MIR-133A REGULATES THE DEVELOPMENT OF ULCERATIVE COLITIS THROUGH THE IL-6/STAT3 SIGNALING AXIS

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ABSTRACT

To find new factors responsible for the development of ulcerative colitis (UC) in model rats to facilitate the development of new therapeutic strategies. Forty rats were separated into four groups, the control (Control), model (Model), negative control (NC) and antisense-miR-133α treated (Treat) groups. UC was induced in rats, except those in the Normal group. Their mental statuses, body weights and bloody stools were scored. The colon tissues were further stained to determine and compare several pathological scores. The myeloperoxidase (MPO) activities were determined for the comparison of neutrophil infiltrations. We also probed the protein levels of STAT3 and TGF-β1 in the colon tissues using immunohistochemistry, and quantitated the transcripts of miR-133α, STAT3, Foxp3 and RoRyt using qRT-PCR. Finally, the Th17 and Treg cells were counted with flow cytometry to analyze the balance between these two important components during immune response. miR-133α was aberrantly upregulated in the rats with UC. Intracolonic injection of antisense-miR-133α inhibitors could suppress the immune responses in the colons and reduce disease severity. The upregulated expression of miR-133α was associated with enhanced STAT3 signaling and immune response in the colon tissues as suggested by the dysregulation of several key factors in this pathway and shifted balance between the Th17 and Treg cells. miR-133α seems to regulate the development of UC through the STAT3 pathway, which can further affect the differentiation of Th17 cells. This microRNA is potentially a new drug target for the treatment of UC.

Keywords: *Ulcerative colitis, TNBS, MiR-133α, MPO, STAT3.*

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Introduction

Ulcerative colitis (UC) is a common digestive system disease that leads to chronic inflammation and ulcers in digestive tracts.1 Although the cause of the disease is unknown, hypotheses have linked it to genetic, environmental, intestinal flora and several other factors. These factors may trigger abnormal immune responses, especially the action of cytokines, and ultimately contribute to the pathogenesis[1-2]. UC can be treated with a number of medications. The associated risks, however, outweigh their benefits, and thus they are not recommended for long-term treatment[3]. In the past few years, the incidence of UC in China has been increasing, prompting new therapeutic strategies[4].

Signal transducer and activator of transcription 3 (STAT3) is a member of the Stat protein family[5]. It is generally considered a growth-promoting, antiapoptotic factor, and can be activated by the entire family of interleukin-6 (IL-6)–type cytokines, growth factors and many other ligands. Constitutive activation of STAT3 by IL-6 is associated with inflammatory bowel diseases including UC[6] Interruption of the IL-6 signaling in animal models led to diminished STAT3 activation and T cell apoptosis, which were likely responsible for the reduced disease severity[7-9]. The role of the elevated IL-6/STAT3 signaling in the development of UC is further supported by the fact that IL-6 is important in regulating the balance between Th17 cells and regulatory T cells (Treg). Th17 is a key player in the pathogenesis of autoimmune diseases, while Treg functions to restrain excessive effector T-cell responses[10]. Collectively, these studies highlight the key role of the IL-6/STAT3 axis in regulating gut immune homeostasis.

MicroRNAs (miRNAs) are short, non-coding RNA molecules, which mainly act as transcription repressors. Several studies demonstrated that differential miRNA expression was associated with UC[11-15].

The miR-133 family has two members, namely miR-133α and miR-133β. They have been best characterized as important regulators of skeletal muscle development[16]. Recent studies have found that neurotensin, an important mediator in colonic inflammation, could upregulate miR-133α in colitis animal models. Increased miR-133α transcription has also been detected in mucosal biopsies of UC patients, further supporting the clinical relevance of miR-133α in the development and progression of UC[17].

Our studies found that miR-133α was upregulated in UC animals and blocking this microRNA using antisense inhibitors reduced the disease severity in all the aspects we have analyzed. Notably, overexpression of miR-133α was associated with aberrant expressions of several key factors in the STAT3 pathway as well as the changed balance between the Th17 and Treg cells. Taken together, the results strongly suggest that the regulatory function of miR-133α is critical for the proper action of the STAT3 pathway, ultimately leading to gut immune homeostasis. Reducing the level of miR-133α in UC patients may be a new therapeutic strategy.

Material and methods

Animals

Forty specific-pathogen-free Sprague-Dawley rats (180-200 g, 6-8 weeks) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (SCXK (Lu) 20140007). The ratio of male to female was 1:1. The rats were raised at 22±1 °C with 60±10% humidity and 12-hour light/dark cycles under the condition of free access to water and food. All animal experiments were performed in strict accordance with the "Guidelines for the Nursing and Use of Laboratory Animals of the National Institutes of Health." The research protocol was approved by the Animal Care Committee at Qianfoshan Hospital.

Establishment of UC model

Briefly, a 3% pentobarbital (40 mg/kg) solution was intraperitoneally injected into a rat. After the animal was anesthetized, a silicone tube (2 mm of diameter) lubricated with paraffin oil was slowly inserted into the intestine from the rat's anus about 8 cm and 0. 25 ml TNBS mixed solution (100 mg/kg) was injected into the rat colon. The rat was then inverted and its anus was closed. After 2-3 min, the animal was returned to the cage for recovery from anesthesia. The rats in the Control group were injected with 0.25 ml saline.

During the induction of colitis, the animals were monitored daily for clinical signs.

After 15 days of colitis induction with or without treatment, peripheral blood was collected using the eyelid sampling method and placed in an anticoagulant tube for flow cytometry. The animal was anesthetized and sacrificed by cervical dislocation. After macroscopic scoring, colon segments were harvested. The samples for H&E and immunohistochemical staining were fixed in 10% formalin for 24 hours at room temperature and embedded in paraffin. The other samples for qRT-PCR analysis were frozen in liquid nitrogen and stored at -80°C.

Animal grouping and Administration

The UC model was established and randomly divided into the Model, NC and Treat groups (n=10) with 1:1 sex ratio. The animals in the Treat group were treated antisense-miR-133α oligoes. The inhibitors (20 μg for each rat) were mixed with 2 μL siPORT NeoFX transfection agent (Invitrogen, USA), incubated in 100 μL Opti-MEM (Invitrogen, USA), and injected into the rat colons. In contrast, random oligoes prepared in the same way were injected into the rats in the NC group. Finally, only siPORT NeoFX and Opti-MEM were injected into the Control and Model animals.

Macroscopical and colon morphological damage index (CMDI) scoring

Macro injury scores were determined according to the previous study[18]. Two pathologists performed double-blind scoring and combined pathological observations by naked eyes and histological changes under light microscopy.

The animals were scored as the following:

- No damage;
- Focal hyperemia without ulceration;
- No congestion or thickening of the bowel wall;
- There is an inflammatory ulcer in one place, but no more than two ulcers and inflammation sites, no more than two major ulcers and inflammation sites, or an ulcer site extending along the length of the colon for more than 1 cm;
- Colon ulcers are more than 2 cm in length.

The CMDI scores were determined as previously described with slight modifications[19].

Briefly, the scoring rule is as follows:

- No damage;
- Mild congestion but no erosion and ulceration;
- Congestion and thickening of the intestinal wall but no ulceration;

• 1 small ulcer of 0~1 cm in diameter;

• Larger ulcer sites of 1~2 cm in diameter, but no adhesion between the bowel and peripheral organs;

• Ulcer sites of 1 ~ 2 cm, thickened intestine with severe adhesion to the surrounding organs.

Microscopic scoring

The colon samples were embedded in paraffin, sliced into 5 μm sections and stained by the H&E method. Two pathologists performed double-blind scoring. According to the level of inflammatory infiltration, they graded the lesions from 0 to 6, determined the levels of ulceration, and confirmed the presence or absence of necrosis[20].

MPO activity determination

The colon tissue samples were mechanically homogenized in a 50 mM phosphate buffer containing 0.5% cetyltrimethylammonium bromide (Sigma-Aldrich, St Louis, MO, USA). The homogenized samples were centrifuged at 4°C (17000 x g, 20 min). The supernatant aliquots (20 µl) were transferred into a 96-well plate and 280 µl of 0.02% dioninetin (50 mM, pH 6.0, containing 0.167 mg/ml Anisidine and 0.0005% H2O2) was added. After 20 min incubation at room temperature, the absorbance was measured at 460 nm. Protein concentrations were determined by Bikinoycin (Pierce Biotechnology, Rockford, IL, USA). The MPO activity was calculated by the following formula: MPO unit/g wet sheet = (OD value of the test tube - OD value of the control tube)/11.3/sampling amount (g).

Quantitation of Th17 and Treg cell frequency using flow cytometry

Th17 cells were stimulated with 50 ng/ml PMA (BioVision, USA), 1 µg/ml ionomycin (Enzo Life Sciences, USA) and 500 ng/ml monensin (eBscience, USA) for 4 h, and stained with FITC anti-mouse CD4 and PE anti-mouse IL-17 antibodies (Bioss, China) at 4°C in the dark for 20 min. The Treg cells were directly stained with FITC anti-mouse CD4 and APC anti-mouse CD25 antibodies (eBioscince, USA) at 4°C in the dark for 20 min. Both types of cells were then washed twice, fixed and permeablized, and further stained with PE anti-mouse Foxp3 (eBioscince, USA). A FACS Canto II flow cytometer (BD, USA) was used to quantitate the labeled cells.

Immunohistochemistry staining and quantitation of Stat3 and TGF-β1

After routine section of the tumor tissues, the slices were roasted, dewaxed using xylene, and hydrated by gradient ethanol solutions. They were further treated with 3% H2O2 in methanol for 20 min, heated in citrate buffer (pH 6.0) for 10 min, and blocked with 5% BSA (Solarbio, Beijing, China) for 20 min. Rabbit anti-mouse STAT3 (1:1000, ABIN2855865, antibodies-online, Aachen, Germany) and TGF-β1 (1:200, ABIN2914257, Abbexa, Cambridge, England) polyclonal antibodies were added for overnight incubation at 4°C. Then, horseradish peroxidase-labeled goat anti-rabbit Ig G (1:1000, #7074, Cell Signaling Technology, USA) was added as the secondary antibody. DAB (Solarbio, Beijing, China) was added for development, followed by hematoxylin counterstaining, dehydration, and sealing. The cells were visualized through a 400x light microscope (Olympus, Japan) and counted using the AperioImagescope 11.1 software. The results were presented as percentages of positive cells (%).

qRT-PCR quantitation of miR-133α, STAT3, Foxp3 and RoRyt

Tissue total RNA was extracted using the Trizol kit (Takara, Japan) (OD260/OD280 of 1.8 - 2.0). RNA was reversely transcribed into cDNA (Applied Biosystems, Waltham, MA, USA). Real-time quantitative PCR was performed using Mastercycler nexus X2 (30 s at 95 °C, 30 s at 95 °C, and 20 s at 60 °C for 45 cycles) (Eppendorf, Hamburg, Germany). Band brightness was quantitated with the 2-ΔΔCt method, and relative expression levels were determined using GAPDH mRNA as an internal reference. The sequences of the primers (Shanghai Shenggong Bioengineering Service Co., Ltd.) are as follows: miR-133α, Forward: 5'- ACACTCCAGCTGGGTTTGGTCCTTCAAC -3' and Reverse: 5'-TGGTGTCGTGGAGTCG -3'. STAT3, Forward: 5'- CAATACCATTGACCTGCCGAT -3', Reverse: 5'- GAGCGACTCAAACTGCCCT -3'. Foxp3, Forward: 5'- TTCACCTATGCCACCCTCAT -3', Reverse: 5'-CCCTTCTCACTCTCCACTCG-3'. RoRyt, Forward: 5'- CCTCCTGCCACCTTGAGTAT-3', Reverse: 5'- TCTGAGCCCTGTTCTGGTTC-3'. GAPDH, Forward: 5'- TGACCTCAACTACATGGTCTACA -3', Reverse: 5'- CTTCCCATTCTCGGCCTTG -3'.

Statistical methods

The SPSS19.0 software was used to analyze the data. The results were expressed as mean±SD. The t-test method was used to analyze the difference between two groups. One-way analysis of variance (ANOVA) was used to test differences between multiple groups, followed by the least significant difference (LSD) test. $P<0.05$ was considered statistically significant.

Results

Rats treated with antisense-miR-133α showed reduced disease severity

Overexpression of miR-133α has recently been found in colitis animal models and mucosal biopsies of UC patients, suggesting that this microRNA is potentially important in the development and progression of the disease.17 In order to further understand the cause of UC and find new therapeutic approaches, we developed UC model in rats and used antisense oligos to suppress miR-133α in rat colons to see if the treatment could suppress the disease symptoms.

After 12 days of treatment with antisense-miR-133α, the animals in the Treatment group manifested improved conditions, such as reduction of bloody stools and increase of body weights. The difference was significant between the Treat and Model groups (P<0.05). Compared with the Control group, the Model and the NC groups showed 24.9% and 22.4% overall reduction in body weight respectively (P<0.05), while the Treatment group showed 13.7% increase (P<0.05) (Figure 1).

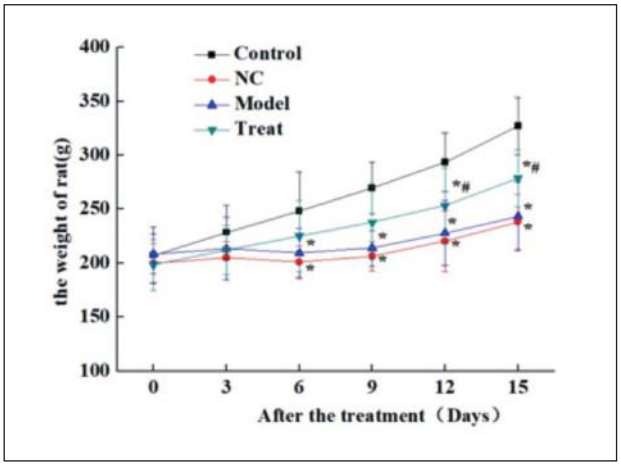

Figure 1: Point plot of the changes of rat body weights. The body weights of the rats in all 4 groups were measured every 3 days over a 15-day period. The data were represented as the mean ± SD. Compared with Control group, *P<0.05; compared with Model group, #P<0.05.

Treated rats showed improved pathological conditions

The rat colon tissues were further scored for comparison of the pathological conditions of all the animal groups. The intestines of the Model rats were enlarged with thickened walls and adhered to the intra-abdominal tissues. Intestinal mucosae appeared to have congestive edema, erosion and ulceration. Under the microscope, we saw inflammation in the entire layer of the colonic walls, infiltration of a large number of inflammatory cells (neutrophils, lymphocytes, plasma cells, and eosinophils), destruction of the intestinal mucosal glandular structure, shedding of intestinal epithelium, and formation of erosion and ulcerative sites (Figure 2A). After antisense-miR-133α administration, the intestinal mucosae of those rats appeared to be normal, the membrane glands were intact, and only a small number of infiltrating inflammatory cells were observed. As shown in Figure 2B, the DAI, Macro rating, CMDI, and histologic scores of the NC group and the Model group were significantly higher than those of the Control group (P<0.05). In contrast, the scores of DAI, Macro, CMDI, and histologic of the treated rats were significantly lower than those of the Model group (P<0.05).

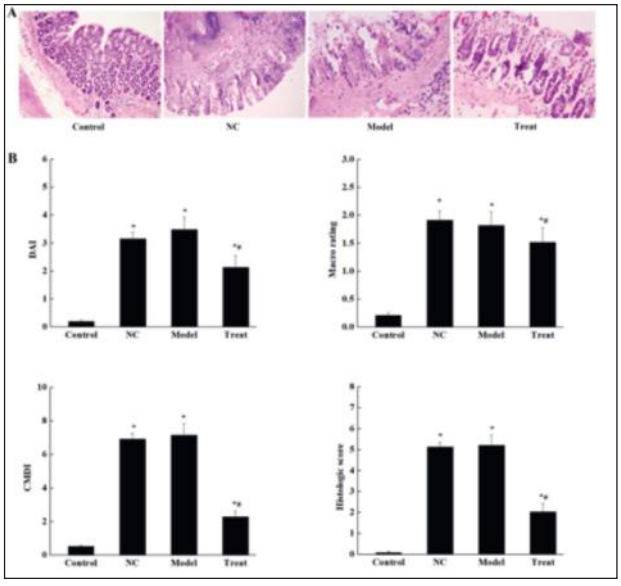

Figure 2: Comparison of the pathological scores. A. HE stained rat colon tissues from all 4 groups. B. Bar plots of the DAI, Macro, CMDI, and Histologic scores of all groups. The data were represented as the mean ± SD. Compared with Control group, *P<0.05; compared with Model group, #P<0.05.

Treatment reduced inflammation in rat colons

Since the pathogenesis of UC has been linked to abnormal immune responses, we tried to understand if suppressing miR-133α could change the inflammatory conditions in the colons of the UC rats. We assessed the infiltration of neutrophils in the colon tissues by comparing the MPO activities. As shown in Figure 3, the MPO activities in the NC and Model groups were higher than that of the Control group (P<0.05), suggesting higher inflammatory infiltration of neutrophils. In contrast, antisense-miR-133α treatment could reduce infiltration to the near normal levels.

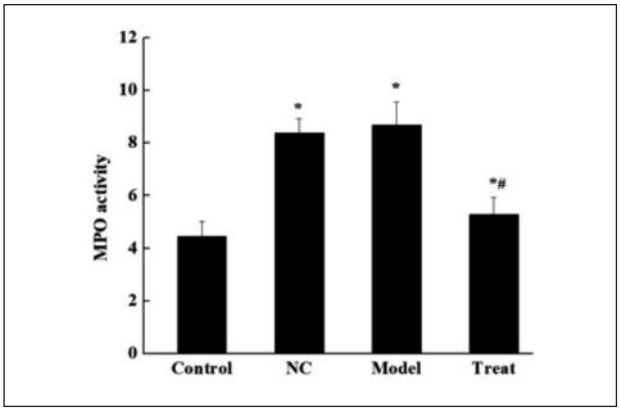

Figure 3: Bar plot of the neutrophil infiltration levels in the colon tissues of all 4 groups. The data were represented as the mean ± SD. Compared with Control group, *P<0.05; compared with Model group, #P<0.05.

Treatment restored the Th17-Treg balance in the peripheral blood

Th17 and Treg cells are two lymphocyte subsets with opposing actions critical for immune homeostasis[10]. The changed balance between these cells has been found to be associated with UC. We thus labeled Th17 and Treg cells and analyzed the percentages of these cells in rat serum using flow cytometry. The results showed that the animals in the NC and Model groups had a larger population of $CD4^+IL\text{-}17^+Th17$ cells but less $CD4^+Foxp3^+$Treg cells than those in the Control group (P<0.05). Again, antisense-miR-133α treatment could partially suppress the colitis symptoms as the Th17 and Treg cell populations were brought back to the near normal levels (Figure 4).

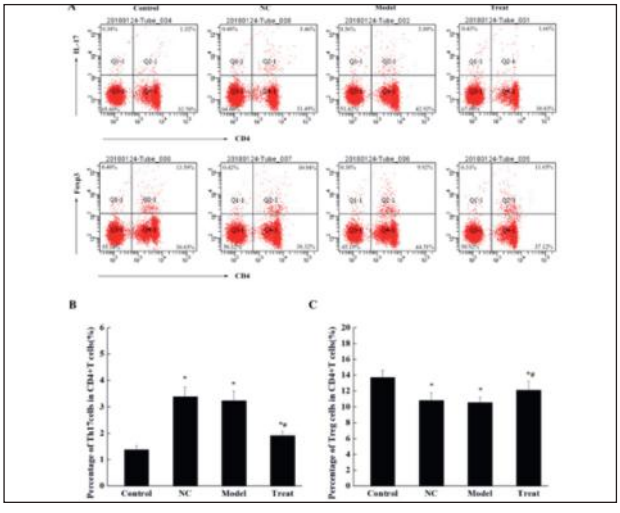

Figure 4: Flow cytometric analysis of Th17 and Treg cells in the peripheral blood. A. Representative flow cytometric results. The top and bottom panels highlight the populations of the $CD4^+IL\text{-}17^+Th17$ and $CD4^+Foxp3^+$Treg cells respectively. B. Bar plots comparing the percentages of the Th17 and Treg cells. The asterisks and hashtags indicate statistical significance (P<0.05) using Control and Model as a reference respectively.

miR-133α affects the STAT3 pathway

Since constitutive activation of STAT3 by IL-6 has been linked to UC, we wondered whether the expression level of miR-133α could affect the STAT3 signaling pathway.

We performed immunochemistry staining against STAT3 and TGF-β1 and found that the proteins were expressed at relatively higher levels in the colon tissues harvested from the NC and Model groups (Figure 5A).

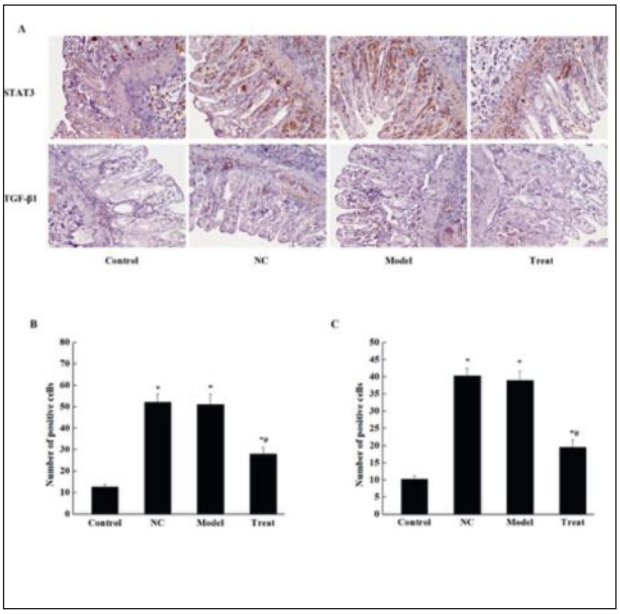

Figure 5: Quantitation of the STAT3 and TGF-β1 protein levels in the colon tissues. A. immunochemistry staining of STAT3 and TGF-β1. The brown spots are stained proteins. B and C. Bar plot of the numbers of STAT3 and TGF-β1 positive cells. The data were represented as the mean ± SD. Compared with Control group, *P<0.05; compared with Model group, #P<0.05.

Furthermore, the numbers of positively stained cells in the tissues from those two groups were apparently higher. In contrast, antisense-miR-133α treatment brought the protein levels back to the near normal levels (Figure 5). Finally, qRT-PCR assays were performed to determine the transcript levels of several genes in the STAT3 pathway.

The upregulated level of miR-133α in UC animals (Model and NC) was associated with overexpression of STAT3 and RoRyt. Meanwhile, the expression of another factor Foxp3 was reduced.

When miR-133α was reduced to the nearly normal level through antisense-miR-133α treatment, the aberrant expression of STAT3, Fox3p and RoRyt were also reversed to the nearly normal levels (Figure 6). These strongly indicated a regulatory impact of miR-133α on the STAT3 pathway.

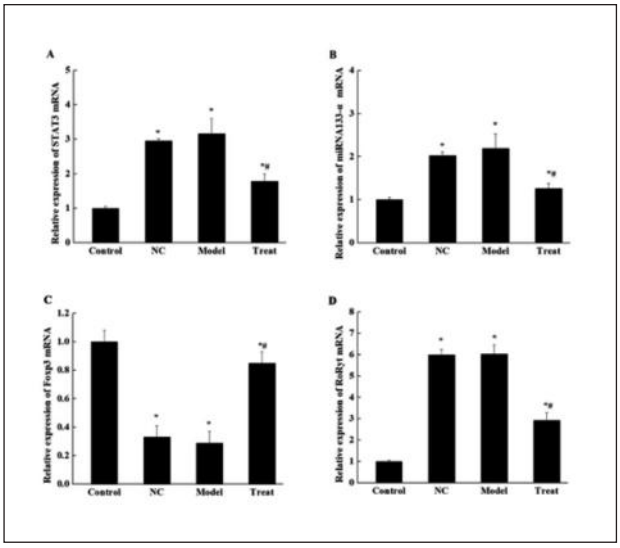

Figure 6: Bar plots of relative levels of relevant genes STAT3 (A), miR-133α (B), Foxp3 (C), RoRyt (D) in the colon tissues. The data were represented as the mean ± SD. Compared with Control group, $^{*}P<0.05$; compared with Model group, $^{\#}P<0.05$.

Discussion

Accumulating evidence supports that miRNAs are key regulators of the immune responses that affect maturation, proliferation, differentiation, and activation of immune system cells, as well as production of antibodies and release of inflammatory mediators[21]. Recently, miR-133α has been found a potentially important factor involved in the development and progression of UC.17 The mechanism of its action and the feasibility of targeting it for therapy, however, remained to be further addressed. In this report, we induced UC in rats and tested the impact of miR-133α on the disease. We think that upregulated expression of miR-133α may play an important role in triggering abnormal immune responses in UC. This is supported by our finding that the UC colon tissues with higher levels of miR-133α showed a stimulated STAT3 signaling cascade which presumably led to a series of abnormal immune reactions in rat colons. This was indeed manifested by the dysregulated Th17 and Treg cell populations. In consistent with this, we also found overexpression of the STAT3 and TGF-β1 proteins, which play important roles in controlling the immune system, and dysregulated mRNA levels of RoRyt and Fox3p, which are master regulators in the development and function of Th17 and Treg cells respectively. The importance of miR-133α was further reinforced by the findings that suppressing the miR-133α level using antisense oligos could reverse all the aforementioned abnormal immune responses back to the nearly normal levels. The treated rats indeed showed improved conditions including good mental statuses, normal body weights and reduced bloody stools.

Although UC can be caused by a variety of factors and there is still no consensus on the molecular basis of the development and progression of the disease, our study reinforced the hypothesis that the dysregulated immune responses in the colon are critical for the pathogenesis. The Th17 and Treg cells are two important components in immune response with opposing effects. They are frequently found at barrier surfaces, particularly within the intestinal mucosa where they function to protect the host from pathogenic microorganisms and restrain excessive effector T-cell responses, respectively[22]. We found that the induction and treatment of UC in rats were strongly associated with the changes of the Th17 and Treg populations, strongly suggesting the importance of the immune system in controlling the disease.

Today, UC can be treated by many medications. Many of them are, however, immunosuppressive reagents which are associated with a number of risk factors, including but not limited to increased risk of cancers.3 Meanwhile, a significant proportion of patients failed to respond to the existing drugs and thus they have to consider surgery to improve the quality of life[23]. The strong need of new treatments in UC has been evident. Our results reported here strongly supported miR-133α as a new drug target. The discovery will also stimulate efforts in searching for other important factor involved in the maturation and regulatory actions of miR-133α, providing new insights into the functional networks responsible for colon immune homeostasis and ultimately opening new opportunities for the development of new therapeutic strategies. miR-133α is a potential driving factor in the development and progression of UC. After treating UC rats with the miR-133α inhibitors, the animals manifested improved mental statuses and increased body weights. Further analysis of the colon tissues found improvements in several pathological scores (DAI, Macro, CDMI, and histologic), reduced expression of proteins (STAT3 and TGF-β1) linked to inflammation, recovery of several gene transcripts in the STAT3 pathway to the near normal levels, reduced neutrophil infiltrations. miR-133α probably regulates colonic immune homeostasis through the STAT3 pathway which can ultimately influence the Th17-Treg balance. We believe that specific inhibitors targeting miR-133α or other factors required for the maturation and function of this non-coding RNA can be a new strategy for the treatment of UC.

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