Original Article

miR-21 antagonist alleviates colitis and angiogenesis via the PTEN/PI3K/AKT pathway in colitis mice induced by TNBS

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Background: The pathogenesis of Crohn's disease (CD) is unknown; however, angiogenesis is known to play an important role in the disease. The present research suggests that microRNA-21 (miR-21) may play a positive regulatory role in disordered angiogenesis in CD.

Methods: C57 wild-type mice were divided into 6 groups. On day 0, all mice in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) group were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol). In the control group, the enema was performed with 50% alcohol. On day 0, 2, 4, and 6, the mice of the agomir-21 + TNBS group and the agomir control + TNBS group were injected with 200 μL, 5 nmol agomir-21 or agomir control [dissolved in ribonuclease (RNase)-free water] by tail vein injection, while the antagomir-21 + TNBS group and the antagomir control + TNBS group were injected with 200 μL, 20 nmol antagomir-21 or antagomir control (dissolved in RNase-free water). The body weight and disease activity index (DAI) score were recorded daily. The colons were obtained to assess macro and microscopic colon damage. The inferior vena cava and the accompanying abdominal aorta were chosen to detect the protein expression of the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/serine/threonine kinase (AKT)/vascular endothelial growth factor (VEGF) axis through western blotting. Serum interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). The distribution and expression of neovascularization were demonstrated by cluster of differentiation 31 (CD31) immunohistochemistry.

Results: Compared with the only-TNBS group, the agomir-21 + TNBS group showed significantly severer colitis symptoms and more abnormal vascular hyperplasia, while the antagomir-21 + TNBS group showed symptom relief and reduced vascular hyperplasia. In addition, agomir-21 obviously inhibited the expression of PTEN and activated the PI3K/AKT/VEGF pathway in mice induced by TNBS, while antagomir-21 effectively antagonized this effect.

Conclusions: miR-21 can promote the progression of colitis in mice induced by TNBS and aggravate the disordered angiogenesis by regulating the PTEN/PI3K/AKT axis. Intravenous injection of miR-21 antagonists can effectively relieve the symptoms of colitis and inhibit colonic angiogenesis.

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Keywords: Colitis mice; TNBS; miR-21; PTEN/PI3K/AKT; angiogenesis

Submitted Jan 27, 2022. Accepted for publication Mar 18, 2022.
doi: 10.21037/atm-22-944
View this article at: https://dx.doi.org/10.21037/atm-22-944

Introduction

Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract with unknown pathogenesis (1). CD causes frequent relapses over a patient's lifetime and can lead to complications such as colon stenosis, abscess, and fistula. At present, there are no specific drugs or programs to cure patients with CD. The main goal of treatment is to relieve colon inflammation through targeted intervention of inflammatory molecules to control the acute onset of the disease and reduce recurrence. Therefore, the treatment of CD requires further research to discover more effective treatment methods. In recent years, scholars have attempted to discover the mechanism of CD and potential treatment strategies from new perspectives such as immunity and vascularity.

Angiogenesis is an important part of the pathogenesis of inflammation in inflammatory bowel disease (IBD) (2). Studies have shown that patients with CD have a higher risk of vascular abnormalities, including vascular dysplasia near the colon tissue (3); abnormalities of the inferior mesenteric artery, inferior epigastric artery, portal vein, and other large vessels (4,5); and thromboembolism (6). One current view characterizes CD angiogenesis as "immune-driven angiogenesis" of the colon microcirculation driven by inflammation and immune responses (7-9); however, this view is not sufficient to explain the occurrence of macrovascular abnormalities and thrombosis in CD.

MicroRNA (miRNA) is a class of endogenous, single-stranded, conserved, non-coding, unique RNA fragments of about 18–22 nucleotides in length (10). miRNAs have attracted much attention as post-transcriptional regulators of gene expression that have epigenetic transcriptional effects on gene regulation. Researchers have found that a variety of miRNAs participate in the pathological immune response to CD (11). In CD, miR-155 and miR-146a inhibit the development and function of colon lamina propria CD103 + CD11b + DC cells (12,13), miR-1246 activates pro-inflammatory nuclear transcription factors to regulate T cell differentiation (14,15), and miR-16 drives nuclear factor kappa B (NF-κB)-related signaling pathways in macrophages (16). In addition, miRNAs play an important role in regulating colon barrier function: miR-155 can enter vascular endothelial cells and impair endothelial integrity (17), and miR-146b improves epithelial barrier function by activating NF-κB (18). This evidence suggests that miRNA plays a non-negligible role in the pathogenesis of CD. However, many of the current studies are still in the initial stages of exploring phenotypes, and there are few reports of overall miRNA intervention or treatment in CD animal models. Therefore, further studies are needed to explore the role of miRNAs in CD.

In our study, the CD animal model [a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced mouse colitis model] was successfully built to investigate the disease-promoting and vascular hyperplasia effects of agomir-21, a type of miR-21 analogue, and the therapeutic effects of antagomir-21, an *in vivo* antagonist of miR-21, on CD colitis mice from a holistic perspective. We found that compared with the pure TNBS model group, mice in the agomir-21 + TNBS group showed significantly severer colitis symptoms and more abnormal vascular hyperplasia, while mice in the antagomir-21 + TNBS group showed symptom relief and reduced vascular hyperplasia. In addition, agomir-21 obviously inhibited the expression of phosphatase and tensin homolog (PTEN) and activated the phosphoinositide 3-kinase (PI3K)/serine/threonine kinase (AKT)/vascular endothelial growth factor (VEGF) pathway in mice induced by TNBS, while antagomir-21 effectively antagonized this effect. Briefly, we improved the previous modeling method of CD mouse model, recorded the usage and dose of miR-21 agonist and antagonist in an exploratory manner. And more importantly, we demonstrated the angiogenesis promoting effect of miR-21 and the antagonism of miR-21 inhibitors *in vivo* in TNBS-induced model. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-944/rc).

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Methods

Experimental animals

C57 wild-type healthy female mice aged 6 weeks and weighing 18–20 g were purchased and raised by the Department of Zoology, Xiangya School of Medicine, Central South University. Experiments were performed under a project license (No. 2022sydw005) granted by the Experimental Animal Ethics Committee of Xiangya School of Medicine, Central South University, in compliance with the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals, 8th edition (19). A protocol was prepared before the study without registration.

Animal grouping and modeling materials

Thirty mice were randomly divided into six groups. All RNAs were designed and synthesized by RiboBio (Guangzhou, China). TNBS-induced colitis showed a pattern similar to that seen in human CD (20).

(I) TNBS (Sigma-Aldrich, St. Louis, MO, USA) group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol).

(II) Control group (alcohol solvent). The back of the mice was shaved and coated with 50% alcohol for 7 days for pre-sensitization. On day 0 and 7, an enema was performed with 50% alcohol.

(III) Agomir-21 + TNBS group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol). On day 0, 2, 4, and 6, the mice were injected with 200 μL 5 nmol agomir-21 [dissolved in Ribonuclease (RNase)-free wate] by tail vein injection.

(IV) Agomir control + TNBS group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol). On day 0, 2, 4, and 6, the mice were injected with 200 μL, 5 nmol agomir control (dissolved in RNase-free water) by tail vein injection.

(V) Antagomir-21 + TNBS group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol). On days 0, 2, 4, and 6, the mice were injected with 200 μL, 20 nmol antagomir-21 (dissolved in RNase-free water) by tail vein injection.

(VI) Antagomir control + TNBS group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol). On day 0, 2, 4, and 6, the mice were injected with 200 μL, 20 nmol antagomir control (dissolved in RNase-free water) by tail vein injection.

Clysis was performed as follows. The mice were mildly anesthetized by a nasal cone of 1.5% isoflurane mixed gas. The anus of the mice was stimulated to expel fecal masses. The 1 mL syringe was inserted into a soft catheter with a diameter of 1 mm and fixed firmly. After fully lubricating the end of the catheter and the perianal area of the mouse, the catheter was inserted through the anus and the catheter was gently upturned to make it smoothly enter the rectum. The catheter was slowly inserted to a position 3–4 cm from the anus, and 0.1 mL of 100 mg/kg TNBS was rapidly injected. The mice were inverted for 2–3 minutes to avoid drug spillover. The mice were then placed on an insulating mat with their heads low and their feet high. After waking up, the mice were fed food and water normally.

The body weight of the mice in the above 6 groups was measured daily; the hair, activity, and mental state were observed; blood or mucus in the stool were recorded; and feces were tested for occult blood. On day 10, the cervical vertebra was dislocated and sacrificed. After dissection, blood was taken quickly from the heart, and the supernatant was isolated by centrifugation at 3,000 rpm and stored at –20 ℃ for preservation. The colon tissues were taken out and measured, and the lesion segment of the colon was inserted into 3% paraformaldehyde. The inferior vena cava and the accompanying abdominal aorta were separated, fully infiltrated, and cleaned by heparinized normal saline and placed in liquid nitrogen for preservation.

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Disease activity index score (DAI)

The mice were scored using the DAI according to the international criteria (21). The formula for the DAI was as follows: (weight loss score + stool shape score + stool blood score)/3.

Macroscopic pathological injury score

The evaluation was conducted according to the macroscopic pathological injury score criteria (22). Colon injury in each group of mice was assessed by 3 observers who were blinded to the grouping of the mice.

Hematoxylin and eosin (HE) staining and microscopic pathological injury score

Formaldehyde-fixed colon tissue was embedded in paraffin and sliced. After the slices were dewaxed in xylene and gradient ethanol, they were stained with hematoxylin and eosin successively, with 1% hydrochloric acid alcohol and saturated lithium carbonate used for bluing. After cleaning, the slices were sealed with gradient ethanol and xylene dehydrated neutral gum, and images were collected under a microscope. The histopathological score was based on the Geboes criteria (23), and each group of slices was assessed by 3 observers who were blinded to the grouping of the mice.

Serum interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α)

IL-1β and TNF-α concentrations in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Absorbance was measured using an ELISA plate reader at 650 nm.

Immunohistochemistry (IHC)

The slices were dewaxed in xylene and gradient ethanol, blocked in 3% H_2O_2, incubated in a wet box at room temperature, rinsed with distilled water, and repaired by microwave for 5–10 minutes. Normal goat serum blocking solution and primary antibody drops [concentration 1:1,000, diluted with phosphate buffer saline (PBS)] were added, and the slices were stored in a wet box at 4 ℃ overnight. The next day, secondary antibody was added, the slices were incubated in a wet box at 37 ℃. The 3, 3'-diaminobenzidine (DAB) chromogenic reaction time was controlled under a light microscope, and the reaction was terminated immediately after chromogenic reaction. The slices were lightly redyed with hematoxylin, with 1% hydrochloric acid alcohol and saturated lithium carbonate used for bluing. They were then washed and soaked with distilled water, dehydrated with gradient ethanol and xylene, and sealed with neutral gum. Microscopic examination was performed for image collection.

Western blotting

Western blotting was conducted using fresh cell lysates, which were separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Sigma, St. Louis, MO, USA) that were then blocked for 1–2 hours with 1% bovine serum albumin (BSA). The blots were probed overnight with appropriate primary antibodies at 4 ℃, followed by probing for 1 hour with secondary horseradish peroxidase (HRP)-conjugated antibodies. Protein bands were then detected with an enhanced chemiluminescence (ECL) kit (Biosharp, Beijing, China). The primary antibodies used were as follows: anti-PTEN (9552, Cell Signaling Technology, Danvers, MA, USA), anti-PI3K (4249, Cell Signaling Technology), anti-AKT (9272, Cell Signaling Technology), anti-P-AKT (4060, Cell Signaling Technology), anti-VEGF (ab46154, Abcam) and anti-GAPDH (10494-1-AP, Proteintech, Wuhan, China).

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Comparisons among the groups were made by using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test or by using a nonparametric rank sum test, as appropriate. A P value of <0.05 was considered to be significant. All analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Agomir-21 aggravates symptoms of TNBS-induced colitis, and antagomir-21 is effective in relieving these symptoms

Compared with those in the alcohol solvent control group (DAI =0), the mice in the TNBS group experienced acute

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colitis and bloody stool on the day of clysis, followed by depression, curled up, weight loss, bloody stool, and continuous mucus or diarrhea the next day. The weight loss peak occurred on the third day, and on day 4–5 the aforementioned symptoms gradually alleviated. The symptoms occurred again on the 7th day following the second TNBS enema. Compared with those in the agomir control + TNBS group and the TNBS group, the mice in the agomir-21 + TNBS group showed significantly worse symptoms (P<0.01), faster weight loss, and more serious bloody stools. Compared with those in the antagomir control + TNBS group and the TNBSgroup, the symptoms of the mice in the antagomir-21 + TNBS group were significantly reduced (P<0.01), the weight loss was not significant, and the occurrence of bloody stool was less. By day 4–5, the stool morphology was basically normal, and the fecal blood was negative. There was no difference between the antagomir-21 + TNBS group and the control group until day 6–7 (P>0.05; *Figure 1A*).

The day of the first TNBS enema was recorded as day 0 of the experiment. The weight change of mice was recorded every day, and the weight loss rate of the mice was calculated according to the following formula: weight loss rate = (weight on that day – initial weight)/initial weight. The results showed that there was no difference in body weight among the groups during the presensitization of TNBS on the back of the mice (Figure S1). After modeling, the body weight of the mice in the TNBS group continued to go down, decreasing by 10.35%±0.67% on day 3, and then gradually increased, reaching its highest value before the second enema on day 7. The body weight of the mice in the agomir-21 + TNBS group decreased by 12.32%±1.74% on day 3 and then gradually recovered, but the recovery trend was still significantly lower than that of the mice in the TNBS group (P<0.01) and the agomir control + TNBS group (10.18%±1.10%, P<0.01). The body weight of the mice in the antagomir-21 + TNBS group decreased by 3.12%±1.35% on day 2 and by –0.53%±2.02% on day 3 and then gradually recovered. The recovery trend was lower than that of the solvent control group (P<0.01) but higher than that of the antagomir control + TNBS group (P<0.01) and the TNBS group (P<0.01). Meanwhile, the body weight of the mice in the solvent control group increased evenly every day (*Figure 1B*).

After the second clysis (on day 7), the weight loss trend and DAI scores in the TNBS, agomir + TNBS, and antagomir + TNBS groups were similar to those on day 1–2. On day 9, the TNBS group decreased by 8.23%±1.06%, the agomir-21 + TNBS group decreased by –15.93%±0.60%, the agomir control + TNBS group decreased by –7.85%±1.89%, the antagomir-21 + TNBS group decreased by –8.31%±2.16%, and the antagomir control + TNBS group decreased by 6.88%±0.33% (Figure S1).

Agomir-21 aggravates the serological inflammatory response in TNBS-induced colitis mice, and antagomir-21 effectively reduces these responses

Serum TNF-α and IL-1β levels in the TNBS group were higher than those in the solvent control group (P<0.01). Serum TNF-α and IL-1β levels in the agomir-21 + TNBS group were significantly higher than those in the agomir control + TNBS group and the TNBS group (P<0.05). Serum TNF-α and IL-1β levels in the antagomir-21 + TNBS group were significantly lower than those in the antagomir control + TNBS group and the TNBS group (P<0.01) but slightly higher than those in the solvent control group, with no statistical difference (*Figure 1C,1D*).

Agomir-21 aggravates TNBS-induced colitis injury, while antagomir-21 can effectively resist these injuries

Compared with those in the solvent control group, the mice in the TNBS group experienced significant shortening of colonic length (P<0.01) and colon injury (P<0.01), which manifested as segmental hyperemia and edema of the colonic wall, severe colonic bleeding, and transmural ulcers. The length of the colon and the severity of colon injury in the agomir-21 + TNBS group were shorter and more serious than those in the agomir control + TNBS group and the TNBS group (P<0.01). Colon length and injury degree in the antagomir-21 + TNBS group were improved compared with those in the antagomir control + TNBS group and the TNBS group (P<0.01) but were similar to those in the solvent control group (*Figure 2A-2C*).

Pathological HE staining results showed that the colonic mucosa of the TNBS group showed severe inflammatory reactions (*Figure 2D*). The results revealed a large number of lymphocytes and neutrophils in the submucosa, crypt fusion or distortion, irregular or atrophic arrangement of glands, and segmental or focal ulcers or bleeding. The degree of colonic injury in the agomir-21 + TNBS group was more serious than that in the agomir control + TNBS group and the TNBS group (P<0.01). The colonic injury degree in the antagomir-21 + TNBS group was improved compared with that in the antagomir control + TNBS

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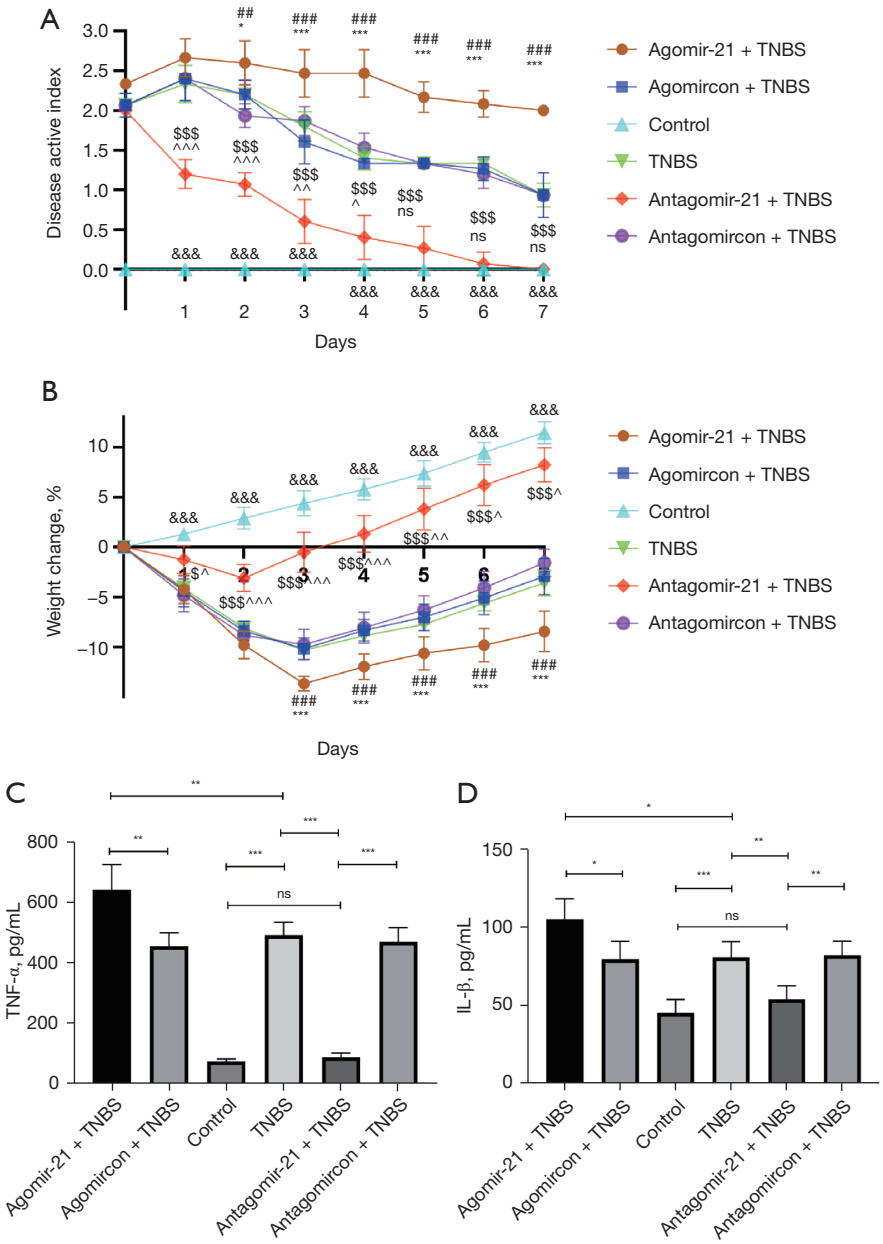

Figure 1 Agomir-21 aggravates the symptoms and inflammatory response of TNBS-induced colitis, and antagomir-21 is effective in relieving colitis and inflammation. (A,B) DAI and weight change rate. $^{\#\#}$, agomir-21 + TNBS group compared with agomir control + TNBS group, P<0.01; $^{\#\#\#}$, P<0.01; *, agomir-21 + TNBS group compared with TNBS group, P<0.05; ***, P<0.001; $^{\$}$, antagomir-21 + TNBS group compared with antagomir control + TNBS group and TNBS group, P<0.05; $^{\$\$\$}$, P<0.001; ^, antagomir-21 + TNBS group compared with control group, P<0.05; ^^, P<0.01; ^^^, P<0.001; ns represents no significant difference; $^{\&\&\&}$, all TNBS groups except antagomir-21 + TNBS group compared with control group, P<0.001. (C,D) Serum IL-1β and TNF-α levels. ns represents no significant difference; *, P<0.05; **, P<0.01; ***, P<0.001; n=5. TNBS, 2,4,6-trinitrobenzenesulfonic acid; DAI, disease activity index; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha. Agomircon, agomir control; Antagomircon, antagomir control.

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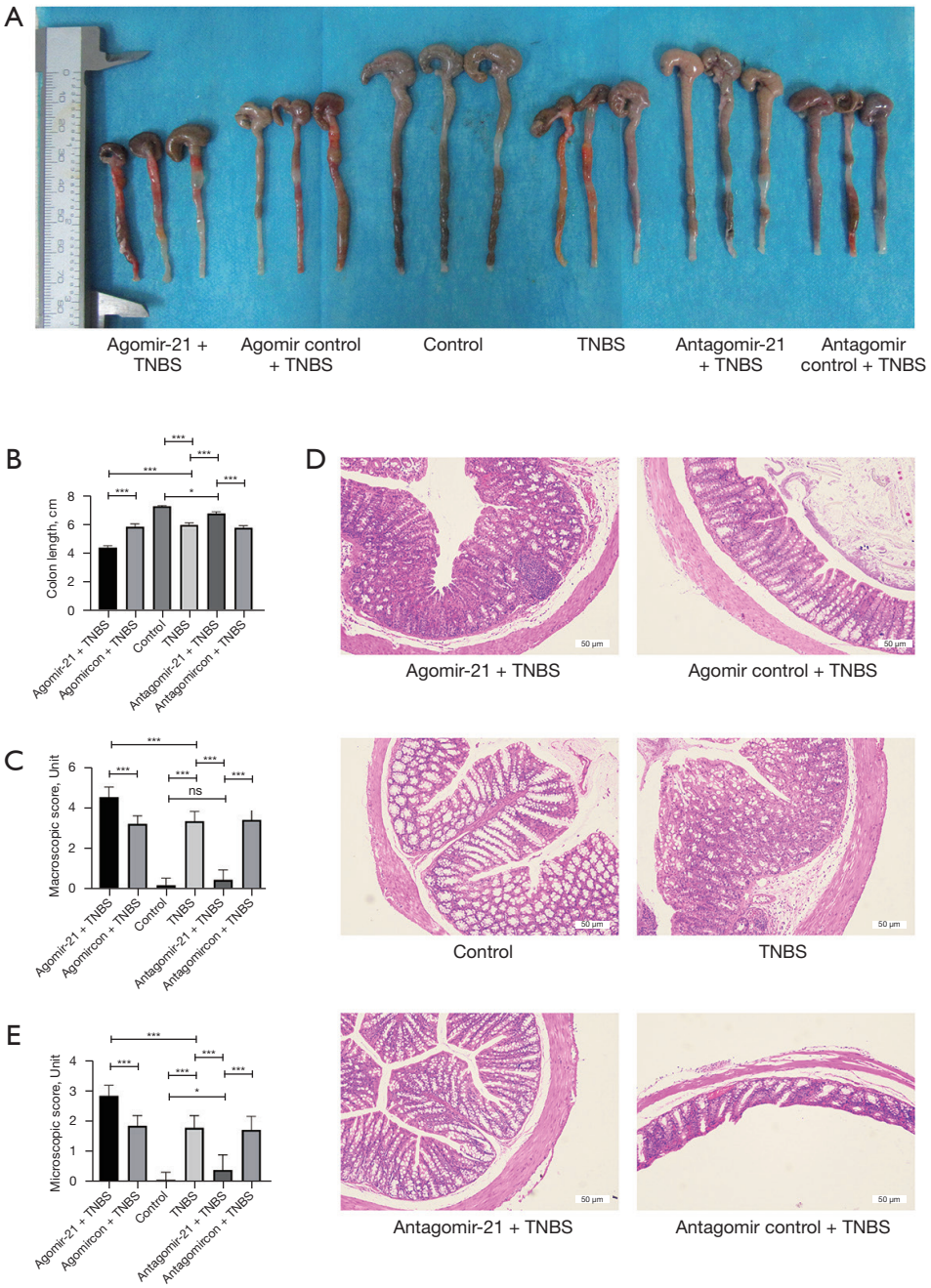

Figure 2 Agomir-21 aggravates TNBS-induced colitis injury, and antagomir-21 can effectively resist these injuries. (A-C) Macroscopic damage of colon. (A) View of the colon after dissection. (B) Colon length change. (C) Macroscopic damage score. (D,E) Microscopic damage of colon. (D) Histopathological HE staining. (E) Microscopic damage score. *, P<0.05; ***, P<0.001; ns represents no significant difference; n=5. TNBS, 2,4,6-trinitrobenzenesulfonic acid; HE, hematoxylin and eosin. In the bar chart, Agomircon represents agomir control; Antagomircon represents antagomir control.

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group and the TNBS group (P<0.01) and was histologically closer to the colons of the solvent control mice (*Figure 2E*).

Agomir-21 aggravates colonic neovascularization abnormality in TNBS-induced colitis mice by regulating the PTEN/PI3K/AKT axis, and this effect was inhibited by antagomir-21

Compared with that in the solvent control group, the expression density of cluster of differentiation 31 (CD31) in the colonic blood vessels of the mice in the TNBS group was increased. The CD31 expression signal in the agomir-21 + TNBS group was more disordered and its density was higher than that in agomir control + TNBS and the TNBS group. The disordered CD31 signal in the antagomir-21 + TNBS group was improved compared with that in the antagomir control + TNBS group and the TNBS group and was similar to that in the solvent control group (*Figure 3A*).

Western blotting results showed that agomir-21 inhibited the expression of PTEN, aggravated the TNBS-induced activation of PI3K/AKT pathway, and increased the production of VEGF (P<0.05). Antagomir-21 can effectively antagonize the PTEN/PI3K/AKT pathway activated by TNBS, and the expression of VEGF in the antagomir-21 + TNBS group was significantly decreased compared with that in the antagomir control + TNBS group and the TNBS group (P<0.01). There was no difference in the expression of PTEN/PI3K/AKT/VEGF axis between antagomir-21 + TNBS group and solvent control groups (*Figure 3B,3C*).

Discussion

In recent years, research on miRNAs has made progress towards clinical application. The role of miRNAs in regulating angiogenesis and immune microcirculation has been proven and applied in the treatment of liver, lung, and heart diseases. Available data indicate that miravirsen miR-122 is the most mature treatment for hepatitis C (HCV), and its antiviral activity has been demonstrated in all HCV genotypes (24). Studies have shown that miR-21 is not only involved in the occurrence and development of colorectal cancer, gastric cancer, breast cancer, and other cancers (25-29), but also seems to carry out its versatile functions differently depending on with the stage of colitis and modulate T-cell function (30). Moreover, miR-21 has been reported to promote angiogenesis in leukemia through targeted regulation of IL-12 (31). miR-21 also participates in the invasion and angiogenesis of renal carcinoma cells through the programmed cell death protein 4 (PDCD4)/C-Jun (AP-1) signaling pathway (32). In addition, miR-21 induces hypoxia inducible factor-1α (HIF-1α) activity to promote angiogenesis after severe limb ischemia (33). Therefore, miR-21 may play a positive regulatory role in angiogenesis. Our previous study found that the level of miR-21 in plasma exosomes of patients with active CD was elevated and the miR-21 can activate the PI3K/AKT pathway and promote the migration and tube formation of vascular endothelial cells by targeting inhibition of PTEN (34). We therefore hypothesized that the promoting effect of exosomal miR-21 on abnormal colonic vascular regeneration in CD model mice could be verified at the overall level so as to reveal a new vascular proliferation mechanism in the pathogenesis of CD, and that the therapeutic effect of antagomir-21, a specific miR-21 antagonist *in vivo*, on CD model mice could be preliminarily verified through the functional loss of miRNA *in vivo*, which could provide a further theoretical basis for the application of miRNA or miRNA antagonists in clinical practice.

Our results showed that an agomir-21 injection through the mouse tail vein could aggravate the symptoms of TNBS-induced colitis in mice, leading to a significantly increased DAI index and significantly high macro and micro pathological scores of colon injury. In contrast, multiple injections of a low-dose of antagomir-21 could alleviate the symptoms of TNBS-induced colitis in mice. Symptom relief manifested as an insignificant weight loss, decreased stool blood and mucus, and a decreased DAI index. Colon length, edema, congestion, and bleeding were also relieved. The HE staining performance of colons from the mice in the antagomir-21 + TNBS group was similar to that in the control group. The levels of serum inflammatory factors TNF-α and IL-1β were also consistent with the above results. These results indicate that miR-21 may play an important role in promoting the progression of CD and that antagonizing miR-21 may effectively alleviate and relieve the symptoms of colitis. To further prove the effect of miR-21 on colonic neovascularization, we performed immunohistochemistry of CD31 in mouse colon tissue. The results showed that the light signal of CD31 in colons from the agomir-21 + TNBS group was significantly enhanced, and the signal distribution was twisted and disordered, suggesting a large amount of abnormal neovascularization. The CD31 signal of colons in the antagomir-21 + TNBS group was similar to that in the control group but

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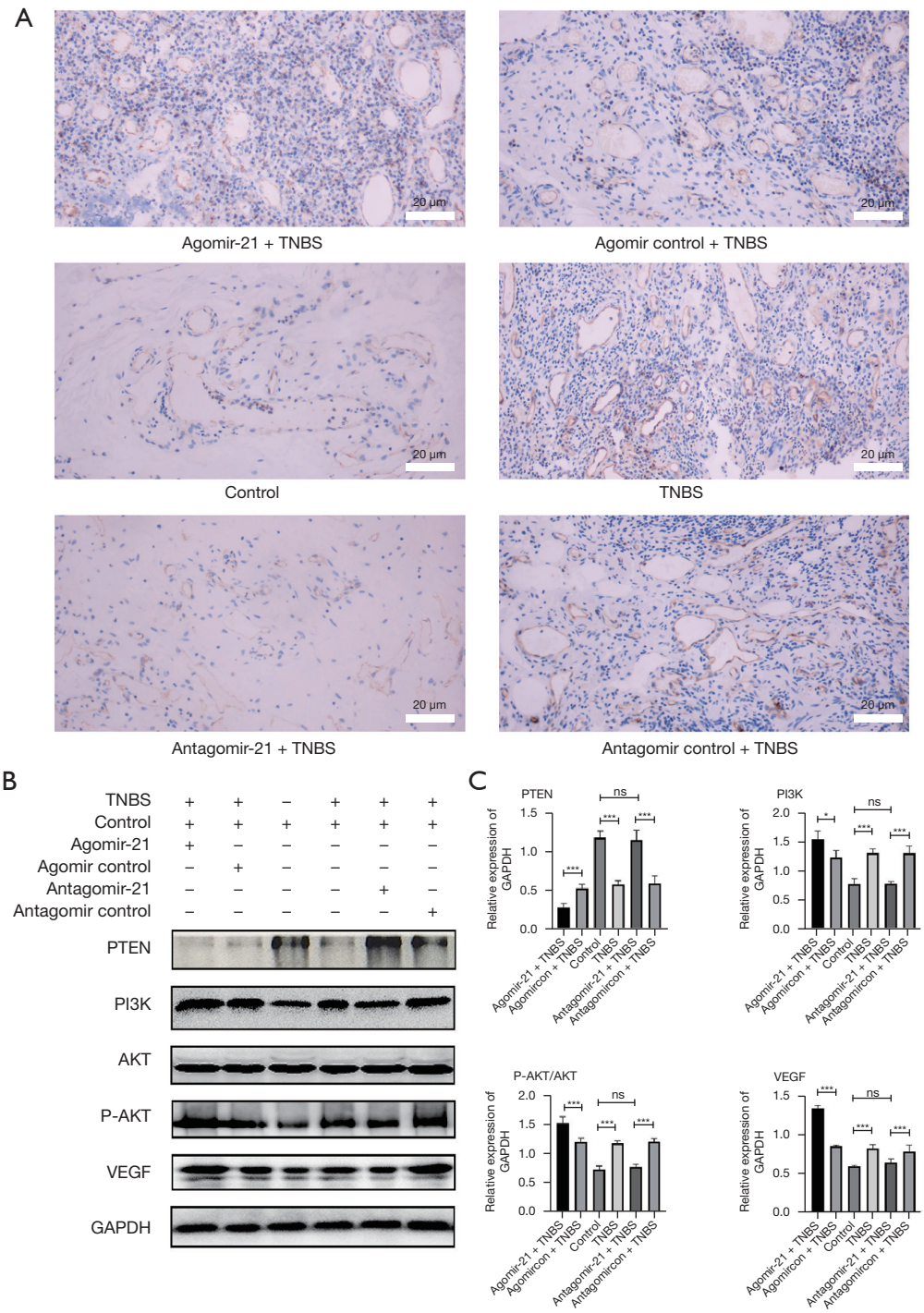

Figure 3 Agomir-21 aggravates colonic neovascularization abnormality in TNBS-induced colitis mice by regulating the PTEN/PI3K/AKT axis, and this effect was inhibited by antagomir-21. (A) Immunohistochemistry. Gray represents a positive signal for CD31 expression. The nuclei are stained blue with hematoxylin. (B,C) The protein level of PTEN, PI3K, P-AKT, AKT, and VEGF were detected by western blotting. + indicates that the mice treatment contains this intervention, – indicates they do not. *, P<0.05; ***, P<0.001; ns represents no significant difference; n=5. TNBS, 2,4,6-trinitrobenzenesulfonic acid; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; AKT, serine/threonine kinase; CD31, cluster of differentiation 31; VEGF, vascular endothelial growth factor. In the bar chart, Agomircon represents agomir control; Antagomircon represents antagomir control.

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significantly lower than that in the TNBS or the agomir-21 + TNBS group, indicating that antagomir-21 could effectively resist the pathological vasculogenic effect of TNBS. The western blotting results showed that agomir-21 could inhibit the expression of PTEN and activate the PI3K/AKT pathway to promote angiogenesis *in vivo*, which was consistent with our immunohistochemical CD31 results, while antagomir-21 could eliminate the changes of relative protein expression induced by TNBS. These results suggest that miR-21 *in vivo* aggravates colonic vascular dysplasia in TNBS colitis mice through the PTEN/PI3K/AKT axis, and antagomir-21 may eliminate those changes.

PTEN/PI3K/AKT signaling pathway plays an important role in both CD and colorectal cancer (35,36). The tissue specific deletion of PTEN can result in autoimmunity and glucose dysregulation, as the PTEN/PI3K/AKT signaling axis plays a central role in metabolism and inflammation under physiological conditions. In addition, PTEN-mediated mitochondrial autophagy was found to be closely related to intestinal mucosal immunity of CD (37). Therefore, researchers believe that targeting PTEN appears to be effective in developing new treatment strategies for CD and several cancer type (38). Current studies mainly focus on the intestinal epithelium or immune system, and rarely involve circulatory pathogenic factors and CD vascular diseases, while which is the main focus of our study. Blood vessels are one of the largest and most important organs in the human body. We have found that exosomal miR-21 targeting *Pten* induced activation of the PI3K/AKT pathway and promoted migration and tube formation of human umbilical vein endothelial cells (HUVECs) in our previous study, which may be an important mechanism of abnormal angiogenesis in patients with CD (34). Therefore, we injected agomir-21, an exosomal miRNA analogue, into the tail vein of mice induced by TNBS and found that the miR-21 analogue could aggravate the manifestation of colitis in the model mice, while antagomir-21, an *in vivo* antagonist of miR-21, could alleviate these manifestations and effectively inhibit mesenteric angiogenesis. Therefore, this study suggests that miR-21 may be a novel biomarker to evaluate the progression and prognosis of CD disease, and that targeted inhibition of miR-21 combined with existing conventional therapies may be a new therapeutic strategy for the treatment or prevention of CD vascular disease and colitis.

Our study has some limitations. Colonic vascular tissue specimens could not be obtained effectively, and large blood vessels such as the inferior vena cava and abdominal aorta cannot completely replace colonic blood vessels. Larger animals such as rats and rabbits should be used as models to further improve the experimental design. Gene knockout animal is also required to exploit pharmacological modulators of the cascade that show efficacy and safety in the future research. The application of miRNA antagonist also faces certain challenges (39,40). miRNA itself is acidic and extremely unstable in the blood, and the off-target effect of miRNA may stimulate the immune system to produce serious immunotoxicity. How to use liposomes or nanoparticles as carriers for safe and targeted delivery is a current research focus.

Conclusions

miR-21 can promote the progression of colitis in mice induced by TNBS and aggravate the disordered angiogenesis in colonic lesions by regulating the PTEN/PI3K/AKT axis, while intravenous injection of miR-21 antagonists can effectively relieve the symptoms of colitis and inhibit colonic angiogenesis.

Acknowledgments

The scientific research platform was provided by the Medical Experiment Center of The Third Xiangya Hospital of Central South University

Funding: This work was supported by the Changsha Municipal Natural Science Foundation (No. kq2014257).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-944/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-944/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-944/coif). All authors report that this work was supported by the Changsha Municipal Natural Science Foundation (No. kq2014257). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

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aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 2022sydw005) granted by the Experimental Animal Ethics Committee of Xiangya School of Medicine, Central South University, in compliance with the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals, 8th edition.

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(English Language Editor: C. Gourlay)

Cite this article as: Xie X, Liu P, Wu H, Li H, Tang Y, Chen X, Xu C, Liu X, Dai G. miR-21 antagonist alleviates colitis and angiogenesis via the PTEN/PI3K/AKT pathway in colitis mice induced by TNBS. Ann Transl Med 2022;10(7):413. doi: 10.21037/atm-22-944

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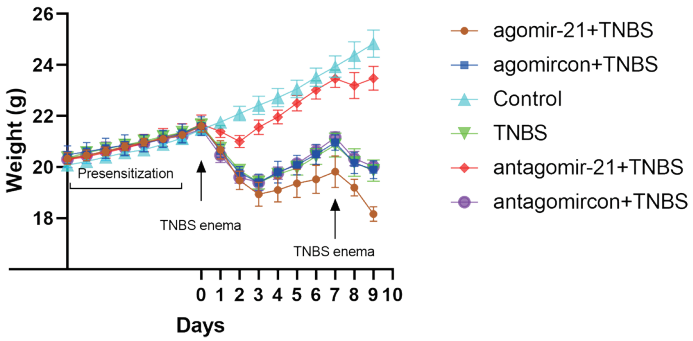

Figure S1 Daily weight of mice in each group. The back of the mice was shaved and coated with 1% TNBS (solvent 50% alcohol) or 50% alcohol for 7 days for pre-sensitization. On day 0 and 7, the mice were given an enema at the concentration of TNBS 100 mg/kg mouse body weight (solvent 50% alcohol) or 50% alcohol. On day 0, 2, 4, and 6, the mice were injected with 200 μL, 5 nmol agomir-21/agomir control (dissolved in RNase-free water) or 200 μL, 20 nmol antagomir-21/antagomir control (dissolved in RNase-free water) by tail vein injection. TNBS, 2,4,6-trinitrobenzenesulfonic acid; RNase, ribonuclease. Agomircon represents agomir control; Antagomircon represents antagomir control.

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https://dx.doi.org/10.21037/atm-22-944