MiR-98-5p expression inhibits polarization of macrophages to an M2 phenotype by targeting Trib1 in inflammatory bowel disease*

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Herein, we unfolded miR-98-5p mechanism in inflammatory bowel disease (IBD). IBD mouse model was established. The severity of colitis was assessed daily using the disease activity index (DAI). Murine peritoneal macrophages were stimulated by lipopolysaccharide (LPS). MiR-98-5p, tribbles homolog 1 (Trib1), M1 and M2 macrophage marker genes mRNA expression was analyzed. The relationship between miR-98-5p and Trib1 was explored using a luciferase reporter assay. The strategy of loss-of-function was used to explore the mechanism of miR-98-5p in macrophage polarization, inflammation and IBD. The results revealed that IBD mice had higher DAI index and miR-98-5p expression when compared to the Sham group. MiR-98-5p and Trib1 displayed a targeted regulation relationship. Knockdown of miR-98-5p transformed LPS-induced M1 macrophage polarization into M2 macrophage polarization and inhibited inflammation via up-regulating Trib1. However, shTrib1 reversed the effects. In vivo experiment, silencing of miR-98-5p, diminished the DAI and promoted M2 macrophage polarization. In conclusion, knockdown of miR-98-5p changed macrophage polarization to the M2 phenotype by increasing Trib1 expression, thereby alleviating IBD symptoms.

Key words: IBD, macrophage polarization, miR-98-5p, Trib1

Received: 03 January, 2020; revised: 08 January, 2020; accepted: 20 January, 2020; available on-line: 03 April, 2020

INTRODUCTION

Inflammatory bowel disease (IBD) is a general term for gastrointestinal diseases caused by inflammatory disorders, which is mainly divided into two categories: Crohn’s disease and ulcerative colitis (UC) (Webkamp et al., 2016). As it is a type of chronic, non-specific intestinal inflammation of unknown etiology, the diagnosis and treatment of IBD are demanding, and it is difficult to completely cure it with existing treatments. The continuous attack of IBD not only seriously affects the quality of life of the patients, but also can cause cancer, which seriously threatens their life and health (Wang et al., 2018). The pathogenesis of IBD is related to genetic, environmental, microbial, and immune factors, but the exact pathogenesis is not clear (Abraham et al., 2017). Recently, the researchers paid extensive attention to the role of immune abnormalities in IBD pathogenesis.

Macrophages are an important class of innate immune cells and antigen-presenting cells, which play a crucial role in anti-infection immunity (Zhang & Wang, 2014). It was reported that macrophages can secrete a large number of cytokines and bioactive substances to participate in the inflammatory response (Grainger et al., 2017). According to the functional characteristics, macrophages are mainly divided into pro-inflammatory macrophages (M1 polarized phenotype) and anti-inflammatory macrophages (M2 polarized phenotype). Previous studies found that macrophages are closely related to the development of IBD (Rubio & Schmidt, 2018; Leonardi et al., 2018; Fries et al., 2013), and it was confirmed that IBD can be alleviated by inhibiting M1 macrophage polarization and promoting M2 macrophage polarization (Daskalaki et al., 2019). Therefore, investigating the molecules that can affect macrophage polarization may provide new targets for IBD therapy.

MicroRNAs (miRNAs) are a class of non-coding single-stranded small RNA molecules with a length of approximately 22 nucleotides (Hu et al., 2019). Previous papers demonstrated the critical role of miRNAs in IBD. For example, miR-665 could promote the progression of IBD by inhibiting XBP1 and ORMDL3 (Li et al., 2017). Besides that, miRNAs are also closely related to the inflammation. Overexpression of miR-146a can diminish M1 macrophage polarization and promote M2 macrophage polarization, and then alleviate the inflammatory response (Huang et al., 2016). miR-98-5p is a type of miRNA which plays a critical role in the inflammatory response (Vergadi et al., 2018). It was revealed that miR-98-5p could negatively regulate the production of IL-10, thereby exerting a pro-inflammatory effect (Liu et al., 2011). In UC, miR-98-5p expression was increased and served as a potential diagnostic target (Coskun et al., 2013). However, the underlying mechanisms of miR-98-5p action in IBD are unclear.

Generally, miRNAs exert their role through the modulation of the target genes expression via binding to their 3'-untranslated region (3'-UTR). Tribbles homolog 1 (Trib1) belongs to Tribbles family, and it is critical for macrophage polarization (Satoh et al., 2013). Silencing of Trib1 can inhibit M2 polarization and induce inflammation (Arndt et al., 2018). Bioinformatics online software predicted a miR-98-5p binding site on Trib1. Thus, we speculated that miR-98-5p might regulate macrophage polarization.
polarization and inflammation through Trib1, and then modulate IBD progression.

In this study, we unfolded the mechanism of miR-98-5p action in macrophage polarization and IBD, aiming to discover a novel target for the treatment of IBD.

METHODS

**IBD mouse model.** All experimental procedures were approved by the Laboratory Animal Welfare and Ethics Committee of Shanghai University of traditional Chinese medicine. C57BL/6 mice (8 weeks old) were purchased from Guangdong Medical Laboratory Animal Center and divided into 4 groups (n=6/group): Sham, IBD, IBD+NC antigen, and IBD+miR-98-5p antagonist. To establish IBD mouse model mice were provided with 4% dextran sulphate sodium (DSS) dissolved in distilled water for one week. Mice in the Sham group were provided with regular water. Mir-98-5p antagonist or NC antigen were injected into the caudal vein of each mouse (Hou et al., 2017). Weight loss, stool consistency, and bleeding of each mouse were monitored daily. The severity of colitis was assessed daily using disease activity index (DAI) scoring system as previously described (Hou et al., 2017). Seven days later, mice were sacrificed and the colonic tissues were collected for quantitative real-time RT-PCR (qPCR).

**Cells.** Brewer’s thioglycollate medium (4%, 1 ml) was injected intra peritoneally into C57BL/6 mice to elicit mouse peritoneal macrophages (Zhu et al., 2016). In order to get adherent peritoneal exudates cells, ice-cold phosphate-buffered saline was used for peritoneal lavage after 4 days. Then, the cells were incubated on dishes for 6 hours. After removing non-adherent cells macrophages were collected.

Mir-98-5p mimic and inhibitor, shTrib1 and their corresponding controls (RiboBio, Guangzhou, China) were transfected into macrophages using Lipofectamine 2000 (Invitrogen). After 48 hours, lipopolysaccharide (LPS, 1 μg/ml) was used to treat the macrophages for 24 hours.

**qPCR.** TRIzol reagent was used to extract the total RNA from colonic tissues or macrophages following the manufacturer's instruction. Reverse transcription system was used for reverse transcription. qRT-PCR was carried out using SYBR Green PCR Master Mix (Takara, Otsu, Japan) with the ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The relative mRNA level of Trib1, monocyte chemoattractant protein 1 (Mcp1), Nitric Oxide Synthase (Nos2), and tumor necrosis factor alpha (Tnfa) was assessed daily using disease activity index (DAI) scoring system as previously described (Hou et al., 2017). Seven days later, mice were sacrificed and the colonic tissues were collected for quantitative real-time RT-PCR (qPCR).

Luciferase reporter assay. Trib1 3’UTR sequence containing wild type (WT) or mutant (MUT) miR-98-5p putative binding region was amplified by RiboBio (Guangzhou, China), and inserted into pGL3-Trib1-3’UTR plasmid (Invitrogen). Then, Lipofectamine 2000 (Invitrogen) was used for co-transfecting the plasmids and miR-98-5p mimics. Dual luciferase reporter gene assay kit (Promega, USA) was used to measure the luciferase signal intensity 48 hours after transfection.

Western blot. RIPA lysis buffer (Beyotime, Beijing, China) was used to extract the total protein from colonic tissues or macrophages. BCA assay was performed to measure the protein concentration. Samples with an equal amount of protein were separated and transferred using 10% SDS-PAGE and PVDF membranes. Then, membranes were incubated with primary anti-Trib1 antibody (1:500, Abcam) at 4°C overnight after blocking in skimmed milk (5%) at room temperature for two hours. Subsequently, the membranes were incubated with secondary antibody (1:2000, Abcam) for one hour at room temperature. The enhanced chemiluminescence detection system (ECL, Roche Molecular Biochemicals) was used to measure the signal. β-actin was used as an internal loading control.

**Enzyme-linked immunosorbent assay (ELISA).** The concentration of TNF-α, interleukin-23 (IL-23), and IL10 in cell culture supernatants was measured by an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, USA) following the manufacturer’s protocol.

**Statistical analyses.** SPSS 19.0 (SPSS, Chicago, IL, USA) was used to perform the statistical analysis. The measurements data were presented using mean ± standard deviation. All assays were performed in triplicate at least. Student’s t-test and ANOVA were used to compare the differences between two groups and between multiple groups, respectively. P<0.05 was considered statistically significant.

### Table 1. The primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
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<tr>
<td>miR-98-5p</td>
<td>Forward</td>
<td>5’-ACACTCCCUAUAACAUUAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GGGAAAGUAGUAGGCTCCGTA-3’</td>
</tr>
<tr>
<td>Trib1</td>
<td>Forward</td>
<td>5’-GCTTCGGCACGACATATAAATAT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAGCGGGTCAGTGGA-3’</td>
</tr>
<tr>
<td>Mcp1</td>
<td>Forward</td>
<td>5’-GGGGAAAGUAGUGAGGCCTCAGA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CGTTTTCGGCTCCGCACATAGGA-3’</td>
</tr>
<tr>
<td>Nos2</td>
<td>Forward</td>
<td>5’-ACATTTCGCTACGCCGATT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CATTTCCAAATGTTCTCTGG-3’</td>
</tr>
<tr>
<td>Tnfa</td>
<td>Forward</td>
<td>5’-CCCCACCGGTCAAGCGATT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GTCTAAGTACTTGGGCAGATTGACC-3’</td>
</tr>
<tr>
<td>Mrc1</td>
<td>Forward</td>
<td>5’-CAGGCCGTTGGAAGTGGAA-3’</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAGCTGATTGGACCTTCTGGAAAC-3’</td>
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<tr>
<td>Ym1</td>
<td>Forward</td>
<td>5’-GGATGCTACATCGGAGAAA-3’</td>
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<td>5’-AGAAGGGTCATCAGGATATA-3’</td>
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<tr>
<td>Arg1</td>
<td>Forward</td>
<td>5’-AACAAGACGGGCTCCCTCCAC-3’</td>
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<td>5’-GGCCTATGGTTACCTCCCCG-3’</td>
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<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5’-CGGAGTCAACGGGATTGGTGTAGAC-3’</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’-AGCCTTCTCCATGGGGTGTAAGAC-3’</td>
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RESULTS

MiR-98-5p expression was increased in IBD mouse model

Firstly, we constructed the IBD mouse model and measured the severity of intestinal inflammation by assessing the DAI score. As shown in Fig. 1A, DAI value was higher in the IBD group than in the Sham group, which indicated the IBD mouse model was successfully established. As expected, the weight of mice in the IBD group was lower than that in the Sham group (Fig. 1B). qPCR results revealed that miR-98-5p was highly expressed in the colonic tissues of IBD group compared to the Sham group (Fig. 1C). Moreover, the expression of M1 macrophage marker genes (Mcp1, Nos2, and Tnfa) was increased in IBD group, while the expression of M2 macrophage marker genes (Mrc1, Ym1, and Arg1) was...
decreased (Fig. 1D). These findings suggested that the aberrantly up-regulated miR-98-5p and M1 macrophage polarization might be associated with the development of IBD.

**MiR-98-5p regulated Trib1 expression by targeting its 3’UTR**

Targetsan (http://www.targetscan.org/vert_72/) predicted miR-98-5p and Trib had binding sites (Fig. 2A). To investigate the relationship between the two molecules, luciferase reporter assay was performed. The results in Fig. 2B showed that co-transfection of miR-98-5p mimic and Trib1-WT diminished the luciferase intensity. Then, we transfected miR-98-5p mimic and inhibitor into macrophage to up-regulate and down-regulate miR-98-5p expression, respectively (Fig. 2C). It was shown that overexpression of miR-98-5p reduced the mRNA and protein level of Trib1 in macrophages, but miR-98-5p knockout brought the opposite effect (Fig. 2D and E). These data indicated that miR-98-5p could negatively regulate Trib1 expression via targeting its 3’UTR.

**Knockdown of miR-98-5p improved IBD symptoms in mice**

Herein, we verified miR-98-5p effects on IBD in vivo. IBD mice were treated with miR-98-5p antagomir. As shown in Fig. 5A, miR-98-5p expression was increased in the colonic tissues of IBD+NC antagomir group but decreased in IBD+miR-98-5p antagomir group. Furthermore, the DAI value was increased in IBD+NC antagomir group (Fig. 5B) and the mice in this group lost weight (Fig. 5C). As expected, miR-98-5p antagomir changed this trend (Fig. 5B and C). In addition, in accordance with the cell experiment, knockout of miR-98-5p inhibited the expression of M1 macrophage marker genes (Fig. 4D). However, shTrib1 diminished the effects (Fig. 4C and D). The above data suggested that the function of miR-98-5p on macrophage polarization and inflammation was related to Trib1.

Knockdown of miR-98-5p improved IBD symptoms in mice

Herein, we verified miR-98-5p effects on IBD in vivo. IBD mice were treated with miR-98-5p antagonist. As shown in Fig. 5A, miR-98-5p expression was increased in the colonic tissues of IBD+NC antagonist group but decreased in IBD+miR-98-5p antagonist group. Furthermore, the DAI value was increased in IBD+NC antagonist group (Fig. 5B) and the mice in this group lost weight (Fig. 5C). As expected, miR-98-5p antagonist changed this trend (Fig. 5B and C). In addition, in accordance with the cell experiment, knockout of miR-98-5p inhibited the expression of M1 macrophage marker genes.
The role of miR-98-5p in IBD

Collectively, these results revealed that miR-98-5p could participate in regulating the development of IBD.

**DISCUSSION**

IBD is an unexplained colorectal inflammatory disease characterized by recurrent intestinal ulcers. Patients with IBD often suffer from diarrhea, mucous bloody stool, and abdominal pain (de Lange & Barrett, 2015). In Europe and the United States, IBD incidence has risen to more than 0.5% of the total population (Molodecky et al., 2012). In China, the number of IBD cases tripled in the last 10 years (Ng et al., 2018). It was pointed out that the risk of cancer increases with the course of IBD (Axelrad et al., 2016). Therefore, it is urgent to find new strategies for the treatment of IBD.
One of the pathogenesis of IBD is the inflammation caused by the altered immune system (Wallace et al., 2014, Rogler, 2017), thus, regulating this process has profound significance for IBD therapy. As immune cells, macrophages play an important role in many aspects of inflammation (Hamidzadeh et al., 2017, Foss et al., 2018). It is proved that the increase of M1 macrophages in the colon is related to the severity and progress of IBD (Leonardi et al., 2018). In the present study, we similarly found that the expression of M1 macrophage marker genes was significantly increased in the colonic tissues of IBD mice, but the expression of M2 macrophage marker genes was reduced. These indicated that colonic M1 macrophage population expanded while the M2 macrophage population shrank during IBD. Thus, exploring the molecules involved in the process of macrophage polarization is expected to provide new ideas for IBD therapy.

It was confirmed that miRNAs are key regulators of macrophage polarization, and miRNAs-mediated macrophage polarization is associated with inflammation-related diseases (Essandoh et al., 2016). For example, over-expression of miR-127 could promote M1 macrophage polarization but diminish M2 macrophage polarization through activating JNK pathway, and then aggravated lung inflammation and injury (Ying et al., 2015). In IBD, 1,25(OH)2D3 could induce a transition from M1 macrophage polarization to M2 polarization by down-regulating miR-125b expression and ameliorated the symptoms of the disease (Zhu et al., 2019).

As a member of let-7 family, miR-98-5p is considered a regulation factor in a variety of tumors. Jiang and others (Jiang et al., 2017) suggested that miR-98-5p overexpression inhibited hepatocellular carcinoma cell growth via targeting IGFB2BP1. Wang and others (Wang et al., 2018) demonstrated that cisplatin resistance in epithelial ovarian cancer could be promoted by miR-98-5p regulating Dicer1. Moreover, miR-98-5p is also involved in other diseases, such as bronchial asthma (Du et al., 2019), Alzheimer’s disease (Li et al., 2016), and cerebral ischemia/reperfusion injury (Sun et al., 2018). In IBD, miR microarray analysis revealed that miR-98-5p was deregulated (Coskun et al., 2013), but miR-98-5p function in IBD remains unclear. In the current study, miR-98-5p was found to be strongly expressed in the colonic tissues of IBD mice. Similarly, LPS treatment increased miR-98-5p expression in macrophages. Moreover, miR-98-5p knockdown could attenuate LPS promoting effect on M1 macrophage polarization and inflammation, as well as the inhibition effect of LPS on M2 macrophage polarization. These finding suggested that miR-98-5p had a critical role in the polarization of macrophages. To clarify this effect, we further studied the mechanism.

Based on the bioinformatics analysis, we found that miR-98-5p and Trib1 had a targeted regulation relationship. The previous study revealed that Trib1 participates in regulating macrophage polarization. For instance, Trib1 induced differentiation of macrophages into M2 in prostate cancer immune microenvironment (Liu et al., 2019); in mice lacking Trib1, the production of proinflammatory cytokine was increased and M2-like macrophages differentiation was reduced (Akira et al., 2013). In this study, we discovered that Trib1 was negatively regulated by miR-98-5p. Knockdown of miR-98-5p could promote M2 macrophage polarization and inhibited M1 macrophage polarization and inflammation response. miR-98-5p function could be diminished by silencing Trib1. This regulatory mechanism was also confirmed in in vivo experiments.

In conclusion, this is the first time to reveal the mechanism of miR-98-5p action in macrophage polarization and IBD, and that the function was related to Trib1. This provides a more theoretical basis for miR-98-5p as a novel therapeutic target for IBD.

Acknowledgements
Not applicable.

Competing interests
The authors state that there are no conflicts of interest to disclose.

Ethics approval
All experimental procedures were approved by the Laboratory Animal Welfare and Ethics Committee of Shanghai University of traditional Chinese medicine.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
WZ conceived and designed the experiments, YHP and QYY analyzed and interpreted the results of the experiments, WY, QYY and YNP performed the experiments.

Patient consent for publication
Not applicable

REFERENCES
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