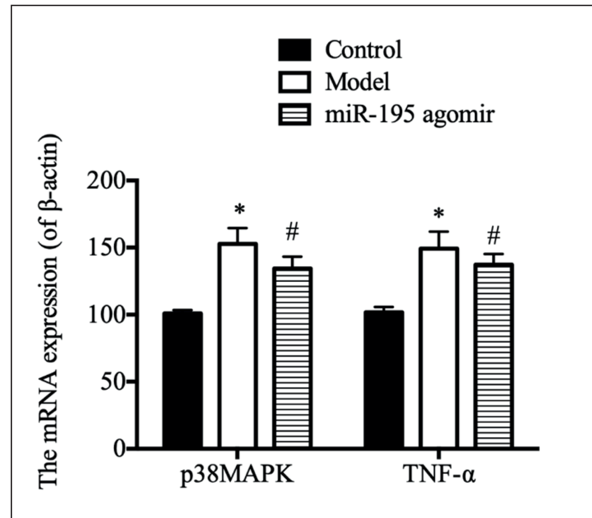

Figure 7. Western blotting bands.

cases, it will cause systemic complications and even deteriorate to colon cancer[15]. Clinically, UC is majorly treated by glucocorticoids, aminosalicylic acids, and antibiotics. However, although these drugs can temporarily relieve the disease, long-term administration will cause adverse reactions. Therefore, finding new safe and effective therapeutic drugs is still a hot topic in research on UC[16].

A great number of literature has verified that abnormal inflammatory responses exert crucial effects in the pathogenesis and development of UC. According to Salem and Wadie[17], the expression level of pro-inflammatory factors in the peripheral blood of UC patients is significantly increased, and the secretion of pro-inflammatory factors has a positive association with the inflammation degree. Elevated levels of IL-6 and IL-1β can influence epithelial cell function in the colon, cause epithelial cell edema and increase permeability, thereby further resulting in neutrophil aggregation, triggering UC, and aggravating its development. MAPK signaling pathway exerts a pivotal effect in the course of UC. It includes extracellular regulated kinase pathway, c-Jun amino-terminal kinase pathway, and p38 MAPK pathway, among which the p38 MAPK signaling pathway is able to stimulate the release of many factors, such as inflammatory factors, growth factors, and cell stress factors. Assi et al[18] used sodium dextran sulfate and

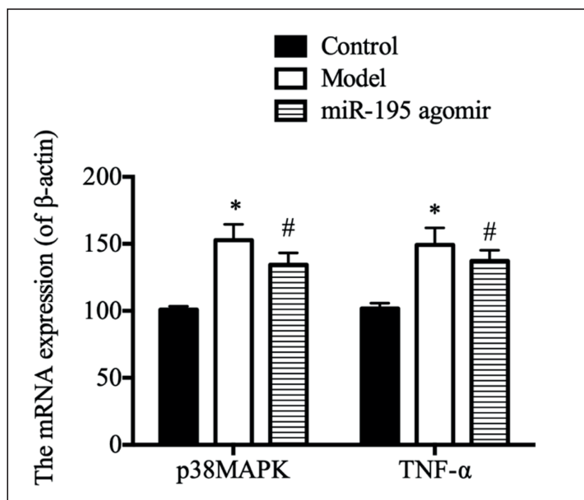

Figure 6. Comparisons of the mRNA levels of p38MAPK and TNF-α in the colon tissues of the rats in each group (*p<0.05: Control group vs. Model group, #p<0.05: Model group vs. miR-195 agomir group).

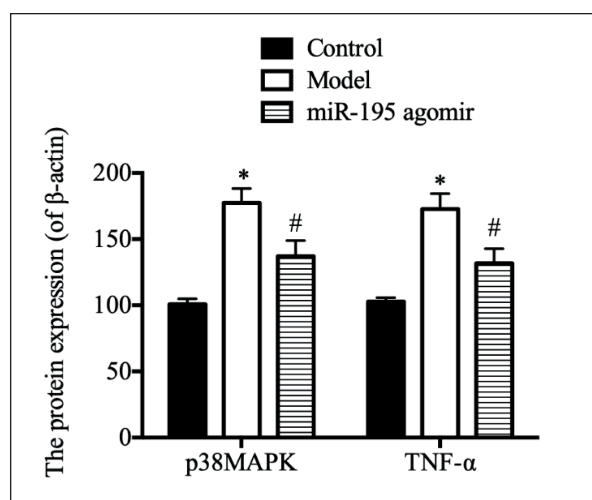

Figure 8. Comparisons of the protein levels of p38 MAPK and TNF-α in the colon tissues of the rats in each group (*p<0.05: Control group vs. Model group, #p<0.05: Model group vs. miR-195 agomir group).

TNBS to prepare the mice model of UC, respectively. After the application of p38 MAPK inhibitor (SB203580), macrophage infiltration in the mouse colon and intestinal mucosa was significantly alleviated, and the pathological score was elevated, suggesting that the p38 MAPK signaling pathway plays a vital role in UC. As the molecular biological technology continuously advances, the discovery of miRNAs has brought new hope for the treatment of UC. Valmiki et al[19] conducted biopsies in inflammatory and non-inflammatory areas in the colon of UC patients, and statistically analyzed the differentially expressed miRNAs using a microarray platform. The results manifested that specific changes appear in the expressions of miR-125, miR-155, miR-223, and miR-138 in the inflammation of patients with UC, thus providing a novel basis for research on miRNAs in UC.

In this investigation, the rat model of UC was first prepared by TNBS/ethanol assay. Literature has pointed out that ethanol can break the intestinal mucosal barrier. TNBS, as a hapten substance, can cause intestinal sensitization to autologous or allogenic proteins, attack the host immune system, and lead to inflammatory cell infiltration, and ulcer. The disease course of UC in rats is similar to that in patients, so rats are ideal models[20]. The pathological damage to the colon tissues of the rats in each group was examined *via* H&E staining. It was discovered that miR-195 agomir could relieve the pathological damage to the rat colon, hematochezia, and infiltration of inflammatory factors. Next, the release of inflammatory factors in each group was determined using the ELISA kit. The results revealed that miR-195 agomir was capable of repressing the release of inflammatory factors, IL-6 and IL-1β, indicating that miR-195 agomir inhibits inflammatory responses. To further explore the regulatory mechanism of miR-195, the mRNA and protein levels of the key targets, p38 MAPK and TNF-α, in the p38 MAPK signaling pathway were detected, respectively. It was found that miR-195 agomir could evidently block the p38 MAPK signaling pathway.

Conclusions

This research showed that miR-195 agomir was able to reduce the pathological damage to the colon of UC model rats, inhibit inflammatory responses, and reduce the release of inflammatory factors. Also, its mechanism may be correlated with the inhibition on the p38 MAPK signaling pathway. Thus we provide new experimental data for the clinical treatment of UC with miRNAs.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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