Protection by microRNA-7a-5p Antagomir Against Intestinal Mucosal Injury Related to the JNK Pathway in TNBS-Induced Experimental Colitis

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ABSTRACT

Background: *Increasing evidence shows that microRNA-7a-5p (miR-7a-5p) plays an important role in regulating the inflammatory process in inflammatory bowel disease (IBD). How miR-7a-5p contributes to this process is poorly defined. The purpose of this study was to examine whether miR-7a-5p regulates 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced inflammatory responses via the JNK pathway.*

Methods: *Colitis was induced in male mice by intracolonic administration of TNBS; mice were divided into 3 groups: normal control (NC), TNBS, and miR-7a-5p antagomir-treated group. Inflammatory responses were estimated by disease activity index (DAI) and histological scores. The relative expressions of miR-7a-5p and tight junction protein, ZO-1, were detected by RT-qPCR. Western blot assays were used to estimate the level of JNK pathway proteins and ZO-1. After miRNA-antagomir injection, the extent of colonic tissue injury and expression levels of ZO-1 and JNK in intestinal tissue were compared.*

Results: *miR-7a-5p and p-JNK expression were higher in the intestinal tissue of the TNBS group as compared to NC. Inhibition of the expression of miR-7a-5p resulted in significantly decreased expression of p-JNK but increased expression of ZO-1 and promoted the recovery of intestinal mucosa.*

Conclusion: *This work demonstrates a correlation between the JNK pathway and miR-7a-5p in TNBS-induced experimental colitis in mice, which may provide a new research direction for the treatment of IBD.*

Keywords: *microRNA-7a-5p, JNK, IBD, microRNA antagomir, tight junction protein*

INTRODUTION

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is a relapsing, remitting, and chronic inflammatory disorder of the colon and rectum. IBD continues to be a major public health problem with increasing incidence and a significant impact on the quality of life. Although its etiology remains elusive, it is generally believed to involve immunological, environmental, and genetic factors. Although there are many improvements in diagnosis, new treatment drugs, and optimized treatment strategies, early diagnosis is challenging due to a lack of convincing markers; furthermore, 20-30% of patients still experience insufficient treatment efficacy.[1] Therefore, it is of great importance to explore the pathogenesis of IBD and search for new therapeutic targets.

MicroRNA (miRNAs) are non-coding, endogenous RNA molecules that are highly conserved and small, about 19-25 nucleotides in length. miRNAs can inhibit protein expression by partial hybridization with the complementary sequence (mainly the 3'UTR) of the target RNA transcript.[2] Each miRNA is estimated to regulate multiple target mRNAs. For example, miR-7a-5p is involved in multiple biological processes including cell proliferation, differentiation, apoptosis, and immune regulation.[3] Previous studies have indicated that miR-7a-5p is related to intestinal diseases.[4] Using both in vivo and in vitro experiments, it was proven that expression of miR-7a-5p was higher in colitis tissue than in normal tissue.[5,6]

Mitogen-activated protein kinase (MAPK) signaling pathways involve a wide range of signal cascades, in which various extracellular stimuli induce inflammation including the production of inflammatory mediators. These targets have naturally become the focus of IBD research.[7,8] Previous studies have shown that MAPK

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subfamilies, including ERK, JNK, and p38 MAPK, play distinct roles in various biological processes. In general, ERK regulates cell growth and differentiation, JNK regulates inflammation, and p38 MAPK regulates apoptosis.[9] The JNK pathway can be activated by stress, differentiation, and growth factors. Recently, many studies have emphasized the anti-inflammatory effect of the JNK regulatory pathway.[10,11] The level of p-JNK in diseased tissue was significantly higher than that of normal tissue in a colitis model induced by LPS, DSS, TNBS, and even in IBD patients.[12,13,14] JNK and miR-7a-5p play important roles in the occurrence and development of IBD, but the exact relationship between them has not yet been reported. Therefore, in this study, we attempt to explain the correlation between JNK and miR-7a-5p, thereby providing new therapeutic targets and an improved theoretical basis for the treatment of IBD.

MATERIALS AND METHODS

Animal Experiments

A total of 30 male 3-week-old Balb/c mice were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China). The mice were given free access to food and water and were individually caged under controlled temperature (25°) and humidity (45-65%) with an artificial 12-h light/dark cycle. The mice were randomly divided into 3 groups as follows: (1) normal control group (NC group, *n* = 10); (2) 2,4,6-trinitrobenzene sulfonic acid (TNBS group , *n* = 10); experimental colitis in this group was induced by TNBS (Sigma, St Louis, USA) via intrarectal injection according to the procedure described by Teng et al.[15]; (3) miRNA-antagomir group (*n* = 10); miR-7a-5p antagomir freeze-dried powder (RIBOBIO, China) was dissolved in saline (0.9%, 200 μL), and 2 hours after TNBS treatment, miR-7a-5p antagomir (100 nmol/kg) was injected into the tail veins of the mice and intestinal tissue was acquired after 7 days.

MAIN POINTS

- *This study reports that miR-7a-5p is closely related to JNK pathway activation in mice with IBD.*
- *miR-7a-5p antagomir alleviates inflammatory responses in mice with TNBS-induced colitis.*
- *We found when miR-7a-5p antagomir was applied, the level of p-JNK in diseased tissues decreased significantly. Expression of tight junction protein and the recovery of intestinal mucosal injury under the microscope suggests a protective effect of inhibitors on the intestinal barrier.*

All mice were evaluated daily for weight, diarrhea, and bloody stool. The disease activity index (DAI) was used to evaluate the degree of colon injury. The DAI was calculated using the mean of the total score: weight loss (0, none; 1, 1-5%; 2, 5-10%; 3, 10-15%; 4, >15%); stool consistency (1, none; 2, loose stool; 4, diarrhea); and bloody stool (0, normal; 2, slight bleeding; 4, massive hemorrhage).

Histological Examination

Mouse colon tissue was quickly removed and washed gently with saline solution after sacrifice. Colon tissue samples were divided into 3 parts and tissue was fixed with 4% formalin, embedded in paraffin, and cut into 4 μm thick sections. Samples were stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, China). Under colonoscopy, the degree of inflammation was classified as 0-4 (0, no sign of inflammation; 1, very low level of leukocyte infiltration; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, thickened colon wall; 4, cross wall infiltration, goblet cell loss, high vascular density, and thickened colon wall). The histological injury score was measured under a high-power field (200×), and at least 5 visual fields were randomly selected for evaluation.

Real-Time qPCR Analysis

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). The qPCR was conducted by SYBR Premix Ex Taq II (Takara Bio Inc.) and a Bio-Rad iQ5 Real-Time system (Bio-Rad Laboratories, Inc., USA). PCR reaction mixtures (20 μL) were performed using a DNA Engine (ABI 7500, Thermo). Detailed procedures can be found in previous reports.[5] The results were calculated using the $2^{-\Delta\Delta ct}$ method and normalized to β-actin (*ActB*) and U6 (*Rnu6*), respectively. The primers (Sangon Biotech, China) used are shown below:

ZO-1 (*Tjp1*): forward 5′-TACCTCTTGAGCCTTGAACTT-3′, reverse 5′-CGTGCTGATGTGCCATAATA-3′; β-actin (*ActB*): forward 5′-GTGACGTTGACATCCGTAAAGA-3′, reverse 5′-GCCGGACTCATCGTACTCC-3′; miR-7a-5p: forward 5′- AGCGGTGGAAGACTAGTGATTTTGTTGT-3′, reverse 5′-GCTGTCAACGATACGCTACG-3′; U6 (*Rnu6*): forward 5′-CTCGCTTCGGCAGCACA-3′, reverse 5′-AACGCTTCACGAATTTGCGT-3′.

Western Blot

Detailed procedures for the western blot assays used in this study can be found elsewhere.[5] In brief, intestinal

tissue samples were lysed in the presence of phosphatase inhibitors and proteins were separated by 10% SDS-PAGE gel. The proteins were electro-transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with primary antibodies against JNK (AHO1362; dilution:1:500; Thermo Fisher Scientific, Inc.), p-JNK (MA5-14943; dilution: 1:250; Thermo Fisher Scientific, Inc.), ZO-1 (40-2200; dilution: 1:1000; Thermo Fisher Scientific, Inc.), and GAPDH (dilution: 1:5000; Wanleibio Shenyang, China). The membranes were incubated with appropriate secondary HRP-conjugated antibodies for 2 h at room temperature. An ECL reagent was used to visualize antibody binding; densities of the bands were quantified with an imaging densitometer.

Statistical Analysis

We performed at least 3 independent experimental replicates for each assay. The experimental data are presented as mean ± SEM and analyzed using Prism version 8.0 software (GraphPad Software, La Jolla, CA, USA) where appropriate. Comparisons between groups were analyzed by one-way analysis of variance (ANOVA), two-way ANOVA, and Dunnett's *t* test. A $P < .05$ was considered statistically significant.

RESULTS

miR-7a-5p Antagomir Alleviates Inflammatory Responses in Mice With TNBS-Induced Colitis

Weight Change and Disease Activity Index (DAI)

In mice with TNBS-induced colitis, weight loss and bloody stools were observed along with decreased activity as compared to that observed NC mice on day 1; severity of symptoms peaked on day 3 after TNBS treatment. The overall health of TNBS-treated mice improved after subsequent injection of miR-7a-5p antagomir; however, changes were not statistically significant until days 6 and 7 after TNBS treatment (Figure 1, $P < .05$).

Histopathological Changes

Destruction of the intestinal epithelium and crypt structure were easily observed in TNBS-treated mice; After treatment with mir-7a-5p antagonist, the intestinal epithelial structure and crypt structure were restored, and the inflammation was reduced, and histological scores between groups were statistically significant (Figure 2, $P < .05$).

Tight Junction Protein Changes

ZO-1 protein and mRNA levels in intestinal mucosa of mice in the TNBS-treated group were significantly lower than in control mice; yet, after injection of mir-7a-5p antagomir, ZO-1 protein and mRNA levels increased as compared with levels in the TNBS-treated group not receiving antagomir (Figures 3 and 4; $P < .05$).

miR-7a-5p Is Highly Expressed in Colon of Mice Treated with TNBS

Compared with the NC group, the relative expression of miR-7a-5p in the colon was significantly increased in mice treated with TNBS (Figure 5, $P < .01$).

JNK Activation in Mice with TNBS-Induced Colitis

As discussed above, JNK is closely related to the inflammatory process. In this study, we used western blots to semi-quantitatively analyze the protein expression and phosphorylation of JNK. Levels of p-JNK in TNBS-treated

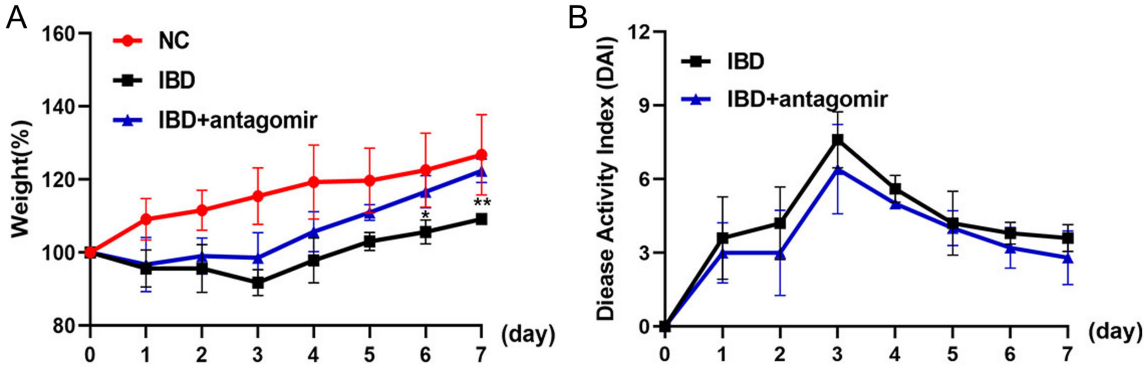

Figure 1. Expression of miR-7a-5p and resulting clinical manifestations in mice. (A) Weight loss in mice with TNBS-induced colitis was observable on day 1 with severe symptoms peaking on day 3 after TNBS treatment. In mice with TNBS-induced colitis, overall health improved after injection of miR-7a-5p antagomir, but did not reach statistical significance until days 6 and 7. (B) There was no significant difference in DAI between the 2 groups. *$P < .05$, **$P < .01$, ns, not significant.

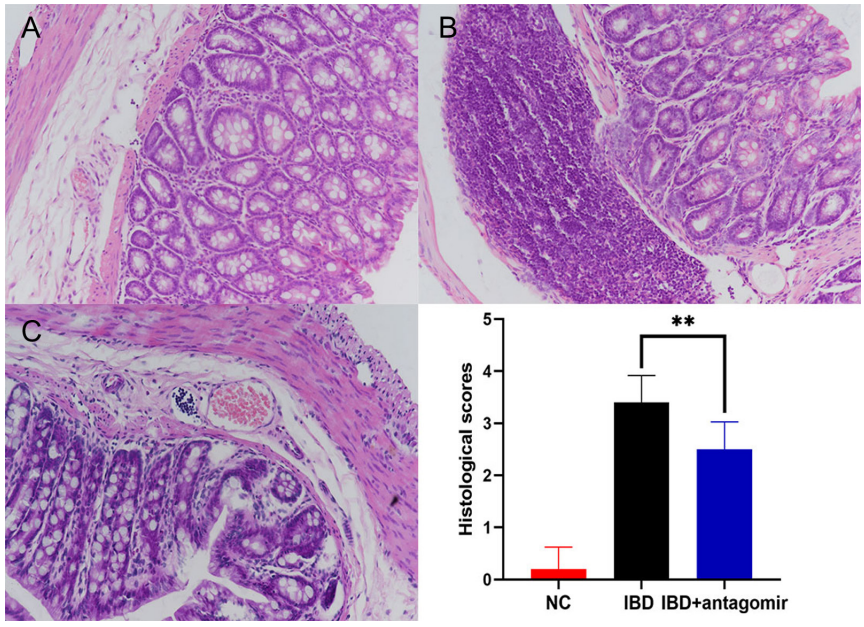

Figure 2. Histopathological changes. Images (A) is of tissue from NC mice. Images in (B) is from mice with TNBS-induced colitis. (C) is images from mice treated with TNBS and antagomir. (D) The histological scores between them were statistically significant. *P < .05, **P < .01, ns, not significant.

mice were significantly higher as compared to control mice (Figure 6, P < .05). Levels of total JNK did not change significantly among groups. In order to explore the relationship between miR-7a-5p and JNK, we compared levels of phosphorylated JNK protein in intestinal tissue from mice in the TNBS-treated group and the miR-7a-5p antagomir-treated group. Levels of p-JNK in the antagomir group were significantly lower as compared to the TNBS-treated mice that did not receive antagomir (Figure 6, P < .05). Therefore, these data indicate a relationship between miR-7a-5p expression and JNK activation.

DISCUSSION

IBD is a serious digestive disease. Patients with IBD begin to develop symptoms in adolescence; they experience life-long impacts on economic status, decreased quality

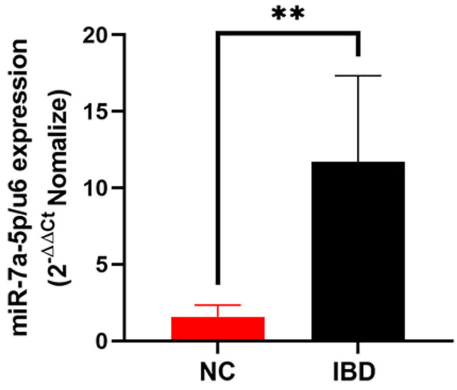

Figure 3. Western blot analysis of ZO-1 expression. mRNA levels of ZO-1 in intestinal mucosa of mice with TNBS-induced colitis were significantly lower than in mucosa of control mice. After subsequent injection of miR-7a-5p antagomir, mRNA levels of ZO-1 increased, which was statistically significant compared with TNBS group. *P < .05, **P < .01, ns, not significant, #P < .05 compared with the NC group.

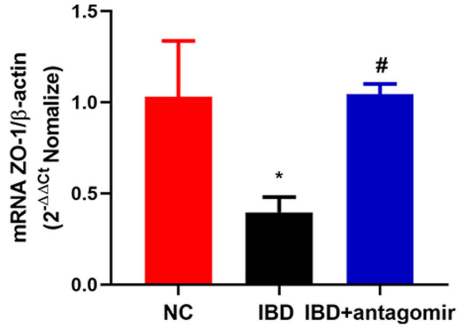

Figure 4. RT-PCR analysis of ZO-1 expression. ZO-1 protein expression in the intestinal mucosa mice with TNBS-induced colitis was significantly lower than that of the control group. However, after subsequent injection of miR-7a-5p antagomir, protein levels of ZO-1 were significantly increased as compared with the TNBS group. *P < .05, **P < .01, ns, not significant, #P < .05 compared with the NC group.

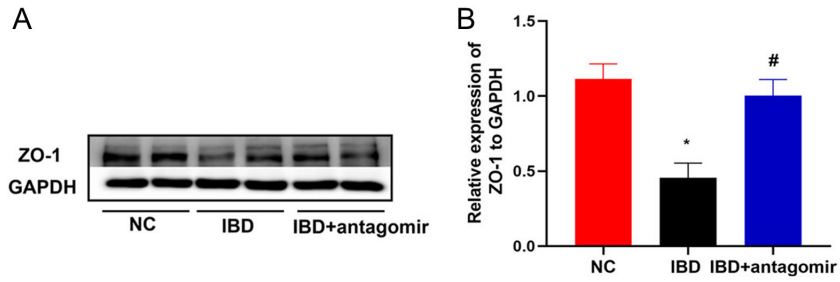

Figure 5. RT-PCR analysis of miR-7a-5p relative expression. Compared with the NC group, miR-7a-5p relative expression in the colon was significantly increased in mice with TNBS-induced colitis ($P < .01$).

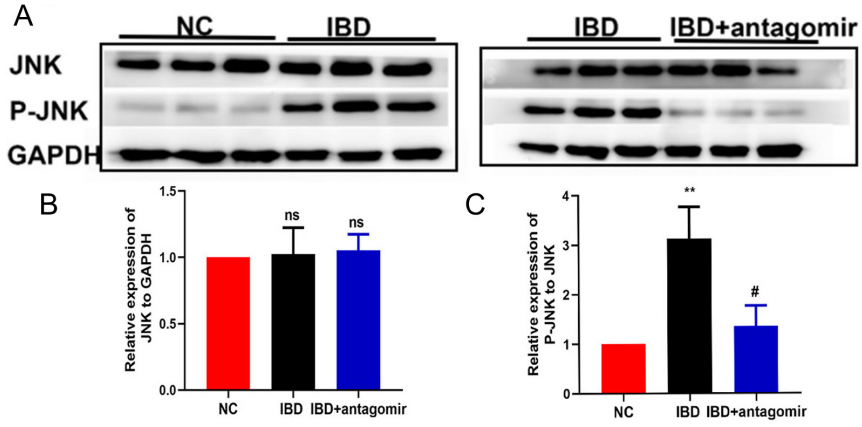

Figure 6. (A) Western blot analysis of JNK and p-JNK protein. (B) The level of total JNK did not change significantly in each group. (C) The level of p-JNK in mice with TNBS-induced colitis was significantly higher than in the control group. *$P < .05$, **$P < .01$, ns, not significant.

of life, increased risk of complications and malignant diseases, and overall shortening of life span.[16] Scientists have made great contributions to understanding the pathogenesis of IBD and finding effective treatment strategies. Although the pathogenesis of IBD is still unclear, its development likely involves multiple factors. Thus, every new piece of information regarding IBD is critical to developing a more complete understanding of this illness.

Previously, we found that miR-7a-5p was closely related to IBD. In both in vivo and in vitro experiments as well as in IBD patients, we found that expression of miR-7a-5p was significantly higher in pathological tissues as compared with normal tissues. These observations were again observed in this study. Therefore, these data indicate that miR-7a-5p may be involved in the occurrence and development of IBD. In addition, previous studies have also shown that trefoil factor 3 (*Tff3*) is one of the targets of miR-7a-5p; Tff3 participates in cell migration, proliferation, and apoptosis through the PI3K/AKT signaling pathway. miR-7a-5p can negatively regulate *Tff3* and aggravate mucosal injury. The inflammatory process and release of inflammatory factors are very important in the pathogenesis of IBD. However, previous studies have not investigated the inflammatory pathway of miR-7a-5p as it relates to IBD. This is the focus of this study.

JNK is a crucial mediator of various pathological signaling pathways involving many inflammatory diseases including rheumatoid arthritis, atherosclerosis, and IBD.[17] Many studies have shown that JNK is activated and mediates the pro-inflammatory cytokine expression in intestinal tissue including TNF-α, IL-1β, and IL-6. In the JNK pathway, p-JNK is activated to initiate downstream signaling events resulting in the release of inflammatory factors and an increase in general inflammation. Therefore, the therapeutic effect of JNK inhibitors on IBD has been an area of considerable research focus in recent years. Here, we report that p-JNK is remarkably higher in diseased tissues as compared to normal tissues. There was no significant change in total JNK levels among groups. When miR-7a-5p

antagomir was applied, the level of p-JNK in diseased tissues decreased significantly. Expression of tight junction protein and the recovery of intestinal mucosal injury under the microscope suggests a protective effect of inhibitors on the intestinal barrier. These data indicated that there was a relationship between miR-7a-5p and the JNK pathway; furthermore, the miR-7a-5p antagomir is involved in the protection of the intestinal mucosal barrier.

In conclusion, this study reports that miR-7a-5p is closely related to JNK pathway activation in TNBS-induced experimental colitis in mice. MiR-7a-5p may act on the JNK pathway directly or may regulate the JNK signaling pathway through other protein molecules. However, the detailed mechanism of their interaction remains unclear and further research is needed. At the same time, this study also has some limitations. Although TNBS-induced experimental colitis mice is widely accepted as a classic model of IBD, IBD is an inflammatory disease with a very complex immune damage mechanism. There must be some differences between acute injury caused by TNBS and IBD. Our results may not be fully applicable to IBD. However, science itself is a process of infinite attempt. We should have the courage to explore, which is the charm of science itself. Only by continuous research can we approach the truth infinitely.

Ethics Committee Approval: The study was approved by the ethics committee of the Affiliated Shengjing Hospital of China Medical University (approval no. 2015PS281K, Shenyang, China).

Informed Consent: This study passed the ethical review of animal experiments.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept- Y.B., L.F.X.; Design- Y.B.; Resource - T.C.H.; Materials - T.C.H.; Data Collection and Processing- Y.B.; Literature Search - Y.B.; Writing - Y.B., T.C.H., L.F.X.; Critical Reviews - Y.B., L.F.X.

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Conflict of Interest: The authors have no conflict of interest to declare.

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