CircRNA malignant fibrous histiocytoma amplified Sequence 1 (MFHAS1) reduced inflammatory responses in a Colitis Model via SIRT1/NF-κB

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Abstract

Ulcerative colitis (UC) is a chronic and non-specific inflammatory bowel disease (IBD). Its pathogenesis remains unclear, but its morbidity shows an increasing trend by year. The pathogenesis of UC may be related to the genetic susceptibility, immune factor and intestinal microflora. In the last few years, an increasing number of studies have examined the relationship between the expression levels of peripheral CircRNA and UC. We aimed to evaluate the efficacy of CircRNA MFHAS1 in colitis and its possible mechanism. The expression of CircRNA MFHAS1 was reduced in colitis, and miR-486-5p expression was also increased in citrobacter rodentium-induced murine colitis. In vitro model, over-expression of CircRNA MFHAS1 reduced inflammatory responses, induced SIRT1 protein expression, and suppressed NF-κB protein expression. However, miR-486-5p CircRNA MFHAS1 suppressed SIRT1 protein expression, and induced NF-κB protein expression in vitro model. The inactivation of SIRT1 reduced the anti-inflammation effects of CircRNA MFHAS1 on inflammatory responses in vitro model. Over-expression of miR-486-5p also reduced the anti-inflammation effects of CircRNA MFHAS1 in vitro model. Our results demonstrated that CircRNA MFHAS1 reduces inflammatory responses in Colitis via SIRT1/NF-κB by miR-486-5p.

Keywords: CircRNA MFHAS1, Colitis, NF-κB, SIRT1, miR-486-5p.

Practical Application: Reduction of inflammatory responses by CircRNA MFHAS1 in colitis.

1 Background

Ulcerative colitis (UC) is a chronic, non-specific, inflammatory disease in which lesions are mainly located in the colonic mucosa (Meade et al., 2008). The precise etiology and pathogenesis of UC remain unclear. UC is associated with chronic disease and easy and repeated recurrence. Therefore, it is difficult to diagnose and treat in clinical practice. The World Health Organization (WHO) classifies UC as a modern refractory disease. In recent years, UC morbidity rates have been increasing (Dhamija et al., 2014) due to the influence of factors such as dietary habit changes and aggravated mental stress. Currently, no specific targeted treatment is available for UC in Western medicine (Meade et al., 2008); comparatively speaking, traditional Chinese medicine (TCM) and its preparations are more effective. TCM UC treatment can not only relieve the clinical symptoms of UC, but can also effectively prevent recurrence (Sandborn et al., 2016).

NF-κB mainly exists in the inactive form under normal physiological conditions. However, it can initiate transcription of multiple genes through various signal transduction pathways in the case of any abnormality (Bing et al., 2017). It plays a vital role in multiple physiopathological activities, particularly in inflammation and immune response (Eissa et al., 2017). It can regulate the expression of immunity- and inflammation-related factors and inflammatory mediators to exert its effects. Excessive NF-κB expression also plays an important role in the genesis and development of various diseases (Bing et al., 2017), including some inflammatory diseases, cardiovascular diseases, and tumors (Gu et al., 2017). Interaction area of NF-κB and profilin IκB, and the nuclear localization sequence are responsible for the binding of NF-κB with DNA, dimerization, and interaction with IκB, respectively. Thus, it allows the activated NF-κB to enter the nucleus to exert its function (Bing et al., 2017).

Silent information regulator 2 (Sir2)-related enzyme is a highly conserved NAD+-dependent histone deacetylase. There are 7 Sir2 homologous genes in humans, referred to as SIRT1–7. Of these, SIRT1 shows the highest homology and has become an important research focus in recent years (Lv et al., 2018). It was reported that SIRT1 protein has anti-inflammatory and anti-oxidative stress effects, and can reduce cell injury (Akimova et al., 2014).

miRNA is a class of small-molecule nucleotides that can regulate gene expression. It is involved in the UC-related gene or protein expression. The single-nucleotide polymorphism (SNP) of miRNA precursor can partly affect the sensitivity and pathogenetic process in UC patients (Bian et al., 2011), and it is of vital importance to the study of UC pathogenesis, as well as diagnosis and prognosis for UC. According to reports, miRNA is expressed in human serum. Moreover, serum miRNA can bind with protein and lipoprotein, thus avoiding being degraded by RNA hydrolase (Ghobadi et al., 2017). Serum miRNA derives from the pathways for cell apoptosis, cell necrosis, and cell secretion, as well as circulating cell lysis. Mature intracellular miRNA can be wrapped by lipoprotein or lipid to form the...
exosome, which is thereby transported out of the cell into the blood. Exosomes that enter the blood can enter the receptor cell again through endocytosis (Netz et al., 2017). Its coating can be removed in the receptor cell to release miRNA, thus exerting its biological function. The present study assessed the efficacy of downregulation of CircRNA MFHAS1 in preventing inflammatory responses in a model of Citrobacter rodentium-induced murine colitis.

2 Material and Methods

2.1 Animals

Fourteen C57BL/6 mice (age 5-6 weeks, weight 20-21 g, male) were housed at 22-23 °C, 55-60% humidity, 12/12 h light/dark cycle, and had free access to water and standard chow. All mice were allocated into 2 groups: sham (n = 6), colitis (n = 8). Mice in the colitis group were administered 5×10⁸ CFU/day of Citrobacter rodentium for 7 days. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Chongming branch of Xinhua Hospital.

2.2 Histopathology assay

Tissue samples were washed with PBS and fixed with 4% paraformaldehyde for 24 h. Tissue samples were deparaffinized, hydrated, and stained using hematoxylin and eosin for 5 min. Tissue samples were examined using an Olympus BX51 polarizing microscope (Olympus Corporation, Tokyo, Japan).

2.3 Enzyme-linked immunosorbent assay (ELISA) KIT

Tissue samples and cells were lysed with RAPA assay containing PMSF at 4 °C for 15 min. We used 10 μg protein to measure the IL-1β, IL-6, TNF-α, and IL-18 levels using ELISA.

2.4 Western blot analysis

Cells were lysed with RAPA containing PMSF at 4 °C for 15 min and were centrifuged at 12 000 g for 10 min at 4 °C. Protein concentrations were estimated by bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL). We used SDS-PAGE to resolve 50 μg of total protein, which was then transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST for 1 h and immunoblotted overnight at 4 °C with p65 NF-κB, SIRT1, and GAPDH (Santa Cruz). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz) at 4 °C. The explored the mechanism and function of CircRNA MFHAS1 in Citrobacter rodentium-induced murine colitis. These results suggest that CircRNA MFHAS1 is involved in the occurrence and development of Citrobacter rodentium-induced murine colitis.

2.6 Immunofluorescence and microscopy

Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min. Then, cells were blocked with 5% BSA containing 0.1% Triton X-100 at 25 °C for 1 h and incubated overnight with SIRT1 (1:100, Abcam; USA) at 4 °C. Cells were then washed with PBS for 15 min and incubated with Alexa Fluor 488 conjugated with anti-rabbit immunoglobulin G (IgG) (1:500 dilution) for 1 h. Cells were then washed with PBS for 15 min and stained with DAPI for 15 min at the dark. Images of cells were captured using an Eclipse TE200 fluorescent microscope (Nikon, Melville, NY).

2.7 Statistical analysis

Data were analyzed using SPSS 17.0 and are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey’s post hoc test were used for comparisons of individual data. p<0.05 was considered to be statistically significant.

3 Results

3.1 The expression of CircRNA MFHAS1 in Citrobacter rodentium-induced murine colitis

The explored the mechanism and function of CircRNA MFHAS1 in Citrobacter rodentium-induced murine colitis. HE staining showed ulcer in Citrobacter rodentium-induced murine colitis, but it did not occur in the sham control group (Figure 1A). We found that body weight was decreased, and there were higher levels of TNF-α, IL-1β, IL-6, and IL-18 in the Citrobacter rodentium-induced murine colitis group compared with the sham control group (Figure 1A-1E). CircRNA MFHAS1 expression was decreased in the Citrobacter rodentium-induced murine colitis group compared with the control group (Figure 1G-1H). These results suggest that CircRNA MFHAS1 is involved in the occurrence and development of Citrobacter rodentium-induced murine colitis.

3.2 CircRNA MFHAS1 regulates inflammation in vitro

We investigated the mechanism underlying the action of CircRNA MFHAS1 in colitis inflammation and assessed whether CircRNA MFHAS1 or CircRNA MFHAS1 inhibitor mimics increased or reduced the expression of CircRNA MFHAS1 in the in vitro model compared with the negative group (Figure 2A-2B). Overexpression of circRNA MFHAS1 reduced the levels of TNF-α, IL-1β, IL-6, and IL-18 in the in vitro model compared with the negative group (Figure 2C-2F), and downregulation of circRNA MFHAS1 increased the levels of TNF-α, IL-1β, IL-6, and IL-18 in the in vitro model compared with the negative group (Figure 2G-2J).
3.3 CircRNA MFHAS1 regulates inflammation in the in vitro model via miR-486-5p.

As shown in Figure 3A, there was increased expression of miR-486-5p in the colitis group compared with the negative group. Overexpression of CircRNA MFHAS1 reduced miR-486-5p expression in vitro compared with the negative group (Figure 3B). Data in Figure 3C show that CircRNA MFHAS1 regulates miR-486-5p to adjust inflammation in vitro. Figures 3D and 3E show that CircRNA MFHAS1 regulates the expression of miR-486-5p by directly targeting its mRNA 3'-UTR, and luciferase assay activity levels were reduced in the overexpression of CircRNA MFHAS1 group compared with the negative group. Overexpression of CircRNA MFHAS1 reduced miR-486-5p expression in vitro compared with the negative group (Figure 3F). CircRNA MFHAS1 inhibitor mimics reduced CircRNA MFHAS1 expression and increased miR-486-5p expression in vitro compared with the negative group (Figure 3G-3H). We found a negative correlation between CircRNA MFHAS1 and miR-486-5p (Figure 3I).

3.4 miR-486-5p regulates the SIRT1/NF-κB signaling pathway

We used gene chip technology to analyze the inflammation signal pathway in the in vitro model. MiR-486-5p mimics increased the expression of miR-486-5p in vitro compared with the negative group (Figure 4A). We found that SIRT1 expression was reduced and NF-κBp65 expression was increased in the
CircRNA MFHAS1 reduced inflammatory responses of colitis in vitro by overexpression of miR-486-5p compared with the negative group (Figure 4B). Our results show that the SIRT1/NF-κB signaling pathway may be regulated by miR-486-5p to further affect the inflammation in colitis (Figure 4C). We also found that miR-486-5p regulates the expression of SIRT1 by directly targeting its mRNA 3'-UTR (Figure 4D). Luciferase assay activity levels were reduced in miR-486-5p over-expression group compared with the negative group (Figure 4E). Overexpression of miR-486-5p suppressed SIRT1 protein expression and increased NF-κB protein expression in vitro compared with the negative group (Figure 4F-4H). It showed that overexpression of CircRNA MFHAS1 suppressed SIRT1 protein expression in vitro compared with the negative group (Figure 4I). Overexpression of CircRNA MFHAS1 suppressed NF-κB protein expression and increased SIRT1 protein expression in vitro compared with the negative group (Figure 4J-4L). Therefore, miR-486-5p regulates the NF-κB signaling pathway by SIRT1 to promote inflammatory responses in colitis.

Figure 2. CircRNA MFHAS1 regulates inflammation in vitro. CircRNA MFHAS1 expression (A and B) using overexpression of MFHAS1 or downregulation of MFHAS1; TNF-α (C), IL-1β (D), IL-6 (E) and IL-18 (F) levels by overexpression of MFHAS1; TNF-α (G), IL-1β (H), IL-6 (I) and IL-18 (J) levels by downregulation of MFHAS1. Negative, negative control group; MFHAS1, overexpression of CircRNA MFHAS1 group; MFHAS1 inhibitor, downregulation of CircRNA MFHAS1 group. **p<0.01 compared with negative control group.
Figure 3. CircRNA MFHAS1 regulates inflammation in vitro model by miR-486-5p. Gene chip results for miR-486-5p expression (A and B) in vivo and in vitro of overexpression of CircRNA MFHAS1; Analysis chart (C), CircRNA MFHAS1 regulates the expression of miR-486-5p by directly targeting its mRNA 3’-UTR (D), luciferase assay activity levels (E), miR-486-5p expression by overexpression of CircRNA MFHAS1 (F); CircRNA MFHAS1 expression (G) and miR-486-5p expression (H) by downregulation of CircRNA MFHAS1 group; negative correlation between CircRNA MFHAS1 and miR-486-5p (I). Negative, negative control group; MFHAS1, overexpression of CircRNA MFHAS1 group; MFHAS1 inhibitor, downregulation of CircRNA MFHAS1 group. **p<0.01 compared with negative control group.
3.5 miR-486-5p reduced the pro-inflammation effects of CircRNA MFHAS1 downregulation in vitro

Next, we used the in vitro model to analyze the mechanism of CircRNA MFHAS1 on inflammation in *Citrobacter rodentium*-induced murine colitis. Overexpression of miR-486-5p increased miR-486-5p expression, suppressed SIRT1 protein expression, and increased NF-κB protein expression in vitro following overexpression of CircRNA MFHAS1 compared with CircRNA MFHAS1 overexpression group (Figure 5A-5D). miR-486-5p reduced the anti-inflammation effects of CircRNA MFHAS1 overexpression on inhibition of TNF-α, IL-1β, IL-6, and IL-18 levels in vitro compared with the CircRNA MFHAS1 overexpression group (Figure 5E-5H).

3.6 The inactivation of SIRT1 reduced the anti-inflammation effects of CircRNA MFHAS1 on inflammatory responses in vitro

The study investigated the mechanism of anti-inflammation effects of CircRNA MFHAS1 in a *Citrobacter rodentium*-induced murine colitis model. Si-SIRT1 suppressed SIRT1 protein expression, and induced NF-κB protein expression in vitro by overexpression of CircRNA MFHAS1 compared with the CircRNA MFHAS1 overexpression group (Figure 6A-6C). The inactivation of SIRT1 reduced TNF-α, IL-1β, IL-6, and IL-18 levels in vitro by overexpression of CircRNA MFHAS1 compared with the CircRNA MFHAS1 overexpression group (Figure 6D-6G). These results show that CircRNA MFHAS1 regulates the SIRT1/NF-κB signaling pathway to affect inflammation in colitis through miR-486-5p.

4 Discussion

UC is most common in Western countries. Recent data indicate that the morbidity of UC in the USA is 200/100 000. Annually, about 0.1-0.15 billion US dollars is spent on UC treatment. In China, the economy has been rapidly developed in the past 10 years (Oliva et al., 2012) and dietary habits have greatly changed (Meade et al., 2008), with lower intake of vegetables and higher intake of processed Western-style foods (Hua et al., 2015). A recent investigation suggests that UC morbidity is negatively correlated with vegetable intake (Sandborn et al., 2012). In addition, ω-6 fatty acid, total fat, and meat intake are positively correlated with UC morbidity (Sandborn et al., 2012). From 2000 to 2012, the annual number of diagnosed cases of UC increased by 250%, with the recurrence rate reaching 72% worldwide. Moreover, USIs affecting a younger population (Hua et al., 2015). In this study, CircRNA MFHAS1 expression was found to be decreased in the *Citrobacter rodentium*-induced murine colitis model compared with the sham control group. Shi et al showed that MFHAS1 suppresses inflammation in Thr239 via inhibition of the TLR4 signaling pathway (Shi et al., 2017).

The etiology and pathogenesis of UC remain unclear. With the development of cytokine research, it was found that cytokines are closely correlated with the pathogenesis of inflammatory bowel disease (Scott & Lichtenstein, 2018). Cytokines can be classified into 2 types-pro-inflammatory...
A cytokine and anti-inflammatory-based on their effects on inflammatory response. The mechanism underlying the association between the difference two types has attracted growing attention (Rosario et al., 2017). The imbalance of the two types of cytokines is considered to be the main cause of the injury of intestinal mucosa (Allamneni et al., 2018). Cytokines are proteins or small-molecule polypeptides (Cho et al., 2016) synthesized and secreted by immune cells (eg, T cells, B cells, macrophages, and monocytes) and non-immune cells (eg, vascular endothelial cells, epidermal cells, and fibroblasts). Cytokines can transfer information between cells and have immunological functions (Rosario et al., 2017). Cytokines can extensively regulate immune response and hematopoietic function, and they participate in pathological processes such as inflammation and injury (Allamneni et al., 2018). Our in vitro results demonstrate that overexpression of CircRNA MFHAS1 reduces levels of TNF-α, IL-1β, IL-6, and IL-18. Zhong et al. (2015) reported that MFHAS1 reduced inflammation in sepsis via the TLR2/NF-κB signaling pathway.

In the absence of external stimulation, NF-κB exists in the cytoplasm in the form of NF-κB-IκB complex. IκBs are degraded in the presence of external stimulation (Galvez-Llompart et al., 2011; Yu et al., 2011). They include cytokine, oxidant, protein kinase C activator, virus, immunological stimulant, ultraviolet rays, and lipopolysaccharide. As a result, the free NF-κB dimer is released (Yu et al., 2011). At this time, NF-κB is translocated from cytoplasm to cell nucleus and binds with the intranuclear target gene κB sequence (Galvez-Llompart et al., 2011). Thus, it
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In addition, it can act on non-histones (such as NF-κB) through deacetylation, and reduce the expression of its downstream genes like iNOS. In addition, it was reported that in NF-κB-treated intestinal epithelial cells, the SIRT1 expression level decreases with increased NF-κB concentration (Wang et al., 2017). The above results suggest that SIRT1 is involved in NF-κB-induced intestinal epithelial cell injury. Moreover, it plays a role in protecting intestinal epithelial cells in this process. CircRNA MFHAS1 reduced inflammation in an in vitro model through reducing the expression of miR-486-5p.

SIRT1 not only reduces the release of inflammatory factors, but can also suppress the interactions between inflammatory factors and the corresponding cell receptors. Thus, it can restrain the inflammatory factor-mediated cytotoxic reaction (Sands et al., 2016). SIRT1 exerts its cell-protection effect through multiple mechanisms. For example, it can silence some apoptosis-inducing proteins through histone modification. In addition, it can act on non-histones (such as NF-κB) through deacetylation, and reduce the expression of its downstream genes like iNOS. In addition, it was reported that in NF-κB-treated intestinal epithelial cells, the SIRT1 expression level decreases with increased NF-κB concentration (Wang et al., 2017). The above results suggest that SIRT1 is involved in NF-κB-induced intestinal epithelial cell injury. Moreover, it plays a role in protecting intestinal epithelial cells in this process. CircRNA MFHAS1 reduced inflammation in an in vitro model through reducing the expression of miR-486-5p.

Figure 6. The inactivation of SIRT1 reduced the anti-inflammation effects of CircRNA MFHAS1 on inflammatory responses in vitro. SIRT1 and NF-κB protein expression (A and B) and Western blot assays of SIRT1 and NF-κB (C), TNF-α (D), IL-1β (E), IL-6 (F), and IL-18 (G) levels. Negative, negative control group; MFHAS1, overexpression of CircRNA MFHAS1 group; miR-486-5p, overexpression of miR-486-5p group. *p<0.01 compared with negative control group, ##p<0.01 compared with overexpression of CircRNA MFHAS1 group.

5 Conclusions

In this study, we demonstrated that CircRNA MFHAS1 reduced inflammatory responses in colitis by inhibiting the SIRT1/NF-κB signaling pathway and reducing the expression...
of miR-486-5p. These results show that CircRNA MFHAS1 has an anti-inflammation effect in colitis and has potential as a new drug or target for treatment of colitis.

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