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MiR-146a regulates the development of ulcerative colitis via mediating the TLR4/MyD88/NF-κB signaling pathway

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Abstract. – OBJECTIVE: To study the effect of micro ribonucleic acid (miR)-146a on the development of ulcerative colitis (UC) and to explore its regulatory effect on the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) and nuclear factor-kappa B (NF-κB) signaling pathways.

MATERIALS AND METHODS: The UC model in rats was established using 2,4,6-trinitrobenzenesulfonic acid (TNBS)/ethanol. A total of 30 male rats were randomly divided into control group, model group and miR-146a inhibitor group, with 10 rats in each group. The disease activity index (DAI) and the macroscopic score of colonic mucosa were measured in each rat. MiR-146a expression in rat intestinal tissues was detected via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Serum levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in rats were detected via enzyme-linked immunosorbent assay (ELISA). Additionally, Western blotting assay was performed to detect protein levels of TLR4, MyD88, and NF-κB in rat intestinal tissues.

RESULTS: Compared with those in control group, rats in model group had notably increased DAI, inflammation score, upregulated expression levels of TLR4, MyD88, NF-κB, and miR-146a, as well as increased serum levels of IL-1β and TNF-α. However, rats in miR-146a inhibitor group exhibited substantially decreased DAI, inflammation score, lowered content of IL-1β and TNF-α and levels of TLR4, MyD88, and NF-κB compared with those in model group.

CONCLUSIONS: We found that miR-146a inhibitor alleviates UC by reducing the release of inflammatory factors through suppressing the TLR4/MyD88/NF-κB signaling pathway.

Key Words:
MiR-146a, Ulcerative colitis, TLR4, MyD88, NF-κB.

Introduction

Ulcerative colitis (UC), a chronic nonspecific inflammatory bowel disease, generally occurs in the colon or left hemicolon, and its pathological manifestations are ulcer and hemorrhage caused by inflammations in the digestive tract mucosa. UC is prevalent in Western developed countries, and its incidence rate in China has been increasing year by year due to changes in the Chinese dietary structure and the improved living standard[1-3]. Acetylsalicylic acid formulation, cortin or immunosuppressant are often clinically applied as the main drugs for UC. However, the efficacy is not very satisfactory, and about 20% severe UC patients still need colectomy. At present, the pathogenesis of UC has not been clarified yet, and researchers believed that UC is resulted from multiple factors, including immunity, heredity and environment, of which the most recognized hypothesis is the abnormal immune response[4,5].

The intestinal dysbacteriosis in UC patients activates the immune system under the antigenic stimulation. Immune reaction is a process in which Toll-like receptor (TLR) family stimulates the activation of the downstream myeloid differentiation factor 88 (MyD88) to form complexes. Subsequently, the downstream nuclear factor-kappa B (NF-κB) is activated, further leading

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to the transformation and secretion of pro-inflammatory factors[6-8]. Hence, inhibition of the TLR4/MyD88/NF-κB signaling pathway may be a novel therapeutic strategy for UC.

In recent years, micro ribonucleic acid (miRNA) is the study focus in medicine. It belongs to a category of highly conserved endogenous non-coding RNAs with about 22 nucleotides in length, and its main function is to promote the degradation of target genes or inhibit their translation into proteins[9]. MiRNAs regulate cellular behaviors and influence the occurrence, development or healing of diseases through various pathways. It has been reported that miRNA dysfunction may be a potential factor for the incidence of diseases. Besides, a great number of studies have revealed that miRNAs possibly participate in the incidence and development of UC[10]. Van der Goten et al[11] discovered that 24 miRNAs were differentially expressed in UC patients, including markedly upregulated hsa-miR-21-5p, hsa-miR-31-5p, hsa-miR-146a-5p, hsa-miR-155-5p, hsa-miR-375, and hsa-miR-650 through microarray analyses. Among these miRNAs, hsa-miR-146a-5p could target on the key factor NF-κB, indicating that miR-146a may treat UC *via* regulating the NF-κB signaling pathway.

In this study, the UC model in rats was established through intrarectal injection with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)/ethanol. We aim to explore the regulatory effect of miR-146a on UC and its potential mechanism, so as to provide fundamental data for the clinical treatment of UC.

Materials and Methods

Experiment Animals

Specific pathogen-free male Sprague Dawley (SD) rats weighing 200-230 g (No.: 61001700002391) were used. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

Reagents and Instruments

Trinitro-benzene-sulfonic acid (TNBS) solution (5% TNBS) was purchased from Sigma (St. Louis, MO, USA), miR-146a inhibitors from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), miR-146a primers from Invitrogen (Carlsbad, CA, USA), enzyme-linked immunosorbent assay (ELISA) kit from Shanghai Fanke Industrial Co., Ltd. (Shanghai, China), polymerase chain reaction (PCR) instrument from ABI (Foster City, CA, USA) and electrophoresis instrument and transfer unit from Bio-Rad (Hercules, CA, USA).

Establishment of the Rat Model of UC

During the adaptive breeding, rats had free access to food and water. A total of 30 rats were randomly divided into 3 groups (n = 10 per group). After fasting overnight, rats received intraperitoneal anesthesia using 10% chloral hydrate, and a silicone tube lubricated by paroline was inserted into the colon through anus for about 8 cm. Rats in model group and miR-146a inhibitor group were injected with TNBS solution (3 mg/kg, containing 50% ethanol), while those in control group were injected with normal saline. After enema, the silicone tubes were pulled out slowly and rat anuses were blocked by cotton swabs, with the tails lifted up until they revived naturally. The UC model in rats was successfully established 3 d later[12].

Pathological Observation of Colonic Mucosa and Tissues

After the rats were executed, colons were taken and washed using normal saline, followed by the observation of changes in colonic mucosa, including the smoothness of colon surface, adhesion to adjacent tissues, hyperemia, and edema.

Detection of the Level of MiR-146a in Rat Colon Tissues Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Colon tissues were taken from the rats to extract the total RNA, quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Then PCR was carried out according to the PCR instructions, with the primer sequences shown in Table I. The reaction conditions were as follows: pre-degeneration at 95°C for 30 s, degeneration at 95°C for 5 s, and annealing at 60°C, for 35 cycles in total. Finally, the relative expression level of miR-146a was analyzed.

Table I. Primer sequence.

Primer	Primer sequence (5′-3′)
MiR-146a	TGAGAACTGAATTCCATGGTT
U6	TTCGTGAAGCGTTCCATATTTT

Determination of the Levels of Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) in the Serum of Rats Via ELISA

Blood was collected from each group of rats and centrifuged for harvesting serum. The contents of IL-1β and TNF-α were determined according to the operation instruction in the ELISA kit.

Measurement of the Levels of TLR4, MyD88, and NF-κB Proteins in Rat Colon Tissues Via Western Blotting

The radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was used to lyse the rat colon tissues, and the supernatant was retained. The concentration of the proteins was determined *via* Bradford assay. The protein sample (50 μg) was loaded for electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at constant current, blocked for 2 h and washed for 3 times (5 min/time) using Tris-Buffered Saline and Tween 20 (TBST) solution. The membranes were incubated with the primary antibodies against TLR4, MyD88, and NF-κB overnight. The day after, membranes were washed and incubated with secondary antibodies. Images were exposed by diaminobenzidine (DAB) developer (Solarbio, Beijing, China). Finally, the optical density of bands was analyzed using ImageJ software.

Statistical Analysis

Data were statistically analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and expressed as ($\bar{x} \pm s$). ANOVA was adopted for the comparison among all groups of data, followed by Post Hoc Test (Least Significant Difference). $p < 0.05$ suggested that the difference was statistically significant.

Results

Colonic Inflammation Scores of UC Rats

After 14 d of treatment, rats in model group exhibited adhesion of intestinal wall tissues, bowel wall thickening, mucosal hyperemia, and proliferation of granulation tissue. The histological score in model group was significantly higher compared with that in control group ($p < 0.01$). After miR-146a intervention, rats had mild adhesion of large intestinal tissues, reduced ulcer and markedly lowered histological scores ($p < 0.01$) (Table II).

MiR-146a Inhibitor Improved the Pathological Morphology

The pathological morphology of rat colons was observed. It was found that, compared with control group, rats in model group showed rough colon surface, adhesion to adjacent tissues, hyperemia, and edema. Compared with model group, rats in miR-146a inhibitor group had relatively smooth colon surface, few adherent adjacent tissues and mild hyperemia and edema (Figure 1).

The Expression of MiR-146a Was Raised in UC Rats

Compared with control group, rats in model group had remarkably increased miR-146a expression in colon tissues (**$p < 0.01$), suggesting that miR-146a expression was significantly upregulated in UC rats (Figure 2). It is indicated that miR-146a inhibitor can be used for the treatment of UC.

MiR-146a Inhibitor Reduced the Levels of IL-1β and TNF-α in Rat Serum

Compared with those in control group, serum levels of IL-1β and TNF-α increased by 3.07 ± 0.32 pg/100 mg protein and 4.53 ± 0.24 pg/100 mg protein, respectively, in model group (**$p < 0.01$). Through the intervention of miR-146a inhibitor, serum levels of IL-1β and TNF-α de-

Table II. Effect of miR-146a on the colonic inflammation scores of TNBS/ethanol rats.

Group	Disease activity index (DAI) score	Score of gross morphological damage	Score of colonic mucosal tissue damage
Control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Model group	1.52 ± 0.28	6.34 ± 0.49**	13.27 ± 3.50*
MiR-146a inhibitor group	0.98 ± 0.23	3.78 ± 1.53$^{\#}$	8.12 ± 3.13$^{\#\#}$

**$p < 0.01$ and *$p < 0.05$, *vs.* Control group. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$, *vs.* Model group.

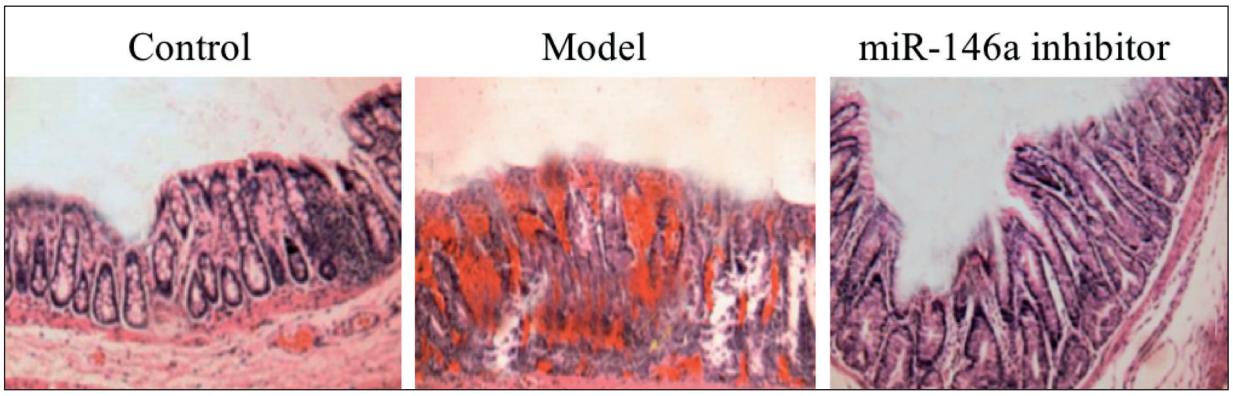

Figure 1. Effect of miR-146a inhibitor on the pathological morphology of rat colons (Magnification × 100).

creased by 1.67 ± 0.33 pg/100 mg protein and 2.14 ±0.32 pg/100 mg protein, respectively ($^{\#\#}p < 0.01$) (Figure 3A-3B).

MiR-146a Inhibitor Repressed the Expressions of TLR4, MyD88, and NF-κB in Rat Colon Tissues

To study the mechanism of miR-146a in regulating the pathogenesis of UC, expressions of three targets in the TLR4/MyD88/NF-κB signaling pathway were detected. According to the results of Western blotting (Figure 4A), protein expressions of TLR4, MyD88, and NF-κB in rat colon tissues of model group were markedly upregulated compared with control group ($^{**}p < 0.01$, $^{***}p < 0.01$, and $^{**}p < 0.01$, respectively). However, compared with those in model group, expressions of TLR4, MyD88, and NF-κB were markedly suppressed in miR-146a inhibitor group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#}p < 0.05$, respectively) (Figure 4B-4D). The above results demonstrated that miR-146a can treat UC in rats through inhibiting the TLR4/MyD88/NF-κB signaling pathway.

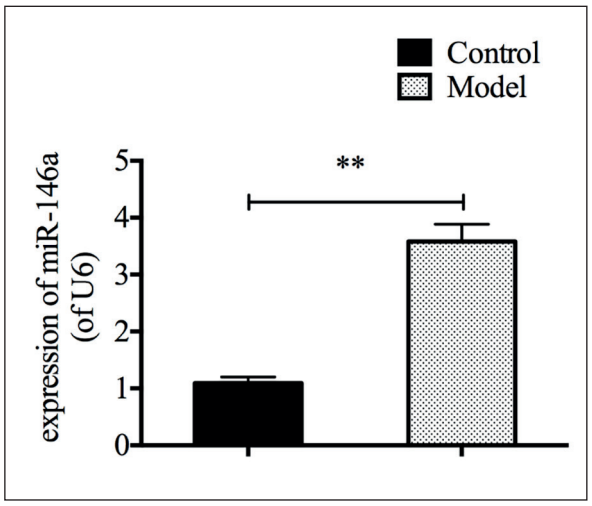

Figure 2. Expression of miR-146a in UC rats ($^{**}p < 0.01$).

Discussion

UC is a kind of chronic recurrent or persistent inflammatory disease of the colon, and its typical onset features include hypogastralgia, diarrhea, and mucous stool with pus and blood. So far, there is no effective treatment regimen in China and foreign countries. Current treatment is only able to alleviate the disease, but long-term medication will contribute to hepatotoxicity and nephrotoxicity[13]. The pathogenesis of UC still remains unclear, and it is believed that UC is correlated with hereditary, dietary and environmental factors, intestinal flora disturbance, and immunological barrier[14]. The incidence rate of UC is relatively high in kinship families and the highest in European developed countries. Studies showed that the toxins produced by microorganisms, such as bacteria, viruses or parasites in intestinal tracts can cause intestinal diseases similar to UC. Pathogen invasion occurs due to the intestinal flora proportion imbalance, namely, the pathogenic bacteria are increased and beneficial bacteria decreased, leading to a series of inflammatory cascade reactions. As a result, antibiotics can mitigate UC symptoms to a certain degree[15]. Some studies have manifested that immunological barrier is an important factor for UC. Toll receptor, a pattern recognition receptor, mediates innate defense reactions. TLR4

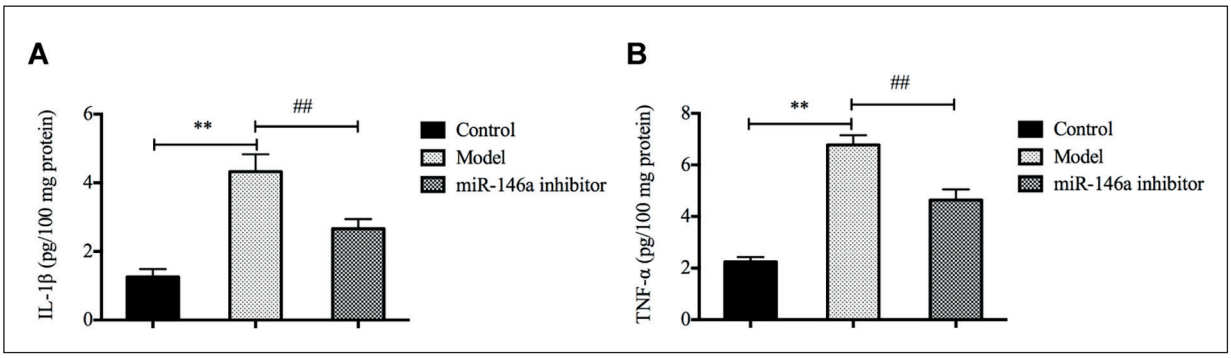

Figure 3. Effect of miR-146a inhibitor on serum levels of IL-1β and TNF-α in UC rat. ***A,*** Content of IL-1β, ***B,*** Content of TNF-α (**$p < 0.01$, ##$p < 0.01$).

mainly recognizes bacterial lipopolysaccharide and after being stimulated, it activates MyD88 factor and the downstream transcription factor NF-κB, further stimulating the release and secretion of inflammatory factors[16]. Feng et al[17] found that baicalin can down-regulate the expression of TLR4 in colon tissues. According to the findings of Chamanara et al[18], the expression level of MyD88 protein is positively correlated with the inflammatory degree in UC rats, suggesting that regulating the TLR4/MyD88/NF-κB signaling pathway plays a crucial role in the occurrence and development of UC. Therefore, developing drugs that can target and regulate the TLR4/MyD88/NF-κB signaling pathway is an urgent issue yet to be solved.

In recent years, miRNAs have become the study focus in the medical field, and with the in-depth studies on genome, it has been discovered that non-coding RNA can also affect the synthesis of coding proteins. Initial miRNAs are cleaved into precursor miRNAs by Drosha enzyme in nucleus, and the latter are transferred *via* transporting proteins from nucleus to cytoplasm. They are cleaved into miRNAs, with 20-25 nucleotides in size, by Dicer enzyme. Mature miRNAs can bind to 3′untranslated region sites, thereby regulating gene encoding. This arouses the attention from scholars, and an increasing number of studies have verified the aberrant expression of miRNAs in the serum, colon tissues and stool of

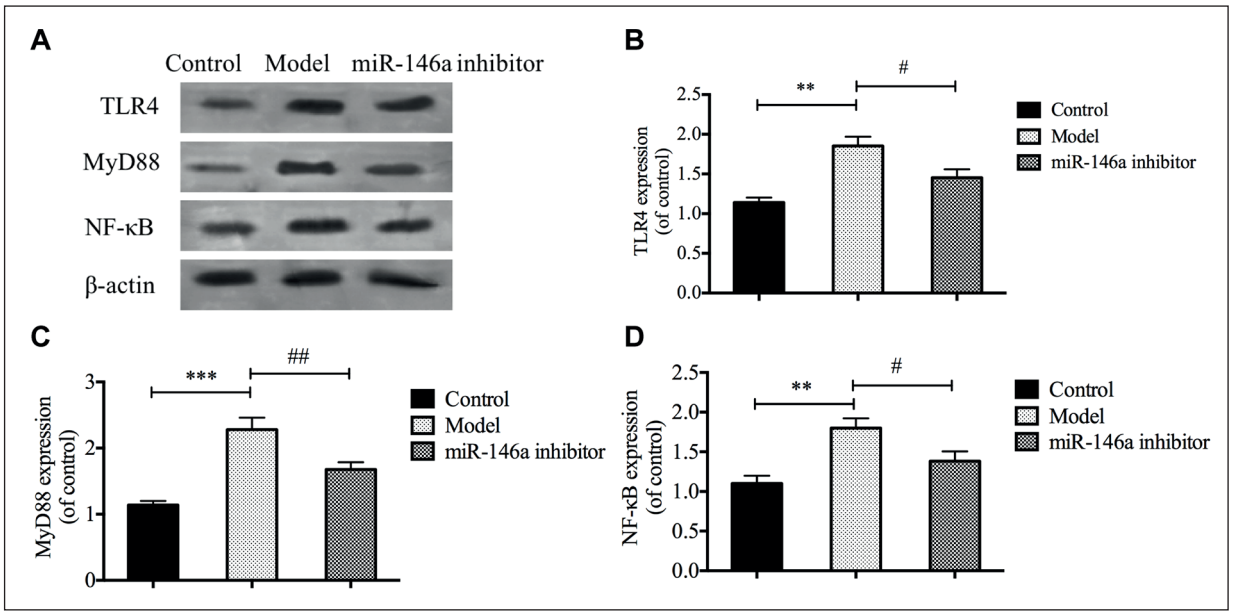

Figure 4. Effect of miR-146a inhibitor on the protein level of the TLR4/MyD88/NF-κB signaling pathway. ***A,*** Western blotting band ***B-D,*** Statistical chart of Western blotting band (*$p < 0.01$, **$p < 0.01$, #$p < 0.05$ and ##$p < 0.01$).

UC patients. We believed that these abnormally expressed miRNAs can cause the occurrence and development of UC or aggravate the disease. For example, Ciccacci et al[19] studied 267 Italian patients with inflammatory bowel diseases and found that miR-122, miR-499, miR-146a, miR-196a-2, and miR-124a were differentially expressed in them, compared with those in normal people. When studying 19 UC patients, Lin et al[20] found through miRNAs screening that miR-147b, miR-194-2, miR-383, miR-615, miR-1826 exhibited differential expressions, compared with those in normal people, and the regulation mechanism may be related to TGF-β, STAT3, IL-8, and the PI3K/AKT/mTOR signaling pathway. The targeted regulation on the differentially expressed miRNAs through gene therapy will provide a new study idea and therapeutic scheme for UC.

In this work, the UC model in rats induced by TNBS/ethanol was utilized for the experiment. This *in vivo* model, similar to the human UC, features long course of disease, simple operation, cost-effectiveness, practicability, and favorable repeatability. It was found that rats in model group exhibited severer pathological injuries of colon tissues than those in control group, with the symptoms of colon adhesion, loose stool and hematochezia. However, rats in miR-146a inhibitor group had improvement in colon adhesion and hemorrhage. To further study the regulative effect of miR-146a inhibitor on the UC rats, expressions of inflammatory factors were detected. Compared with control group, expression levels of inflammatory factors increased in model group, which were notably declined after the intervention of miR-146a inhibitor. Furthermore, the target proteins in the TLR4/MyD88/NF-κB signaling pathway were detected to explore the regulation mechanism of miR-146a. The results revealed that miR-146a inhibitor could evidently repress the expressions of TLR4, MyD88, and NF-κB proteins, suggesting that the mechanism of miR-146a inhibitor in treating UC may be correlated with the inhibition of the TLR4/MyD88/NF-κB signaling pathway.

Conclusions

We found that miR-146a inhibitor can exert a therapeutic effect on UC rats *via* suppressing the TLR4/MyD88/NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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