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Down-regulation of miR-215 attenuates lipopolysaccharide-induced inflammatory injury in CCD-18co cells by targeting GDF11 through the TLR4/NF-κB and JNK/p38 signaling pathways

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Key words: Ulcerative colitis, miR-215, GDF11, TLR4/NF-κB p65 signaling pathway, JNK/MAPK signaling pathway.

Abstract. Ulcerative colitis (UC) is a risk factor for carcinogenesis of colorectal cancer, which is associated with disruption of the epithelial barrier and disorder of the
inflammatory response. It has been reported that the expression of microRNA (miR)-215 is upregulated in patients with long-term UC. The present study aimed to investigate the effects of miR-215 on lipopolysaccharide (LPS)-induced inflammatory injury in CCD-18Co cells, as well as to identify the underlying possible molecular mechanisms. CCD-18Co cells were treated with 1 µg/ml LPS to induce inflammatory injury. Reverse transcription-quantitative PCR was performed to determine the expression of miR-215 in LPS-treated CCD-18Co cells. Moreover, a dual luciferase reporter system assay was used to evaluate the interaction of miR-215 and growth differentiation factor 11 (GDF11) in CCD-18Co cells. The expression of miR-215 was significantly upregulated in LPS-treated CCD-18Co cells. Knockdown of miR-215 significantly alleviated the inflammatory response and oxidative stress in LPS-treated CCD-18Co cells. In addition, GDF11 was identified as a direct binding target of miR-215 in CCD-18Co cells. Knockdown of miR-215 significantly increased the expression of GDF11, but decreased the expression levels of Toll-like receptor (TLR)4, phosphorylated (p)-p65, iNOS, p-p38 and p-JNK in LPS-treated CCD-18Co cells. Collectively, the present findings indicated that knockdown of miR-215 alleviated oxidative stress and inflammatory response in LPS-treated CCD-18Co cells by upregulating GDF11 expression and inactivating the TLR4/NF-κB and JNK/p38 signaling pathways.

Introduction

Ulcerative colitis (UC) is recognized as a chronic inflammatory disorder of the colon, which is a well-established risk factor for carcinogenesis of colorectal cancer (Farraye et al., 2010; Chen et al., 2014b). UC is categorized into chronic, idiopathic and relapse, and the repetition of epithelial cells impairment and repair is the main cause of developing colorectal carcinoma (Terzic et al., 2010). The primary aim of the treatment of UC is to rapidly improve the symptoms of patients with UC and maintain the improvement of the disease in the long term. Steroids, hormones, immunosuppressants or their derivatives are major therapeutic methods for patients with UC; however, modest effects and serious side effects are observed. In recent
years, 5-aminosalicylates (5-ASAs) are considered as a potential standard therapeutic method for improving the symptoms of patients with mild-moderate active UC (Hanauer, 2016). However, 80% of patients with UC are moderate-to-severe. Although there is an increasing number of studies investigating the etiology of UC, the molecular mechanisms of pathogenesis of UC remain unknown. Emerging evidence has revealed that the expression levels of inflammatory cytokines, including IL-1β, IL-6 and TNF-α, in colon tissues, regulated by the Toll-like receptor 4 (TLR4)/NF-κB p65 signaling pathway or the JNK/MAPK signaling pathway, serve vital roles in the development of UC (Zhang et al., 2015; Min-Seok et al., 2018; Wang et al., 2019a).

MicroRNAs (miRNAs/miRs), a class of non-coding transcripts <25 nucleotides, serve essential roles in a variety of biological processes by regulating the expression of targets genes by binding to their 3’-untranslated regions (UTRs) (Bartel, 2009; Zhang and Li, 2018). Aberrant expression of miRNAs has been reported to be involved in immune and inflammatory responses (Fabbri et al., 2012; Chen et al., 2014a). For instance, inflammatory factors and lipopolysaccharide (LPS) can induce the upregulation of miRNAs, including miR-155 and miR-146a, by modulating the activation of the NF-κB signaling pathway (Taganov et al., 2006; Williams et al., 2008).

It has been revealed that miRNAs exert critical roles in the pathogenesis of UC (Qu et al., 2019). For example, Feng et al observed that miR-126 was significantly upregulated in patients with UC, and overexpression of miR-126 could activate the NF-κB signaling pathway (Feng et al., 2012). In addition, high expression of miR-15 in patients with UC has been shown, and upregulation of miR-15 induced nuclear translocation of p-65 and increased the expression levels of inflammatory cytokines, such as IL-8 and IFN-γ (H. Zhang and Li, 2018). Moreover, miR-215 is upregulated in patients with long-standing UC and the abnormal expression of miR-215 is associated with UC-related colorectal cancer (Pekow et al., 2018). However, the role of miR-215 in the pathogenesis and progression of UC is yet to be fully elucidated (Tan et al., 2013; Pekow et al., 2018). Therefore, the present study was
conducted to determine the role of miR-215 in the progression of UC and investigate its underlying molecular mechanisms, in order to provide novel targets for treatment of UC.

**Materials and methods**

**Cell culture**

The normal human colon epithelial cell line, CCD-18Co, was obtained from American Type Culture Collection. CCD-18Co cells were cultured in Eagle's Minimum Essential Medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.).

**Cell transfection**

miR-215 agomir, miR-215 antagonir and GDF11 small interfering (si)RNA were purchased from Shanghai GenePharma Co., Ltd. The miR-215 agomir, miR-215 antagonir or GDF11 siRNA were transfected into CCD-18Co cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. The culture medium containing 10% FBS was changed at 6 h after transfection, and the cells were then incubated for 42 h at 37°C.

**Inflammatory analysis**

The culture media of CCD-18Co cells was collected and centrifuged at 1,400 rpm/min to obtain the cell-free supernatant. Expression levels of IL-6, TNF-α, IL-10, IL-1β, SOD and GSH were detected using ELISA, in accordance with the manufacturer’s instructions (ELK Biotechnology, Wuhan, China).

A Lipid Peroxidation (MDA) Assay Kit (Nanjing JianCheng Bioengineering Institute, Nanjing, China) was used to detect the level of MDA in CCD-18Co cells.

**Real-time quantitative reverse transcription PCR (RT-qPCR)**

TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNAs from CCD-18Co cells. Subsequently, the total RNAs were used for reverse
transcription with a QuantiTect Reverse Transcription kit (Qiagen, NY, USA). qPCR was conducted using the SuperRT One Step RT-PCR kit (CWBIO, Beijing, China). The relative expression of miR-215 was evaluated via the $2^{-\Delta\Delta Cq}$ method, and the expression of U6 was used as the internal control. Primers were used as follow: miR-215-forward: 5'-GGGACCTATGAATTGAGACC-3', miR-215-reverse: 5'-CTCAACTGCTGTCGTGGAGTC-3'; U6-forward: 5'-AACGCTTCACGAATTTGCGT-3'.

Dual-luciferase reporter system

The 3'UTR sequence of GDF11, including wild-type (WT) or mutant (MT) miR-215 binding site, was sub-cloned into pmirGLO vectors (Promega Corporation). The miR-215 or NC was co-transfected with pmirGLO-GDF11-WT or pmirGLO-GDF11-MT vector into CCD-18Co cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, luciferase activity was detected at 48 h using a dual-luciferase assay kit (Promega Corporation) according to the manufacturer’s instructions.

Western blotting

Cells were collected, washed three times with 1X PBS and suspended with RIPA buffer to extract proteins. Then, the concentration of protein was detected with a BCA Protein Assay (Bio-Rad Laboratories, Inc.), and 30 µg proteins of each sample were separated by electrophoresis. Western blotting was performed as previously described (Luan et al., 2018). The primary antibodies were used as follows: GDF11 (1:2,000; cat. no. ab239515; Abcam), TLR4 (1:1,000; cat. no. ab22048; Abcam), phosphorylated (p)-p65 (1:500; cat. no. ab53489; Abcam), p65 (1:1,000; cat. no. ab16502; Abcam), iNOS (1:1,000; cat. no. ab213987; Abcam), p-p38 (1:1,000; cat. no. 4511; Cell Signaling Technology, Inc.), p-JNK (1:1,000; cat. no. 9255; Cell Signaling Technology, Inc.), p-JNK (1:1,000; cat. no. 3708; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.)
Reactive oxygen species (ROS) analysis

ROS generation was assessed by staining cells with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFHDA; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol.

Data statistics

All of the data in the present study are presented as the mean ± standard deviation. Data analysis was conducted using SPSS 22.0 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference. Results were obtained from at least three independent experiments.

Results

Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells

To investigate the role of miR-215 in the progression of UC, the expression of miR-215 in CCD-18Co cells treated with LPS was detected. As shown in Fig. 1A, the expression of miR-215 was significantly increased in LPS-treated CCD-18Co cells, compared with the control group. In addition, overexpression of miR-215 markedly increased miR-215 expression in CCD-18Co cells, while knockdown of miR-215 notably decreased the expression of miR-215 in cells (Fig. 1B).

Knockdown of miR-215 significantly reduced the expression of miR-215 in LPS-stimulated CCD-18Co cells, compared with LPS + NC group (Fig. 1C). In addition, LPS significantly increased the expression levels of TNF-α, IL-6 and IL-β, while decreasing the expression of IL-10 in CCD-18Co cells. However, these LPS-induced changes were reversed in the presence of miR-215 antagonir (Fig. 1D-G). The results suggested that downregulation of miR-215 could inhibit LPS-induced inflammatory response in CCD-18Co cells.
GDF11 was a direct binding target of miR-215

Next, the present study predicted the target gene of miR-215 using Starbase 2.0. As presented in Fig. 2A, GDF11 was identified as a potential target of miR-215. A dual luciferase assay was conducted to confirm the direct interaction between miR-215 and GDF11. As shown in Fig. 2B, miR-215 agomir suppressed the luciferase activity of GDF11-WT, but it did not affect the luciferase activity of GDF11-MT. In addition, overexpression of miR-215 significantly inhibited the expression of GDF11 in CCD-18Co cells, while knockdown of miR-215 notably upregulated the expression of GDF11 in cells (Fig. 2C). Thus, GDF11 was a direct binding target of miR-215.

Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells via regulation of GDF11/TLR4/NF-κB p65 signaling

To determine whether the TLR4/NF-κB p65 signaling pathway exerts a role in the progression of UC mediated by the miR-215/GDF11 axis, western blotting was performed. As shown in Fig. 3A-E, LPS significantly reduced the expression of GDF11, but increased the expression levels of TLR4, p-p65 and iNOS in CCD-18Co cells. However, these LPS-induced changes were reversed in the presence of miR-215 antagonir. Additionally, LPS-induced NO production in CCD-18Co cells was reversed by miR-215 knockdown (Fig. 3F). It was found that GDF11 knockdown markedly downregulated the expression of GDF11, but upregulated the expression of TLR4 in CCD-18Co cells (Fig. 3G-I). These results demonstrated that downregulation of miR-215 inhibited the LPS-induced inflammatory response in CCD-18Co cells via inactivation of TLR4/NF-κB p65 signaling by targeting GDF11.

Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells via inactivation of JNK/p38 signaling pathway

To examine whether the JNK/p38 signaling pathway contributes to the development of UC, the phosphorylation and expression levels of JNK and p38 were detected using western blot analysis. As shown in Fig. 4A-C, treatment with LPS resulted in increased phosphorylation of p38 and JNK proteins in CCD-18Co cells,
but these LPS-induced changes were reversed by miR-215 knockdown. Therefore, it was revealed that knockdown of miR-215 was able to inhibit LPS-induced inflammatory response in CCD-18Co cells via inactivation of the JNK/p38 signaling pathway.

**Downregulation of miR-215 alleviated LPS-induced oxidative stress in CCD-18Co cells**

Next, the present study investigated the effects of miR-215 on LPS-induced oxidative stress by detecting intracellular ROS generation. As shown in Fig. 5A, treatment with LPS significantly increased ROS generation in CCD-18Co cells. However, knockdown of miR-215 reversed the production of ROS caused by LPS (Fig. 5A). Furthermore, LPS treatment significantly increased the level of MDA, but decreased the activities of SOD and GSH in CCD-18Co cells (Fig. 5B-D), but these changes were markedly reversed by miR-215 knockdown. Collectively, the results suggested that knockdown of miR-215 could alleviate LPS-induced oxidative stress in CCD-18Co cells.

**Discussion**

It has been reported that miRNAs are closely associated with the development and progression of UC (Polytarchou et al., 2015; Yang et al., 2019). Ando et al found that the expression of miR-21 was downregulated in CD3 T cells during the remission phase of UC, indicating that miR-21 may be a novel target candidate to trigger remission in UC (Ando et al., 2016). In addition, knockdown of miR-223 alleviates intestinal barrier in inflammatory bowel disease (IBD) mice (Wang et al., 2016; Li et al., 2020). Meng et al also indicated that miR-129 could ameliorate intestinal inflammation in trinitrobenzene sulphonic acid-induced colitis mice by targeting FBW7 and inhibition of the NF-κB pathway (Meng et al., 2020). The present results indicated that knockdown of miR-215 upregulated the expression of GDF11, resulting in the inactivation of TLR4/NF-κB signaling pathway and amelioration of inflammatory response in LPS-treated CCD-18Co cells. Moreover, knockdown of
miR-215 abolished the activation of the JNK/p38 signaling pathway in LPS-treated CCD-18Co cells. The present study demonstrated that miR-215 serves an essential role in the progression of UC.

Increasing evidence has revealed that miR-215 is involved in the development of a variety of cancer types. For instance, increased expression of miR-215 is associated with the poor prognosis of patients with high-grade glioma, and overexpression of miR-215 significantly promotes the proliferation of glioma cells by inhibiting the expression of RB1 (Meng and Xi, 2017). In addition, miR-215 is considered an oncogene in gastric cancer (Li et al., 2016). However, it has been reported that miR-215 also acts as a tumor suppressor in cancer types, including breast cancer, colon cancer and non-small cell lung cancer (Cai et al., 2017; Gao et al., 2019a; Ullmann et al., 2019). Nonetheless, the complex function of miR-215 in the progression of cancer, its roles in inflammatory disease, are yet to be fully elucidated. The differential expression of miR-215 has been reported in multiple inflammatory diseases, including diabetic nephropathy, hepatitis and UC (Huang et al., 2019; Wang et al., 2019b). Previous studies have also identified the upregulation of miR-215 in patients with UC, while its function on the progression of UC and the underlying molecular mechanisms remain unknown.

In the present study, it was found that the expression of miR-215 was significantly upregulated in LPS-treated CCD-18Co cells. In addition, GDF11 was identified as a binding target of miR-215. It has been reported that GDF11 is involved in embryogenesis, metabolic disturbance and a variety of cancer types (Shimajiri et al., 2011; Sinha et al., 2014). Moreover, a recent study revealed that treatment with GDF11 could decrease the levels of pro-inflammatory factors in apolipoprotein E-null mice and inhibit neuroinflammation in Alzheimer’s disease (Mei et al., 2016; Zhang et al., 2018). Upregulation of GDF11 can suppress inflammatory response in inflammatory arthritis and psoriasis by inactivating the NF-κB signaling pathway (Li et al., 2019; Wang et al., 2019c). In DSS-induced colitis, GDF11 significantly suppresses the secretion of IL-1β and inactivates NLRP3 inflammation by inhibiting the activation of the TLR4/NF-κB p65 signaling pathway (Wang et al., 2018). In the
present study, it was demonstrated that knockdown of miR-215 significantly increased
the expression GFD11, but decreased the expression levels of TLR4 and NF-κB p65
in LPS-treated CCD-18Co cells. Consistent with a previous study (Wang et al., 2018),
the current results suggested that knockdown of GDF11 could increase the expression
of TLR4 in CCD-18Co cells. Therefore, these data indicated that knockdown of miR-
215 could alleviate LPS-induced inflammatory response in CCD-18Co cells via
regulating the GDF11/TLR4/NF-κB p65 signaling pathway.

Emerging evidence has demonstrated the vital roles of the JNK/MAPK signaling
pathway in the pathogenesis of UC (Gao et al., 2018). Activation of the JNK/MAPK
signaling pathway is involved in the regulation of inflammatory response, apoptosis
and oxidative stress (Panahi et al., 2018). It has also been shown that increasing the
phosphorylation level of JNK/MAPK signaling transduction and overproduction of
malondialdehyde (MDA), iNOS and TNF-α, IL-1β, and IL-6 were responsible for the
progression of UC (Shi et al., 2016). Another study revealed that treatment with
chlorogenic acid could attenuate the development of UC induced by dextran sodium
sulfate by inhibiting the activation of the JNK/MAPK signaling pathway, as well as
decreasing the level of MDA and increasing the activities of SOD and GSH (Gao et
al., 2019b). Consistent with previous studies, the present findings demonstrated that
treatment with LPS significantly activated the JNK/MAPK signaling pathway,
enhanced the level of MDA and decreased the activities of SOD and GSH in CCD-
18Co cells. However, these changes were markedly reversed by miR-215 knockdown.

In conclusion, the present results suggest that knockdown of miR-215 inhibited the
inflammatory response and oxidative stress in LPS-stimulated CCD-18Co cells by
inactivating the TLR4/NF-κB p65 and JNK/p38 signaling pathways. Therefore, miR-
215 may be a potential biomarker and therapeutic target for the treatment of UC.

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Author’s contribution
Study design, literature research, experimental study was performed by Boyang Sun and Kai Xing. Data acquisition, data analysis and statistical analysis was performed by Chen Qi and Ke Yan. Yan Xu was responsible for guarantor of integrity of entire study, manuscript preparation and manuscript editing.

Ethics approval and consent to participate
The study was carried out in accordance with the World Medical Association Declaration of Helsinki was and approved by the Ethics Committee at The Affiliated Stomatological Hospital of Nanjing Medical University.

Competing interests
The authors declare that they have no competing interests.

Data Availability
The data sets used in this study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1 Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells. (A) CCD-18Co cells were treated with 1µg/ml LPS for 48 h. The level of miR-215 in CCD-18Co cells was detected by RT-qPCR. (B) CCD-18Co cells were transfected with NC, miR-215 agomir and miR-215 antagonir for 48 h. The level of miR-215 in CCD-18Co cells was detected by RT-qPCR. (C) CCD-18Co cells were transfected with miR-215 antagonir for 48 h, then exposed to 1µg/ml LPS for 24 h. The level of miR-215 in CCD-18Co cells was detected by RT-qPCR. (D, E, F, G) CCD-18Co cells were transfected with miR-215 antagonir for 48 h, then exposed to 1µg/ml LPS for 24 h. The levels of TNF-α, IL-6, IL-1b and IL-10 in the cells-free supernatant was determined using ELISA kit. **P<0.01 vs. control group. ##P<0.01 vs. LPS + NC group.

Figure 2 GDF11 was a direct binding target of miR-215. (A) Gene structure of GDF11 at the position of 1070-1076 indicates the predicted target site of miR-215 in its 3'UTR. (B) Luciferase activity was detected following co-transfecting with WT/MT GDF11 plasmid and miR-215 in CCD-18Co cells using the dual luciferase reporter assay. **P<0.01 vs. vector-control group. (C) CCD-18co cells were transfected with NC, miR-215 agomir and miR-215 antagonir for 48 h. The level of GDF11 in CCD-18Co cells was detected by RT-qPCR. **P<0.01 vs. NC group.

Figure 3 Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells via regulation of GDF11/TLR4/NF-κB p65 signaling. (A) CCD-18Co cells were transfected with miR-215 antagonir for 48 h, then exposed to 1 µg/ml LPS for 24 h. Expression levels of GDF11, TLR4, p-p65, iNOS in CCD-18Co cells were detected with western blotting. β-actin was used as an internal control. (B, C, D, E) The relative expression of GDF11, TLR4 and iNOS in CCD-18Co cells was normalized to β-actin; The relative expression of p-p65 in CCD-18Co cells was normalized to p65. (F) ELISA was adopted to determine the level of NO in the cell-free supernatant. (G) CCD-18Co cells were transfected with GDF11 siRNA
for 48 h. Expression levels of GDF11 and TLR4 in CCD-18Co cells were detected with western blotting. β-actin was used as an internal control. (H, I) The relative expression of GDF11 and TLR4 in CCD-18Co cells were normalized to β-actin. **P<0.01 vs. control group. #P<0.05, ##P<0.01 vs. LPS + NC group.

Figure 4 Downregulation of miR-215 inhibited LPS-induced inflammatory response in CCD-18Co cells via inactivation of JNK/p38 signaling pathway. CCD-18Co cells were transfected with miR-215 antagomir for 48 h, then exposed to 1 µg/ml LPS for 24 h. (A) Expression levels of p-p38 and p-JNK in CCD-18Co cells were detected with western blotting. β-actin was used as an internal control. (B) The relative expression of p-p38 in CCD-18Co cells was normalized to p38. (C) The relative expression of p-JNK in CCD-18Co cells was normalized to JNK. **P<0.01 vs. control group. ##P<0.01 vs. LPS + NC group.

Figure 5 Downregulation of miR-215 alleviated LPS-induced oxidative stress in CCD-18Co cells. CCD-18Co cells were transfected with miR-215 antagomir for 48 h, then exposed to 1 µg/ml LPS for 24 h. (A) Flow cytometry was used to detect the generation of ROS. (B, C, D) The production of MDA, SOD and GSH in cell-free supernatant was detected by ELISA. **P<0.01 vs. control group. ##P<0.01 vs. LPS + NC group.
A

GDF11 1070-1076 5’-...CCAGCCUCUAUCUUCAGGUCAAU...-3’ (WT)

miR-215 3’-CAGACAGUUAAGUAUCCAGUA-5’

GDF11 5’-...CCAGCCUCUAUCUUCGAUAUGAU...-3’ (MT)

B

Relative luciferase activity

Control Vector-control miR-215 agomir

WT MT

C

Relative GDF11 level (fold change)

Blank NC miR-215 agomir miR-215 antagonir
HISTOLOGY AND HISTOPATHOLOGY

**Figure 1.**

- **A:** Western blot analysis showing expression levels of GDF11, TLR4, p-p65, p65, and iNOS.
- **B-E:** Bar graphs illustrating relative expression levels of GDF11, TLR4, p-p65, and iNOS under different conditions: Control, LPS, LPS + NC, LPS + mir-215 antagonir. Significant differences are marked with **,** **,** and ###.
- **F:** Graph showing NO production under control, LPS (1 μg/ml), LPS + NC, LPS + mir-215 antagonir conditions. Significant differences are marked with **.*
- **G-H:** Western blot and bar graph for GDF11 and TLR4 expression under control, siRNA-ctrl, GDF11 siRNA conditions. Significant differences are marked with **.*
- **I:** Bar graph for relative GDF11 expression under control, siRNA-ctrl, GDF11 siRNA conditions.
A

B

C

<table>
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Relative p-p38 expression

Control: 0.2, LPS: 0.4, LPS + NC: 0.6, LPS + miR-215 antagonist: 0.8

Relative p-JNK expression

Control: 0.2, LPS (1μg/ml): 0.4, LPS + NC: 0.6, LPS + miR-215 antagonist: 0.8

**p < 0.05, ***p < 0.01, ###p < 0.001