Ginsenoside Rh2 Reduces Inflammation of Ulcerative Colitis via Elevating microRNA-30a-5p

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ABSTRACT

Ulcerative colitis (UC) is a kind of chronic inflammatory bowel disease, closely related to colon cancer. Ginsenoside Rh2 (GRh2) is a rare protopanaxadiol type triterpene saponins in ginseng, which has anti-inflammatory/cancer/diabetic impacts. To investigate the molecular mechanism in which GRh2 reduced inflammation of UC via upregulating microRNA (miR)-30a-5p, we detected miR-30a-5p in the tissues and serum of patients with active UC and in NCM460 cells after dextran sulfate sodium (DSS) treatment. Inflammation factors, nuclear factor-κB (NF-κB) and p65, were detected in NCM460 cells introduced with disparate concentrations of GRh2 after DSS treatment or transfection. We found that GRh2 increased expression levels of miR-30a-5p to inhibit inflammation and promote to advancement of NCM460 cell. In conclusion, enhancive miR-30a-5p further elevated GRh2’s repression UC’s progression. GRh2 drives miR-30a-5p to repress inflammation, NF-κB pathway and cell advancement, thereby mitigating UC.

INTRODUCTION

Ulcerative colitis (UC) is a kind of chronic inflammatory bowel disease (Jung et al., 2021). The incidence is increasing year by year, and patients with disease degree are prone to recurrence, which is also supposed to result in colon cancer (Li et al., 2021). Therefore, the harm of UC to the human body cannot be ignored. Nowadays, the best clinical treatment of UC is drug therapy. Current drugs for the treatment of UC include mesalazine (Watanabe et al., 2013), 5-aminosalicylic acid (Fukuda et al., 2020), steroids and immunosuppressive agents. However, long-term application of the drugs could result in diversified side impacts like drug resistance, liver and kidney toxicity, and allergic reactions, while traditional Chinese medicine has obvious advantages like memorable efficacy, reduced recurrence rate, and fewer side impacts in treating UC. New Chinese medicine treatment of UC has an extremely extensive prospect.

Ginsenoside Rh2 (GRh2) is a rare protopanaxadiol type triterpene saponins in ginseng (Hu et al., 2020). Many reports have confirmed GRh2 has anti-inflammatory/cancer/diabetic effects (Wang et al., 2021). For instance, GRh2 reduces allergic airway inflammation by controlling activation of nuclear factor-κB (NF-κB) in a mouse model of asthma (Li et al., 2015). A study reveals the molecular mechanism of GRh2 in UC. GRh2 alleviates UC by regulating the STAT3/miR-214 pathway (Chen et al., 2021). However, mechanism of GRh2 in treatment of UC requires to be further clarified.

MicroRNA (miRNA) is a class of tiny endogenous single-stranded non-coding RNA molecules with the function of regulating gene expression, participating in various immune-linked illnesses (Van der Goten et al., 2021). There is increasing evidences manifesting miRNAs can affect the disease activity in UC patients (Malham et al., 2021), and are dysregulated in the inflammatory environment of the intestinal mucosa of UC patients (Dubois-Camacho et al., 2019). MiR-20b, miR-98, miR-125b-1 and let-7e are specifically elevated in UC patients, and can be used as new biomarkers of UC (Coskun et al., 2013). MiR-141 plays an important role in intestinal inflammation in patients with active UC by inhibiting CXCL5 (Cai et al., 2017). Plentiful studies have manifested miR-30a-5p is reduced in illnesses (Zhang et al., 2021), and is considered a regulator of inflammation (Xiang et al.,...
MiR-30a-5p silencing targeting SOCS1 promotes macrophages M2 polarization and improves cardiac injury behind viral myocarditis (VMC) (Zhang et al., 2021). However, the role of miR-30a-5p in UC remains unclear. In this study, human normal colonic epithelial cells NCM460 were studied in vitro to verify the effect of GRh2 on UC. The results originally identified the molecular mechanism in which GRh2 alleviating the inflammation of UC by upregulating miR-30a-5p, and provided a theoretical basis for development GRh2 as a new target for UC treatment.

**MATERIALS AND METHODS**

**Human tissue samples**

24 active UC patients and 20 healthy individuals undergoing colonic mucosal biopsy within colonoscopy at Affiliated Hospital of Zunyi Medical University was enrolled (Li et al., 2017) from 2016 to 2017. After blood collection, serum was extracted by centrifuge and stored in a PAXgene™ tube covering RNA stabilization solution (Fisher Scientific, Waltham, MA) (Wu et al., 2011). Colon tissues of 11 patients undergoing surgical resection and corresponding healthy controls were collected, and biopsy tissues were obtained by mucosal dissection (Pekow et al., 2017). Biopsy tissues were obtained by mucosal dissection RNA later (Ambion, Austin, TX, USA) with quick-freezing in liquid nitrogen, and then the serum was stored with the biopsy tissue in a refrigerator at -80°C (Van der Goten et al., 2014). Pathological analysis via a pathologist was further confirmed as active UC. All patients in the study signed informed consent forms. This study was approved by the Ethics Committee of the Ethics Committee of Affiliated Hospital of Zunyi Medical University and performance of all operations were carried out according to the guidelines permitted by the ethics Committee.

**Cell culture**

Normal colonic epithelial cell NCM460 (ATCC, Manassas, VA, USA) were in Dulbecco’s Modified Eagle Medium (DMEM) appended with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, which was changed every other day. 80% confluent cells were selected for subsequent experiments (Liu et al., 2019).

NCM460 cells were treated with 2% dextran sulfate sodium (DSS) (MP Biomedicals, Santa Ana, USA) at 37°C for 4 d for establishment of an in vitro UC model (Nighot et al., 2013).

**Cell transfection**

NCM460 cells were seeded in a 24-well plate (5 × 10^4 cells/well) and cultured in antibiotic-free DMEM. When cell confluence reaches 80%, miR-30a-5p mimic/inhibitor and their negative controls (NC-mimic/inhibitor) were transfected into NCM460 cells, according to the method of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) manufacturing. The transfection efficiency was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR). All oligonucleotides were derived from Ribobio (Guangzhou, China) (Zhang et al., 2019).

**Cell counting kit (CCK)-8 detection of cell viability**

The viability of NCM460 cells treated or transfected with different concentrations of GRh2 (0, 10, 20, 50 μM, purity ≥ 98%, Merck KGaA, Darmstadt, Germany) was determined by CCK-8 method. NCM460 cells were seeded into 96-well plates (8000 cells/well) for culture. Addition of 10 μL CCK-8 reagent (Guanzhou Tianti Biotechnology Co., Ltd., Guangzhou, China), and incubation were implemented (Tian et al., 2020). Then a BioTek PowerWave spectrophotometer (Thermo Fisher, USA) was employed for detection of the absorbance at 450 nm. The inhibition rate was calculated as (the control - the experimental/the control) OD × 100% (Liu et al., 2019).

**Flow cytometry**

Aptosis of NCM460 cells was determined by detection and analysis (Ding and Wen, 2018). According to the manufacturer’s protocol, cells were isolated with trypsin and stained with Annexin V-luciferin isothiocyanate/Propyl iodide (Beyotime Institute of Biotechnology). The results were analyzed applying FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (version 7.6.1; Tree Star, Inc.) (Deng et al., 2020).

**RT-qPCR**

Total RNA was extracted from NCM460 cell and tissue samples using TRIzol reagent (Invitrogen) according to the instructions, with miRNA Reverse Transcription Kit (Promega, USA) or PrimeScript RT-PCR Kit (Takara, Japan) for reverse transcription of miRNA and mRNA into cDNA. StepOne real-time PCR system (Applied Biosystems, USA) was used for RT-qPCR detection (Luo et al., 2020). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as a loading control for mRNA, with U6 for miRNA. The primers were from GeneCopoeia (California, USA). PCR primer sequences applied in the research were shown in Table I.

**Enzyme-linked immunosorbent assay (ELISA)**

GRh2 with different concentrations or transfected into serum-free DMEM cell culture medium was incubated. The supernatant was sucked by a straw, the impurities were removed by centrifugation at 300 g, and...
the supernatant was taken for subsequent experiments (Kraskiewicz et al., 2020). Concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in cell culture supernatant were determined using ELISA kits (eBioscience, Thermo Fisher Scientific, USA) according to manufacturer’s instructions. Absorbance at 450 nm was measured with a microplate meter (Biotek Instruments, Inc., Winooski, VT, USA) (Goudarzi et al., 2020).

**Table I. Primer sequence design.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’-3’)</th>
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<tbody>
<tr>
<td>miR-30a-5p</td>
<td>F: ACACTCCAGCTGGGTGTAACATCCTCGAC; R: CAGTGCGGTCTCGTGAGT</td>
</tr>
<tr>
<td>U6</td>
<td>F: CGCGCTTGGCAGCACATAACT; R: ACGCCCTCAATGCGGCCTGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: CCGGAGAGGAGGGCTTACG; R: CAGAAATGCGCAATGCAAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: GTCTGGGCCATAGGCTGA; R: CAGCCTCTTCTATTCCTGC</td>
</tr>
<tr>
<td>IL-1p</td>
<td>F: TGTGTTAGTGCTGCTGTGA; R: TGTGAAATGCCACCTTTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AGGAGCGAGACCCCACCTAACA; R: AGGGGGGCTAAGCAGTTGGT</td>
</tr>
</tbody>
</table>

**Western blot**

Total protein of NCM460 cells was extracted by radioimmunoprecipitation analysis of a mixture of lysate buffer (Beyotime Biotechnology, China) and protease and phosphatase inhibitors (MedChemExpress, China). Quantitative analysis was carried out by Bicinchoninic Acid protein analysis kit (Thermo Scientific, USA). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrical imprinting was carried out on polyvinylidene fluoride membrane. Then the membrane was blocked with 5% skim milk powder (Guo et al., 2021). Then, incubation of the membrane was with the following primary antibodies: anti-GAPDH (1: 2000; Abbkine, Inc., USA), anti-NF-κB p65 and anti-NF-κB p-p65 (both 1: 1000) (Cell Signaling Technology, USA). After washing with Phosphate Buffered Saline Tween-20 buffer solution, membranes were incubated with diluted horseradish peroxidase-conjugated secondary antibody (1: 500, 00, Santa Cruz Biotechnology, USA) (Rezayat et al., 2018). After washing with Tris-buffered saline with Tween 20, the protein bands were observed using the enhanced chemiluminescence kit (Beyotime) according to the manufacturer’s instructions (Peng et al., 2020).

**Statistical analysis**

All data are presented as mean±standard error. Prizm GraphPad V6 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. A t test was used to compare differences between the two groups, and one-way ANOVA and Bonferroni multivariate comparison test were used to compare differences between three groups and more than three groups. \( P < 0.05 \) was considered a significant difference (Tambuwala et al., 2019).

**RESULTS**

**GRh2 inhibits inflammation and the NF-κB pathway**

In order to explore the mechanism of GRh2 action in UC cells, NCM460 cells were treated with 2% DSS to construct UC model in vitro, and then treated with different concentrations of GRh2 (0, 10, 20, 50 μM). In chronic intestinal inflammation, multiple immune and non-immune and intestinal epithelial cells (NCM460) produce a variety of pro-inflammatory/anti-inflammatory factors, such as TNF-α, IL-1β and IL-6, which can affect apoptosis of NCM460 cells (Zeng et al., 2020). TNF-α, IL-1β and IL-6 were determined in the supernatant of GRh2 and NCM460 cells with different concentrations (Fig. 1A, B). Results demonstrated that GRh2 inhibited TNF-α, IL-1β and IL-6 in a dose-dependent manner. Therefore, NF-κB was detected to clarify that GRh2 memorably repressed NF-κB p-p65 (Fig. 1C). Results manifested GRh2 inhibited the inflammation response and the NF-κB pathway in UC cells.

**GRh2 drives UC cell advancement**

Next, we explored expression of GRh2 in UC tissues. Cell progression was detected (Fig. 2A-B), indicating that GRh2 had a concentration-dependent effect on NCM460 cell progression after DSS treatment. These results confirmed that GRh2 can effectively promote UC cell process in a concentration-dependent manner. Among the above treatments, 50 μM GRh2 had the most obvious effect on UC cells. 50 μM GRh2 was selected for subsequent experiments.

**Mir-30a-5p has a significant inhibitory effect on active UC patients**

To understand the difference of miR-30a-5p between active UC patients and healthy controls, miR-30a-5p was detected in serum of 24 active UC patients and 20
healthy controls, as well as in colon tissues of 11 active UC patients and 11 healthy controls. The results showed that miR-30a-5p was significantly decreased in active UC patients compared with healthy controls ($P < 0.05$) (Fig. 3A, B). Relative miR-30a-5p was detected in normal or DSS-introduced NCM460 cells to ensure that Mir-30a-5P was significantly inhibited in DSS-treated cells compared with normal ($P < 0.05$) (Fig. 3C).

Characterization of MiR-30a-5p in UC cells

To investigate the characteristics of miR-30a-5p in UC cells, miR-30a-5p mimics/inhibitors and NC-mimic and NC-inhibitor were transfected into NCM460 cells.
without introduction of GRh2, respectively. miR-30a-5p was detected in the transfected NCM460 cells (Fig. 4A), revealing that vs. the NC-mimic/inhibitor, miR-30a-5p in the cells introduced with miR-30a-5p mimic/inhibitor was clearly enhanced or declined (P < 0.05). Detection of inflammatory-linked factors was in NCM460 cells behind transfection manifested enhancive miR-30a-5p inhibited the inflammation (P < 0.05) (Fig. 4B, C). It came out in the transfected NCM460 cells, augmented miR-30a-5p restrained NF-κB p-p65 (P < 0.05) (Fig. 4D), while NF-κB p65 did not change memorably. The transfected NCM460 cell progression was then measured to ensure that inhibition of Mir-30A-5P significantly inhibited NCM460 cell progression (P < 0.05), while upregulating one enhanced that (P < 0.05) (Fig. 4E, F).

GRh2 inhibits UC progression via elevating miR-30a-5p

To explore the effect of GRh2 on Mir-30a-5P in NCM460 cells, we detected miR-30a-5p in NCM460 cells treated with different concentrations of GRh2. The results showed that GRh2 drove miR-30a-5p (P < 0.05) (Fig. 5A). To further investigate the molecular mechanism of GRh2 controlling UC progression by increasing miR-30a-5p, we transfected miR-30a-5p mimic/inhibitor and NCs into NCM460 cells introduced by GRh2 (50 μM). NCM460 cells were then detected to progress behind GRh2 import and transfection. The results showed that the enhancement of miR-30a-5p further enhanced the effect of GRh2 by inhibiting inflammatory response, phosphorylation of NF-κB p65, and enhancing UC cell progression, while mir-30a-5p inhibitors attenuated GRh2 inhibition of UC progression by enhancing miR-30a-5p (Fig. 5B-F). In conclusion, GRh2 alleviates UC by upregulating miR-30a-5p.

DISCUSSION

UC is a long-term intestinal inflammatory illness. Its pathogenesis refers to diversified genes and pathways (Wang et al., 2021). Nowadays, little is known about its pathogenesis. There is a popular belief that the illness is stimulated via environmental, immune, genetic and intestinal flora disorders and other reasons (Zhou et al., 2021). Chinese medicine has been employed for treating UC for a long time in China. Recently, numerous pharmaceutical companies have started to pay attention to the research and development of natural products owing to considerations like drug resistance, cost and safety. It has become an important direction for the treatment of UC to discover the main active ingredients from Chinese traditional medicine (Zhou et al., 2021).

Studies have confirmed that GRh2 is an intermediate metabolite of ginseng (Zhang et al., 2020), which has anti-inflammatory/cancer/diabetic/obesity functions (Wang et al., 2021). For instance, Rh2 enhances immune cell activity but inhibits tumor cell growth (Lee et al., 2018). Rh2 inhibition is used to treat the cellular progression of cancer and can also be used to alleviate the side effects of chemotherapy or radiotherapy (Li et al., 2020). Rh2 also plays a role in the treatment of UC. In previous studies, GRh2 alleviates DSS-stimulated colitis by enhancing TGF-β signaling (Ye et al., 2014). In this study, we found that GRh2 alleviates DSS-stimulated UC by elevating miR-30a-5p. In addition, other studies have shown that the ingredients contained in other Chinese medicines also have a considerable impact on the treatment of UC. For example, pomegranate polyphenols reduce intestinal inflammation of UC in vivo and in vitro via Mir-145/p70S6K1/HIF1α axis (Kim et al., 2017). Anemoside B4, a compound with anti-inflammatory activity, prevents acute UC by inhibiting the TLR4/NF-κB/MAPK pathway (Ma et al., 2020). Resveratrol enhances T-regulatory cells by inhibiting Mir-31 to prevent colitis stimulated by 2, 4,
6-nitrobenzene sulfonate solution (Alrafas et al., 2020).

To reveal the mechanism of GRh2 action in UC, NCM460 cells were treated with 2% DSS to establish an inflammatory model, and then treated with different concentrations of GRh2 (0, 10, 20, 50 μM). The results showed that GRh2 inhibited inflammation in a concentration-dependent manner, which is consistent with previous studies (Chen et al., 2021). GRh2 also has anticancer effects in UC related colorectal cancers. However, there are only 2 reports on the function of GRh2 in UC, and no studies have been conducted to clarify the therapeutic effect of GRh2 in human UC patients. Therefore, the molecular mechanism of GRh2 in UC needs to be further studied, and a large number of studies are needed to verify and ensure its safety.

A large amount of evidence suggests that miRNA is involved in the pathogenesis of UC. For instance, miR-155 is involved in the pathogenesis of UC by targeting FOXO3a (Ji et al., 2014). MiR-141 participates in UC pathogenesis via targeting CXCL5 (Cai et al., 2017). Meanwhile, miRNA can control the biological behavior of UC. For instance, miR-155 targets Est-1 and stimulates UC via the Th17 pathway mediated by IL-23/17/6 (Hou et al., 2017). Inhibition of miR-330-3p alleviates DSS stimulated UC and apoptosis by enhancing er stress component XBP1 (Chen et al., 2020). This study initially found that miR-30a-5p was inhibited in tissues and serum of patients with active UC and in DSS-introduced NCM460 cells. Enhanced Mir-30a-5p drives UC cell progression, which is inhibited by inhibition of Mir-30a-5p. Differences in miR-30a-5p also affect inflammatory responses, NF-kB and p65 phosphorylation. Considering previous reports, in UC, elevated Mir-31 and -155 control the IL-13 pathway and p65 phosphorylation. Considering previous reports, in UC, miR-30a-5p also affect inflammatory responses, NF-κB is inhibited by inhibition of Mir-30a-5p. Differences in enhanced Mir-30a-5p drives UC cell progression, which is inhibited by inhibition of Mir-30a-5p. Differences in miR-30a-5p was inhibited in tissues and serum of patients (Chen et al., 2017). Inhibition of miR-330-3p alleviates DSS stimulated UC and apoptosis by upregulating the endoplasmic reticulum stress components XBP1. Hereditas, 157: 18. https://doi.org/10.1186/s41065-020-00135-z


CONCLUSION

In conclusion, GRh2 promotes the progression of UC cells and controls the progression of UC in a dose-dependent manner. Enhancement of miR-30a-5p inhibits the progression of UC by inhibiting inflammation and activation of the NF-κB pathway. GRh2 alleviates UC by elevating miR-30a-5p. The results of this study once again confirmed the characteristics of GRh2 in UC, and provided a theoretical basis for GRh2 to be used as a new UC treatment drug in the future.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Ginsenoside Rh2 Reduces Inflammation of Ulcerative Colitis


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