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ORIGINAL ARTICLE

miR-223-3p Promotes Ulcerative Colitis Progression in Rhubarb Decoction-Induced Rats with the Spleen-Kidney-Yang Deficiency Model by Regulating the TGF-β/Smad3 Pathway

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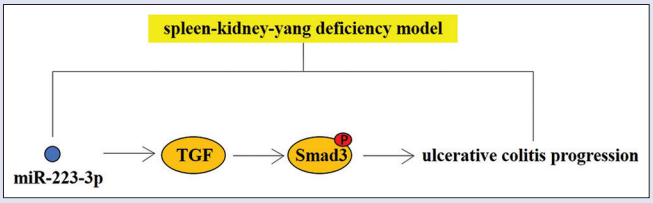
ABSTRACT

Background: Ulcerative colitis (UC) is a prevalent case which needs more detailed exploration. We attempted to illustrate how miR-223-3p acted in the development of UC. **Materials and Methods:** The level of miR-223-3p in serum from UC patients was detected using quantitative reverse-transcriptase polymerase chain reaction. The *in vivo* experimental spleen-kidney-yang deficiency mice model was established to get a better understanding of the action of miR-223-3p in UC development. Hematoxylin and eosin staining was applied to observe the intestinal epithelial barrier function. Western blotting was applied to measure the expression of ZO-1, Occludin, TGF-β, and Smad3. **Results:** Enrichment of miR-223-3p was observed in serum from UC patients. miR-223-3p antagomir could lower the shortening of colon length, alleviating the intestinal epithelial barrier function damage caused by UC. Under the UC conditions, TNF-α, IL-6, and IL-1β were up-regulated, whereas with the miR-223-3p antagomir, the up-regulation was less pronounced; ZO-1 and Occludin protein levels were decreased, whereas the decrease was less obvious in the miR-223-3p antagomir group. Additionally, miR-223-3p regulated UC development through the TGF-β/Smad3 pathway. **Conclusion:** miR-223-3p promoted UC development with spleen-kidney-yang deficiency through the TGF-β/Smad3 pathway.

Key words: Intestinal, miR-223-3p, spleen-kidney-yang deficiency, TGF-β/Smad3 pathway, ulcerative colitis

SUMMARY

- Collectively, the function of endophytic flora in the health of the body is a new hotspot in the current medical field. The research on the efficacy and mechanism of traditional Chinese medicine based on the regulation of intestinal flora is a new direction of the basic research of traditional Chinese medicine. Of note, it is the first time to elucidating the mechanism of tonifying the spleen and kidney decoction in treating UC with spleen-kidney-yang deficiency syndrome by regulating the flora and TGF-β/Smad3 pathway. Here, the influence of drugs on intestinal flora and the balance axis of Th17/Treg in UC were combined to illustrate the therapeutic effect of traditional Chinese medicine.



Abbreviations used: Ulcerative colitis (UC); Transforming growth factor-β (TGF-β); SFDA (State Food and Drug Administration); Hematoxylin and eosin (HE); Analysis of variance (ANOVA); Trinitrobenzene sulfonic acid (TNBS).

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INTRODUCTION

Ulcerative colitis (UC) is a chronic condition associated with the disorder of gut microbial communities.[1-3] The etiology of UC is not clear, and in recent years, the incidence and prevalence of UC are increasing worldwide, which has a negative impact on all aspects of life of people of all ages.[4,5] In spite of great progress in the therapy of UC, the clinical symptoms frequently re-occur, along with persistent side effects.[6] In this regard, novel effective therapeutic methods, such as biomarkers for UC, need to be identified to improve the treatment and prognosis of patients with UC.

UC belongs to the category of "chronic diarrhea" and "dysentery" in traditional Chinese medicine. The disease is mainly located in the spleen, complicated and prolonged. The spleen-yang deficiency can easily affect the kidney, so the spleen-kidney-yang deficiency is the common symptom of UC. Based on the theory of spleen-yang deficiency in UC, spleen deficiency is the origin of the onset of UC, and the spleen-kidney-yang deficiency can be caused by prolonged diarrhea. However, the specific mechanism of action of the tonifying spleen and tonifying kidney formula in the treatment of UC is still unclear.

One of the major classes of molecules that regulate UC development is miRNAs.[7-10] They are endogenous non-coding small RNAs that are about 22 nucleotides in length, typically functioning by degrading mRNAs.[11] To facilitate more comprehensive investigations of miRNAs' function in UC development, here, we focused on

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investigation about what impacts miR-223-3p had on the progression of UC.

Transforming growth factor-β (TGF-β) is a kind of cytokine with multiple physiological functions.[12] Smads protein is a signal-transmitting molecule that can be activated by the complex generated after TGF-β binding to its receptor in the cell.[13] The TGF-β/Smad3 signal pathway is closely related to immuno-suppression and inflammatory response.[14] Th17 is the immune-promoting cell, whereas Treg is the immune-suppressing cell. Abnormalities of the immune system, together with Th17/Treg immune balance, are recognized to be essential in the progression of UC.[15,16] The activation of Treg cells can be combined with a high concentration of TGF-β antibody specificity, blocking the immuno-suppressive effect of Treg cells. At the same time, TGF-β secreted by Treg cells can bind to Smad3 cell membrane receptor so as to exert its immuno-suppressive effect. All in all, TGF-β plays a crucial role in Th17 cell differentiation and is a major factor for maintaining the balance axis of Th17/Treg.[17]

Based on the above understanding, we hypothesized that the formula of the invigorating spleen and nourishing kidney can adjust intestinal flora of UC rats. It may affect the metabolites of intestinal flora, thereby initiating the TGF-β/Smad3 pathway. TGF-β is promoted to act on intestinal epithelial mucosal cells, promoting differentiation into Treg cells and restoring the balance of the Th17/Treg axis, thus playing a therapeutic role.

MATERIALS AND METHODS

Serum collection

Serum samples were harvested from 27 UC patients in the Third Affiliated Hospital of Henan University of Traditional Chinese Medicine from 2019 to 2020. Twenty-seven healthy volunteers who underwent colonoscopy at the same time were chosen as controls. The CONSORT diagram of the study. A 5 mL peripheral blood sample was withdrawn from each subject and placed in a plain tube for serum separation. Serum was stored at -80°C for RNA extraction. The conduction of this present study gained approval from the Ethics Committee of the Third Affiliated Hospital of Henan University of Traditional Chinese Medicine (Approved Number: 2019HL-114-01). Informed consent was obtained from all UC patients and healthy volunteers. The research protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki.[18] The clinical study is reported according to the CONSORT statement.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using an SV Total RNA Isolation System (Promega, USA), and cDNA was generated using a Reserve Transcription Kit (QuantiTech, USA). U6 or GAPDH acted as an internal control. Data were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in the study are listed in Table 1.

UC animal model with spleen-kidney-yang deficiency establishment

A total of 40 SPF Wistar rats with a body weight of 180 ± 20 g were randomly divided into the control group and model group (20 rats in the blank group and 20 rats in the model group). Rats in the sham group (control group) were given distilled water 2 mL/d, and rats in model groups were given rhubarb decoction 13.5 $mg \cdot kg^{-1} \cdot d^{-1}$ in the morning and distilled water 2 $mL \cdot {}^{-1} \cdot d^{-1}$ in the afternoon for 14 days. On day 15, model rats were injected with hydrocortisone injection, 25 $mg \cdot kg^{-1} \cdot d^{-1}$, at the left and right buttocks alternately every other day, and the blank group was in gavage with a constant volume of distilled water for 10 days. On the 25th day, the rats were not restrained water but fasted for 24 h. On day 26, the rats were first anesthetized with 10% chloral hydrate and then were given an enema. Briefly, a polypropylene tube with a diameter of 2 mm and a length of 10 cm was inserted into the anus 8–10 cm reach the colon of rats, and then 100 $mg \cdot kg^{-1}$ trinitrobenzene sulphonic acid (TNBS) and 50% ethanol mixture volume were slowly injected into the colon. Rats in the blank group were treated with the same volume of distilled water.[19]

On the third day, 100 µl of miR-223-3p antagomir solution or miR-223-3p antagomir negative control obtained from Ribobio (USA) was injected into the colon of rats from the control and model groups, respectively. On day 10, all rats were sacrificed and the whole colon was taken for follow-up analysis.[20]

Table 1: The sequences of the qRT-PCR primers in this study

Primer	Sequences
Human	
miR-223-3p	Forward: 5′-CGCUAUCUUUCUAUUAACUGACCAUAA-3′
	Reverse: 5′-CGCUAUCUUUCUAUUAUGACUCCAUAA-3′
GAPDH	Forward: 5’-GCACCGTCAAGGCTGAGAAC-3’
	Reverse: 5’-GGATCTCGCTCCTGGAAGATG-3’
U6	Forward: 5’-CTCGCTTCGGCAGCACA-3’
	Reverse: 5’-AACGCTTCACGAATTTGCGT-3’
Mouse	
TNF-α	Forward: 5′-CCCCAAAGGGATGAGAAGTT-3′
	Reverse: 5′-CACTTGGTGGTTTGCTACGA-3′
IL-1β	Forward: 5′-GGATGAGGACATGAGCAACCT-3′
	Reverse: 5′-AGCTCATATGGGTCCGACAG-3′
IL-6	Forward: 5′-CCGGAGAGGAGACTTCACAG-3′
	Reverse: 5′-CAGAATTGCCATTGCACAAC-3′
TGF-β	Forward: 5′-GACCGCAACAACGCCATCTA-3′
	Reverse: 5′-GGCGTATCAGTGGGGGTCAG-3′
GAPDH	Forward: 5′-TGCACCACCAACTGCTTAG-3′
	Reverse: 5′-GGATGCAGGGATGATGTTT-3′

Western blotting

Colon tissues were lysed with RIPA Lysis Buffer (Beyotime, China) containing 1% PMSF (Beyotime, China). Samples were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transfer was performed to PVDF membranes. 5% non-fat milk was used to block the membranes, which were then treated with the primary antibodies at 4°C overnight: anti-ZO-1 (1:1000, Cat#: 5406, CST, USA), anti-Occludin (1:1000, Cat#: 91131, CST, USA), anti-TGF-β (1:1000, Cat#: 3711, CST, USA), anti-Smad3 (1:1000, Cat#: 9513, CST, USA), anti-p-Smad3 (1:1000, Cat#: 9520, CST, USA), and anti-GAPDH (1:1000, Cat#: 5174, CST, USA). After rinsing with TSBT three times, 10 min each time, and a 1 hour incubation of secondary antibodies at 37°C, the protein bands were visualized using the ECL reagent (Beyotime, China).

Colonic mucosal tissue pathology observation

The rat colon mucosa tissue at 1–2 cm was selected and placed in a 10% formalin liquid for fixation. Then decalcification, dehydration, transparent wax immersion, and embedding were carried out. Tissue sections with a thickness of about 5 um were then cut using a paraffin microtome. Then xylene and anhydrous ethanol were used to dewax into water. Hematoxylin and eosin (HE) staining was used to seal the seal, and the results were observed under a microscope (Olympus, Japan).

Statistical analysis

Experimental results were processed using SPSS19.0, and measurement data were expressed as mean ± standard deviation. Independent sample t-test was used for comparison between the two groups. One-way analysis of variance (ANOVA) was used for comparison of multiple groups. The LSD method was used for test of homogeneity of variances, and Dunnett's T3 test was the method analyzing heterogeneity of variances. The Chi-square test was the method analyzing the grade data, and repeated measurements were used to analyze the variance. We considered $P < 0.05$ to be statistically significant.

RESULTS

The effect of miR-223-3p in UC progression

To observe the potential role of miR-223-3p in UC development, the expression of miR-223-3p in 27 serum specimens from UC patients was detected by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). It is shown that the level of miR-223-3p in the serum specimen from UC patients was significantly up-regulated, 2.5-fold as high as the normal counterpart [Figure 1a]. Given that miR-223-3p exhibited a high expression level in the serum specimen from UC patients, which indicates that miR-223-3p might attribute to UC progression, we designed the spleen-kidney-yang deficiency model in mice injected with TNBS to get a better understanding of the action of miR-223-3p in UC progression with spleen-kidney-yang deficiency. The Mir-223-3p antagomir was designed to inhibit the level of miR-223-3p. As shown in Figure 1b, the miR-223-3p antagomir could protect the intestine from the damage caused by UC and the reduction of colon length was less obvious in the miR-223-3p antagomir group [Figure 1c], suggesting that miR-223-3p could accelerate the progression of UC with spleen-kidney-yang deficiency. Moreover, immuno-histochemical staining also proved that the miR-223-3p antagomir was able to alleviate the intestinal epithelial barrier function damage cause by UC [Figure 1d]. The results above indicated that miR-223-3p had the potential to promote UC development with spleen-kidney-yang deficiency.

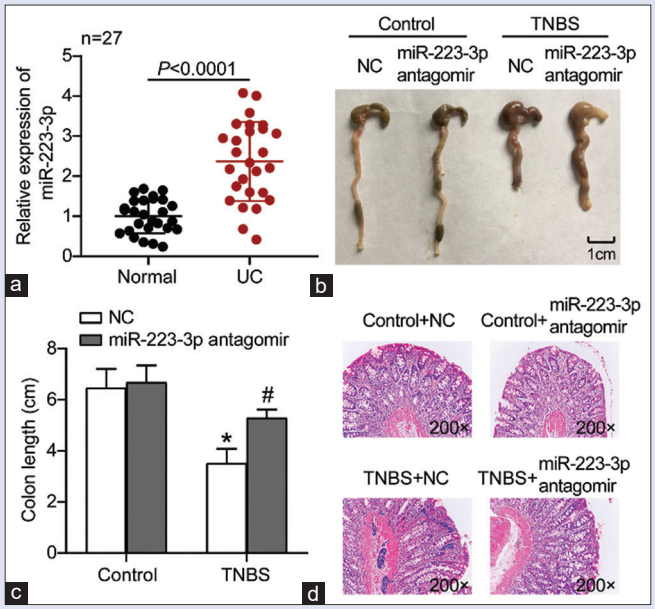

Figure 1: The effect of miR-223-3p in UC progression. (a) The comparison of expression of miR-223-3p in 27 UC serum samples and 27 healthy serum samples by qRT-PCR. $P < 0.0001$ versus healthy serum samples. (b and c) The colon length was measured in the spleen-kidney-yang deficiency mice model with treatment of the miR-223-3p antagomir. $^{*}P < 0.05$ versus NC group; $^{\#}P < 0.05$ versus miR-223-3p antagomir group. (d) Histopathological changes in the colon tissue were examined by HE staining. Scale bar, 50 mm. (N = 10, repetition = 3)

MiR-223-3p regulated UC development through pro-inflammatory cytokines

Emerging data have suggested that intestinal mucosal immunity possesses an essential role in the regulation of intestinal flora.[21,22] Normally, intestinal flora and intestinal immunity are in a dynamic balance. However, because of a variety of factors such as susceptibility genes and environment, the action of dynamic balance will be destroyed, and then the inflammation occurs.[16] The imbalance of intestinal flora will result in abnormal secretion of related inflammatory cytokines and intestinal mucosal inflammation, which can be a contributor to the pathogenesis of UC. During the intestinal mucosal immune response, a variety of inflammatory cytokines are involved. In this regard, we measured the expression levels of several pro-inflammatory cytokines such as TNF-a, IL-6, and IL-1β in the spleen-kidney-yang deficiency mice model when miR-223-3p was inhibited by the antagomir. By this effort, we can find out if miR-223-3p regulates UC development with spleen-kidney-yang deficiency through pro-inflammatory cytokines. As a result, reduction of several pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) were observed with the existence of the miR-223-3p antagomir [Figure 2a-c]. To be more specific, under the TNBS conditions, the expression level of TNF-α was up-regulated by 8-fold relative to that of the control group, whereas when the miR-223-3p antagomir was introduced, the expression level of TNF-α was only up-regulated by 5-fold relative to the control group [Figure 2a]. Similar trends were observed in the expression levels of IL-6 and IL-1β. Under the TNBS conditions, the expression level of IL-6 was up-regulated by 6-fold relative to the control group, whereas when the miR-223-3p antagomir was introduced, the level of IL-6 was only up-regulated by 4-fold relative to that of the control group [Figure 2b]. Under the TNBS conditions, the level of IL-1β was up-regulated by 3.5-fold relative to that of the control group, whereas when the miR-223-3p antagomir was introduced, the level of IL-1β was only up-regulated by 2.5-fold relative to that of the control group [Figure 2c]. What is more, the protein levels of tight junction proteins (ZO-1 and Occludin) in the spleen-kidney-yang deficiency mice model responding to the miR-223-3p antagomir were detected using western blotting. The observation was that ZO-1 and Occludin protein levels were decreased in the spleen-kidney-yang deficiency mice model, whereas the decrease was less obvious in the miR-223-3p antagomir treatment group [Figure 2d]. Collectively, the results suggested that miR-223-3p had a negative effect on the maintenance of gut barrier functions through regulation on TNF-α, IL-6, IL-1β, ZO-1, and Occludin expression levels.

miR-223-3p regulated UC through the TGF-β/Smad3 pathway

To explore the relationship between miR-223-3p and TGF-β in the development of UC with spleen-kidney-yang deficiency, we also examined both mRNA and protein TGF-β expression in an experimental mouse colitis model. Subjected to TNBS, the mRNA expression level of TGF-β was increased by more than 2-fold relative to that of the control group [Figure 3a], suggesting that TGF-β had the potential to promote the progression of UC. Besides, when it was treated with the miR-223-3p antagomir, the increase of the mRNA expression of TGF-β was less pronounced [Figure 3a], which suggested that miR-223-3p had the same

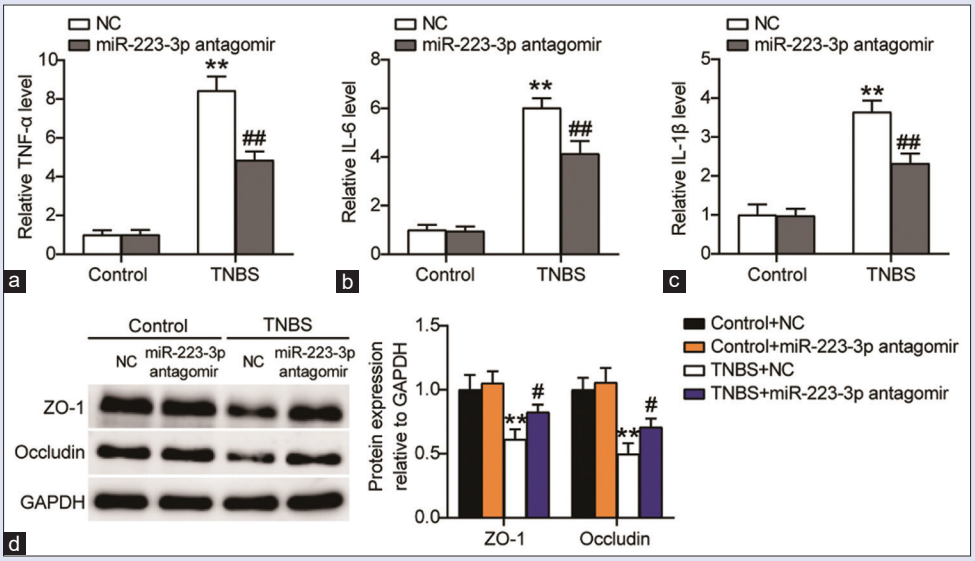

Figure 2: MiR-223-3p regulated UC development through pro-inflammatory cytokines. (a-c) The mRNA expression of TNF-α, IL-6, and IL-1β in the spleen-kidney-yang deficiency mice model with treatment of the miR-223-3p antagomir by qRT-PCR. $^{**}p < 0.001$ versus blank control group. $^{\#\#}p < 0.001$ versus miR-223-3p antagomir. (d) The protein expression of ZO-1 and Occludin in the spleen-kidney-yang deficiency mice model with treatment of the miR-223-3p antagomir by western blotting. $^{**}p < 0.001$ versus blank control group. $\#p < 0.05$ versus control + miR-223-3p antagomir. (N = 10, repetition = 3)

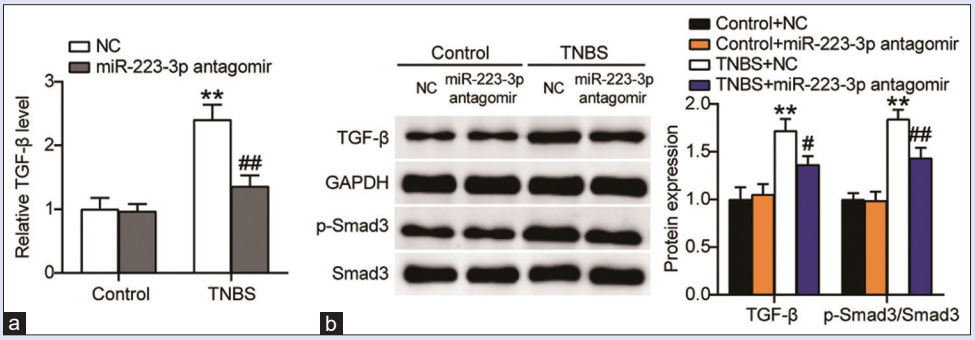

Figure 3: miR-223-3p regulated UC through the TGF-β/Smad3 pathway. (a) The mRNA expression of TGF-β in the spleen-kidney-yang deficiency mice model with treatment of the miR-223-3p antagomir by qRT-PCR. $^{**}p < 0.001$ versus blank control group. $^{\#\#}p < 0.001$ versus miR-223-3p antagomir. (b) The protein expression of TGF-β, Smad3, and p-Smad3 in the spleen-kidney-yang deficiency mice model with treatment of the miR-223-3p antagomir by western blotting. $^{**}p < 0.001$ versus blank control group. $\#\#p < 0.001$ versus control + miR-223-3p antagomir. (N = 10, repetition = 3)

trend as that of TGF-β in the regulation of UC development, and miR-223-3p promotes UC development through regulation on TGF-β. In addition, it exhibited the same result in the protein expression level of TGF-β. The protein expression level of TGF-β was increased by about 1.7-fold relative to that of the control group [Figure 3b], and when the miR-223-3p antagomir was introduced, the increase of the protein expression of TGF-β was less pronounced [Figure 3b]. Additionally, the protein level of Smad3 and the phosphorylation level were increased by about 1.8-fold compared to the control group [Figure 3b], and when the miR-223-3p antagomir was introduced, the increase of the protein expression of Smad3 and the phosphorylation level was less pronounced [Figure 3b]. Taken together, miR-223-3p promoted UC progression with spleen-kidney-yang deficiency by regulating the TGF-β/Smad3 pathway.

DISCUSSION

UC has been widely accepted as an important risk factor for people health with unclear molecular mechanisms.[23,24] This study measured miR-223-3p expression in UC patients and healthy controls. The miR-223-3p expression level was significantly elevated in UC patient serum compared to healthy controls, suggesting the promotional action of miR-223-3p in UC progression. A spleen-kidney-yang deficiency mice model was constructed here, and further *in vivo* experiments demonstrated that the miR-223-3p antagomir was able to lessen the shorting of colon length, alleviating the intestinal epithelial barrier function damage caused by UC. What is more, under the UC conditions, the expressions of inflammatory cytokines such as TNF-α, IL-6, and IL-1β were up-regulated, but when the miR-223-3p antagomir was introduced, the up-regulation of their expression levels were less pronounced. In addition, under the UC conditions, the protein levels of tight junction proteins (ZO-1 and Occludin) were decreased, whereas the decrease was less obvious with the miR-223-3p antagomir treatment. Moreover, it is observed that the level of TGF-β was increased under the UC conditions, whereas the elevation was reduced by the introduction of the miR-223-3p antagomir. Besides, miR-223-3p could promote the phosphorylation of Smad3 according to the western blotting result. Thus, we came to a conclusion that miR-223-3p was able to promote UC progression with spleen-kidney-yang deficiency through the TGF-β/Smad3 pathway.

Expression of the miR-223 has been reported to be up-regulated in the colonic mucosa of patients with IBD,[25,26] suggesting a facilitative action of miR-223 in the progression of IBD. In 2016, serum miR-223 has been identified to be a biomarker for IBD treatment.[27] Then in 2017, myeloid-derived miR-223 was reported to be involved in intestinal inflammation in IBD.[28] More recently, miR-223-3p was revealed to be related to the inflammatory process of the intestine.[29] In addition, Wang *et al.*[30] treated colonic epithelial cell lines with IL23 to induce the colitis model *in vitro* and found increased expression of miR-223. In this study, we tried to further demonstrate the role of miR-223-3p in the regulation of UC progression. Similar to the previous report, our results revealed that the miR-223-3p expression level in the serum sample of UC patients was significantly up-regulated. This finding implicated that miR-223-3p might be a key factor for UC development. Interestingly, Wang *et al.*[30] analyzed the effect of miR-223 on colitis *in vivo*, revealing that miR-223 antagonism increased the body weight of TNBS-induced colitis mice and alleviated the intestinal pathology. Based on these results, therefore, we conducted further *in vivo* experiments to illustrate the action of miR-223-3p in a spleen-kidney-yang deficiency mice model, analyzing the levels of inflammatory cytokines (TNF-α, IL-6, IL-1β) and tight junction proteins such as ZO-1 and Occludin. Results indicated that the miR-223-3p antagomir restrained the expression levels of TNF-α, IL-6, and IL-1β under the UC conditions. However, the miR-223-3p antagomir promoted the levels of ZO-1 and Occludin under the UC conditions. It was proved that miR-223-3p could worsen spleen-kidney-yang deficiency in UC.

UC is caused by abnormal and excessive local immune responses to bacterial microbiota components poorly controlled by endogenous counter-regulatory mechanisms such as immuno-suppressive factor TGF-β1.[31] It has been reported that TGF-β1 expression is up-regulated in the inflammatory bowel of patients with inflammatory bowel disease.[32] Similarly, in this study, TGF-β1 expression was also up-regulated in the UC spleen-kidney-yang deficiency model induced by TNBS. In addition, Smad3, as a molecule related to the TGF-β signaling pathway, was also found to be highly expressed with TGF-β1 in UC.[33] Interestingly, our study found an increase in Smad3 phosphorylation in UC rats. All these indicated that the TGF-β/Smad3 pathway was activated in UC. Furthermore, the miR-223-3p antagomir was capable of repressing the protein level of TGF-β and the phosphorylation of Smad3 according to the western blotting result, indicating that miR-223-3p was capable of contributing to UC progression with spleen-kidney-yang deficiency by regulating the TGF-β/Smad3 pathway.

However, there were shortcomings in the present study. For example, by what target genes miR-223-3p exerts its effect in the UC development has not been verified, which can be further investigated in the future study. Besides, more attempts are required to make to assess the mechanisms underlying the TH17/Treg balance between miR-223-3p and the TGF-β/Smad3 pathway.

CONCLUSION

Collectively, the function of endophytic flora in the health of the body is a new hotspot in the current medical field. The research on the efficacy and mechanism of traditional Chinese medicine based on the regulation of intestinal flora is a new direction of the basic research of traditional Chinese medicine. Of note, it is the first time to elucidating the mechanism of tonifying the spleen and kidney decoction in treating UC with spleen-kidney-yang deficiency syndrome by regulating the flora and TGF-β/Smad3 pathway. Here, the influence of drugs on intestinal flora and the balance axis of Th17/Treg in UC were combined to illustrate the therapeutic effect of traditional Chinese medicine.

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Conflicts of interest

There are no conflicts of interest.

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