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MicroRNA-21-5p promotes the inflammatory response after spinal cord injury by targeting PLAG1

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Abstract. – **OBJECTIVE:** The aim of this study was to investigate the expression level of microRNA-21-5p and its target gene PLAG1 in the serum of patients with spinal cord injury (SCI), as well as the role and mechanism of microRNA-21-5p in lipopolysaccharide-induced primary microglial inflammation.

PATIENTS AND METHODS: The levels of microRNA-21-5p and PLAG1 in the peripheral blood of 24 patients with spinal cord injury and 24 healthy people were examined by quantitative Polymerase Chain Reaction (qPCR) analysis. The expressions of inflammatory factors, including IL-1, IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA). After microglial BV2 cells were treated with different concentrations of LPS, microRNA-21-5p, and PLAG1 expressions were examined by qPCR, while IL-1, IL-6, and TNF-α levels in cell supernatant were measured by ELISA. Finally, the binding relationship between microRNA-21-5p and its target gene was analyzed by Luciferase reporter assay.

RESULTS: The data showed that microRNA-21-5p was upregulated in the serum of SCI patients, and also increased in LPS-treated cells, with a certain concentration dependence. On the contrary, PLAG1 was remarkably downregulated, suggesting that the above two genes were negatively correlated in SCI patients. ELISA results indicated that the knockdown of microRNA-21-5p or overexpression of PLAG1 reduced the levels of IL-1, IL-6 and TNF-α in BV2 cells. Meanwhile, microRNA-21-5p might be involved in regulating the inflammatory response of BV2 cells by modulating PLAG1.

CONCLUSIONS: MicroRNA-21-5p promotes the inflammatory response after SCI by targeting PLAG1.

Key Words:
Spinal cord injury, Inflammatory response, MiRNA, PLAG1.

Introduction

Spinal cord injury (SCI) refers to the dysfunction of movement and sensation under the plane of complete or incomplete SCI caused by external forces on the spinal cord[1]. Epidemiological data show that traffic accidents and high-altitude falls are the main causes of injuries, and the number of patients is increasing by nearly 10,000 every year[2]. SCI includes primary and secondary injury mechanisms. On the basis of primary injury, the inflammatory damage of progressive and self-destructive cascade amplification of spinal cord tissues caused by multiple factors is called secondary SCI, and the damage degree is far greater than that of primary one[3]. The dysfunction caused by SCI has no clear therapeutic impact revealed by studies in China and foreign countries. Hence, it is of great significance to explore the immune mechanism of secondary SCI to preserve residual spinal cord function to the greatest extent and promote the reconstruction of injured spinal cord structure and function.

Microglia are immune cells of the central nervous system. As part of the innate immunity of the central nervous system, microglia can perform the functions of phagocytosis and scavenging of damaged neurons and cell debris, providing a suitable living environment for other cells[4]. Within a few minutes of SCI, microglia begin to migrate to the site of injury and change morphologically, becoming indistinguishable from mononuclear macrophages. This process is caused by microglia sensing the molecular stimuli associated with injury (myelin sheath, cell debris, laminin, etc.), which are activated through toll-like receptor recognition and show significant morphological and

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functional changes[5]. SCI is capable of causing the production of other secretory factors, such as cytokines, which bind to receptors on the cell surface to further activate microglia. The activation of immune receptors upregulates the signaling pathways of transcription factors, immunoregulatory receptors and secretory factors, involving microglia, which constitute a strong pro-inflammatory response, amplifying inflammation and exacerbating pathological responses (secondary injury)[6]. Various inflammatory molecules secreted by microglia can promote inflammatory cell infiltration and mediate the apoptosis of nerve cells, thus leading to the aggravation of secondary SCI. Some studies have found that proinflammatory cell mediators, such as IL-1, IL-6, and iNO produced by activated microglia can damage neurons[7]. Therefore, regulating the activation of microglia cells and controlling the corresponding acute inflammatory response have important clinical significance for the treatment of SCI[8].

MiRNAs are associated with central nervous system injury. A large number of miRNAs are expressed abnormally in the cerebral cortex in rat models of brain injury[9]. In addition, the expressions of 97 miRNAs are remarkably changed in the spinal cord of SCI rates. Bioinformatics analysis has shown that target genes regulated by these differentially expressed miRNAs may be closely bound up with SCI pathophysiological process[10]. The above studies have shown that miRNA with abnormal expression in injured site of central nervous system may be involved in its pathological process.

MicroRNA-21-5p is highly expressed in many tissue and organ injuries[11,12], such as brain trauma, cerebral infarction, myocardial infarction, muscle injury, and renal ischemia reperfusion, indicating a close relationship between microRNA-21-5p and tissue injury. In addition, Lu et al[13] have reported that in Wistar rats induced by dextran sulfate sodium (DSS), the degree of intestinal inflammation, intestinal epithelial cell apoptosis, and its pathology score were remarkably attenuated by microRNA-21-5p gene knockout.

In this study, a significant increase was detected in microRNA-21-5p expression in peripheral blood of SCI patients, and *in vitro* investigations also indicated that microRNA-21-5p expression in Lipopolysaccharide (LPS)-stimulated microglia was increased with the concentration. Therefore, this study explored the possible role of microRNA-21-5p in the development of SCI and its potential mechanism from the perspective of inflammatory response.

Patients and Methods

Clinical Specimen

From May 2017 to June 2019, peripheral blood samples were collected from 24 acute traumatic SCI patients and 24 healthy volunteers recruited at Shandong Provincial Hospital Affiliated to Shandong First Medical University. Inclusion criteria: patients with SCI at different degrees after trauma confirmed by MRI, CT or surgery. Exclusion criteria: patients with neurological diseases, cerebrovascular diseases, spinal cord compression or other related diseases. All individual participants included in the study signed informed consent. This study was approved by Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Injury classification of patients with SCI was based on the American Spinal Cord Injury Association (ASIA) guidelines.

Cell Culture and Cell Transfection

The mouse microglia cell line BV2 provided by Shanghai Institute of Cells (Chinese Academy of Sciences) (Shanghai, China) was cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 1% penicillin and streptomycin and 10% fetal calf serum (Gibco, Rockville, MD, USA) in an incubator at 37°C with suitable humidity and 5% CO_2. Transfection was performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in strict accordance with the instructions.

Construction of LPS-Stimulated Microglia Cell Line

BV2 cells were treated with LPS at different concentrations (0, 1, 5, 10 μg/mL). After 24 hours, the concentrations of the inflammatory factors IL-1, IL-6 and TNF-α were measured to select an optimal LPS concentration for the next experiment.

Quantitative Polymerase Chain Reaction (qPCR) Assay

Total RNA was extracted from the plasma of SCI patients following the instructions of the Stabilized Blood-to-CT™ Nucleic Acid Preparation kit (Thermo Fisher Scientific, Waltham, MA, USA). Complementary

deoxyribose nucleic acid (cDNA) was obtained using M-MLV reverse transcriptase, and qPCR was conducted with LightCycler 480 Probes Master Kit, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as internal reference. The primer sequences used in qPCR reaction PLAG1: F: 5'-ATCCCTCTCACCACCTTTCTTT-3', R: 5'-GCCACCTTGTAACTCCATCAG-3'. MicroRNA-21-5p: F: 5'-TAGCTTATCAGACTGATG-3', R: 5'-CAGTGCGTGTCGTGGAGT-3'. GAPDH: F: 5'-TCAAGATCATCAGCAATGCC-3', R: 5'-CGATACCAAAGTTGTCATGGA-3'. U6: F: 5'-ATACAGAGAAAGTTAGCACGG-3', R: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Luciferase Assay

The sequence of PLAG1 [including microRNA-21-5p binding site (PLAG1-WT) and PLAG1 mutant (PWRN2-MUT) containing mismatched microRNA-21-5p binding sequences] was cloned into pmirGLO Luciferase vector (Promega, Madison, WI, USA). 20 µL of Stop & Glo stop solution was added to the reaction tube, and the fluorescence intensity used to detect the internal reference (Renilla fluorescein) was measured again.

Western Blotting Assay

Cells were lysed, shaken on ice for 30 minutes, and centrifuged at 4°C, 14000 × g for 15 minutes. Total protein concentration was calculated by bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blotting analysis was carried out based on standard procedures.

Enzyme Linked Immunosordbent Assay (ELISA)

Specific ELISA kits (Abcam, Cambridge, MA, USA) were performed to measure IL-1, IL-6, and TNF-α concentrations in the serum of SCI patients, and supernatant collected from cultured cells.

Statistical Analysis

Measurement data were presented as mean ± standard deviation (SD). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Expression of MicroRNA-21-5p and Inflammatory Factors in Serum of SCI Patients

QPCR detected that microRNA-21-5p expression was remarkably upregulated in patients with SCI compared with that in normal controls (Figure 1A). Then, ELISA results manifested that the levels of inflammatory factors IL-1, IL-6, and TNF-α in SCI patients' plasma were markedly elevated (Figure 1B-1D). After treating BV2 cells with LPS, the levels of microRNA-21-5p and IL-1, IL-6, and TNF-α were all gradually upregulated as the LPS concentration was increased (Figure 1E-1H). The above results indicated that microRNA-21-5p expression was bound up with the inflammatory response induced by SCI.

PLAG1 was a Target Gene of MicroRNA-21-5p

It was found that microRNA-21-5p could be remarkably upregulated or downregulated by transfection of microRNA-21-5p mimics or inhibitors (Figure 2A). In addition, bioinformatics analysis revealed that PLAG1 was a potential target gene of microRNA-21-5p, and the interaction between the two genes was showed by Luciferase assay (Figure 2B). To further verify their regulatory relationship, PLAG1 expression was examined after transfection of microRNA-21-5p inhibitor in BV2 cells. Figures 2C-2D showed that both PLAG1 mRNA and the protein levels were significantly increased. Next, a significant decrease was detected in PLAG1 expression in peripheral blood of patients with SCI (Figure 2E), and Pearson analysis indicated that microRNA-21-5p was markedly negatively correlated with PLAG1 expression, with R2 = 0.3757, $p=0.0015$ (Figure 2F). Moreover, in BV2 cells administrated with LPS, PLAG1 expression was found to be downregulated with a certain concentration dependence (Figure 2G). At the same time, the transfection efficiency of PLAG1 overexpression plasmid and siRNA was tested for subsequent research (Figure 2H).

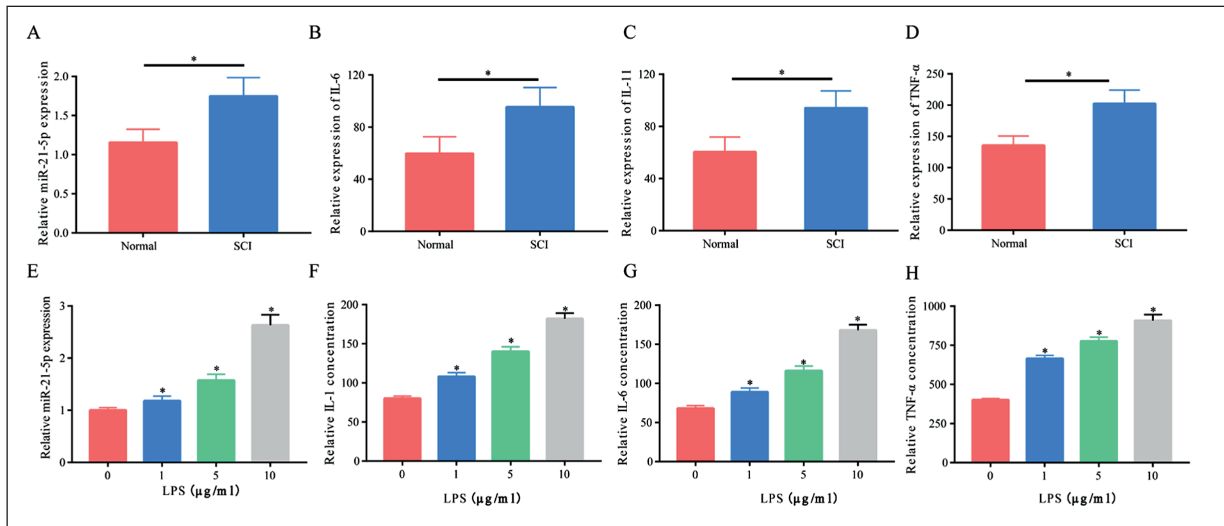

Figure 1. Expressions of miR-21-5p and inflammatory factors in peripheral blood and BV2 cells in patients with SCI. **A,** qRT-PCR showed that miR-21-5p was upregulated in peripheral blood of 24 pairs of SCI patients compared with that of healthy controls. **B-D,** The expressions of IL-1, IL-6 and TNF-α in peripheral blood of 24 pairs of SCI patients and healthy controls were increased. **E,** qRT-PCR detected the expression of miR-21-5p in microglia stimulated by LPS increased with increasing concentration. **F-H,** Under LPS stimulation, the expressions of IL-1, IL-6 and TNF-α in microglia were detected increased by ELISA, with a certain concentration dependence. (*$p<0.05$).

Aberrantly Expressed MicroRNA-21-5p or PLAG1 Promoted Inflammatory Response

To further explore the impact of abnormal expression of microRNA-21-5p or PLAG1 on SCI, BV2 cells were treated with 5 μg/mL LPS for subsequent experiments. It was found that LPS administration induced an increase in microRNA-21-5p expression, which was reversed by microRNA-21-5p inhibitor (Figure 3A). Meanwhile, a same tendency in the levels of IL-1, IL-6 and TNF-α was observed in BV2 cells of each group (Figure 3B-3D). In addition, under the action of LPS, a decrease was detected in PLAG1 expression, which was then increased by transfection of PLAG1 overexpression plasmid (Figure 3E). Meanwhile, ELISA results showed that upregulating PLAG1 expression partially reversed the stimulating effect of LPS on inflammatory factors (Figure 3F-3H).

MicroRNA-21-5p Functioned by Regulating PLAG1

Since PLAG1 is a target gene of microRNA-21-5p, it was speculated that microRNA-21-5p functions by acting on PLAG1. To verify this, the expressions of two proteins were simultaneously overexpressed or knocked down, and ELISA was performed to measure inflammatory response. Consequently, after LPS acted on BV2 cells, simultaneous upregulation of microRNA-21-5p and PLAG1 reversed the promoting effect of microRNA-21-5p mimics on the levels of inflammatory factors, measured by ELISA (Figure 4A-4C). Meanwhile, the opposite results were observed after simultaneous downregulation of microRNA-21-5p and PLAG1 (Figure 4D-4F). The above observations suggested that microRNA-21-5p could regulate the inflammation induced by SCI by modulating PLAG1.

Discussion

The pathophysiological process of SCI can be divided into primary injury stage and secondary injury stage. The primary injury is mainly related to the cause of the SCI and the severity of the violence that caused the injury. The secondary injury is based on the primary SCI with local bleeding, intracellular enzyme release, free radical generation in the spinal cord tissues induced by local internal environmental disturbances, such as cell necrosis, mainly include processes, such as inflammatory response, axon demyelination, nerve cell death, and glial scar formation, which determines the outcome of patients with SCI[14]. The secondary injury stage is characterized by

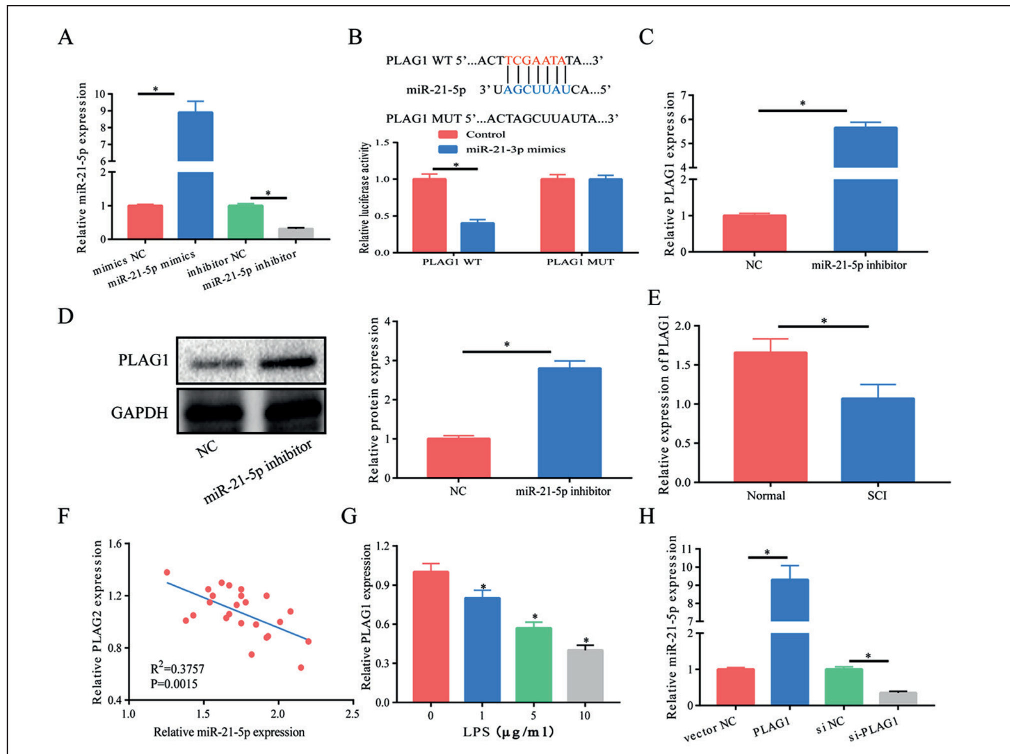

Figure 2. PLAG1 was a target gene of miR-21-5p. **A,** The expression of miR-21-5p was significantly increased after transfection with miR-21-5p mimics, but was decreased after transfection with miR-21-5p inhibitor. **B,** Dual-Luciferase reporter gene experiment confirmed a potential binding relationship between miR-21-5p and PLAG1. **C, D,** After down-regulating the expression of miR-21-5p, the mRNA and protein expressions of PLAG1 were significantly increased. **E,** PLAG1 was significantly reduced in peripheral blood of patients with SCI. **F,** miR-21-5p expression was negatively correlated with PLAG1 expression. **G,** PLAG1 expression was significantly downregulated in microglia stimulated by LPS. **H,** PLAG1 overexpression plasmid increased PLAG1 expression significantly, which was decreased by si-PLAG1 (* $p<0.05$).

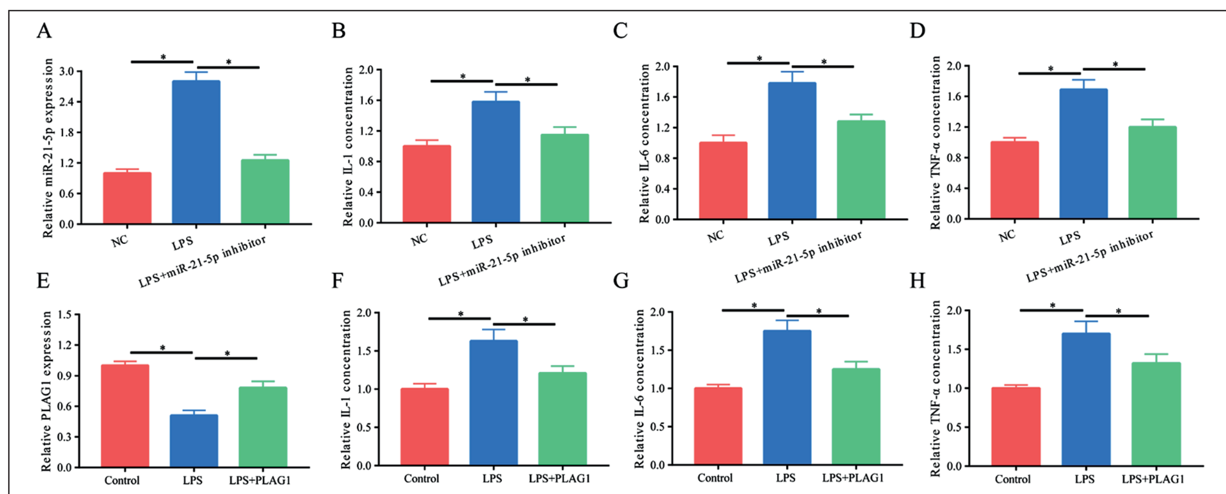

Figure 3. Aberrantly expressed miR-21-5p or PLAG1 promoted inflammatory response. **A,** 5 μg/mL LPS stimulation induced an increased expression of miR-21-5p in BV2 cells, which could be reversed by miR-21-5p inhibitor. The expressions of IL-1 (**B**), IL-6 (**C**) and TNF-α (**D**) in microglia were significantly increased under LPS stimulation, and simultaneously down-regulating the expression of miR-21-5p partially reversed this effect. **E,** 5 μg/mL LPS stimulation induced a reduction in miR-21-5p expression in BV2 cells, which could be reversed by miR-21-5p mimics. The expressions of IL-1 (**F**), IL-6 (**G**) and TNF-α (**H**) in microglia were significantly increased under LPS stimulation, while simultaneously upregulating the expression of PLAG1 partially reversed this effect (*$p<0.05$).

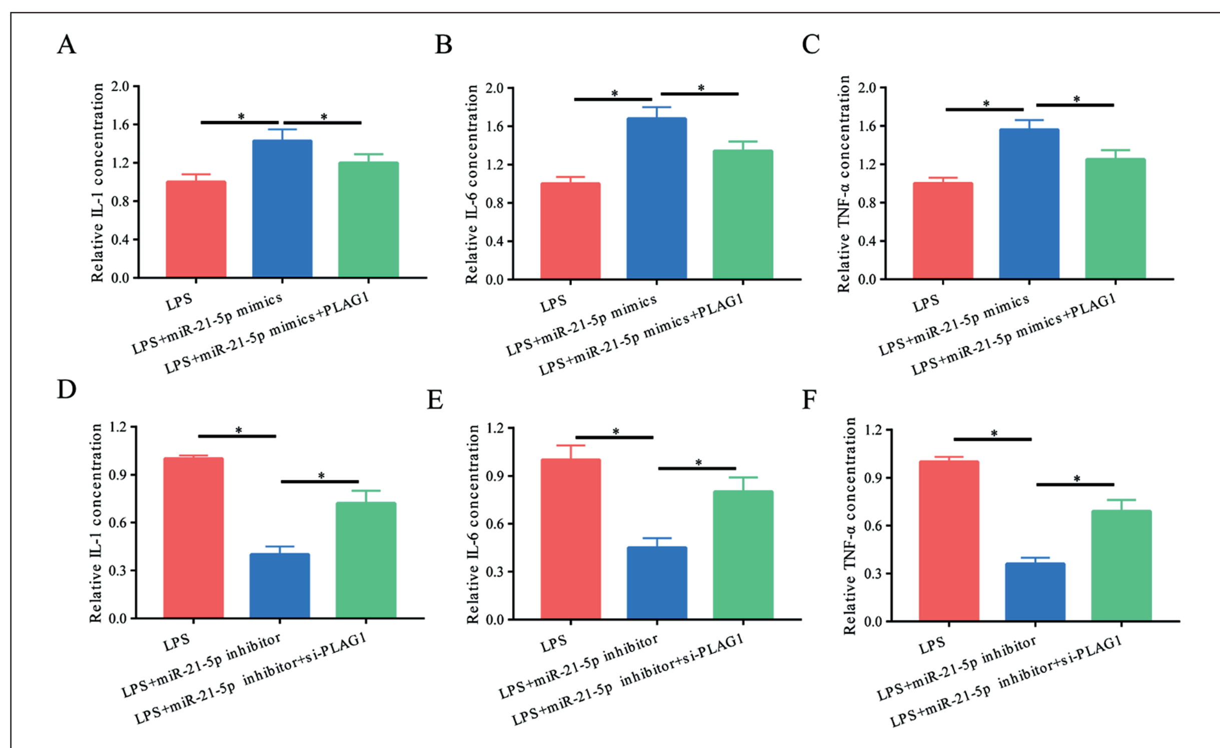

Figure 4. MiR-21-5p functioned by regulating PLAG1. **A-C,** After up-regulating the expression of miR-21-5p in microglia stimulated by LPS, the expressions of IL-1, IL-6 and TNF-α were significantly increased, while simultaneously up-regulating PLAG1 partially reversed this effect. **D-F,** In the microglia stimulated by LPS, the expressions of IL-1, IL-6 and TNF-α were decreased significantly after down-regulating the expression of miR-21-5p, and this effect was partially reversed after the simultaneously down-regulation of PLAG1 (*p<0.05).

further injury of neurons and glial cells, resulting in remarkably expanded SCI and involvement of higher spinal cord segments. Therefore, SCI firstly manifests as direct damage to the spinal cord structure caused by traumatic violence, followed by a series of pathophysiological changes, such as ischemia, hypoxia, apoptosis, and post-traumatic inflammatory response in the center of the SCI, resulting in degeneration of local nerve cells necrosis and inhibition of glial cell repair function, which finally leads to neurological dysfunction[15]. Therefore, it is necessary to explore the prevention and treatment of SCI from the perspective of inflammatory response.

Bak et al[16] have confirmed that miRNAs are highly expressed in the central nervous system, including the brain and spinal cord. Meanwhile, 350 types of miRNAs are discovered in adult rats, of which 269 are highly expressed in the spinal cord of SCI rats and 36 in that of normal rats[17]. Although the specific role and mechanism of miRNAs in the nervous system are still unclear, it has been largely shown that miRNAs are engaged in the development of nervous system injury, neurodegenerative diseases and intracranial tumors. Nakamishi et al[18] showed that in the mouse SCI model, microRNA-223 and microRNA-124a were both abnormally expressed, among which the former was associated with severe responses after injury, while the latter was affected by cell death after injury. In spinal cord transection mice model, inhibiting microRNA-20a improves the apoptosis of motor neurons and promotes nerve regeneration and nerve function restoration by regulating Ngnl (neurogenin 1)[19]. Therefore, the changes in miRNAs suffering from SCI may induce certain pathophysiological processes, but the underlying mechanism remains to be further studied, especially the correlation with inflammatory response after SCI, which needs further exploration. In this research, a significantly increased microRNA-21-5p expression was detected in plasma samples of SCI patients, and elevated levels of inflammation factors were revealed at the same time. In addition, in BV2 cells administrated with LPS, microRNA-21-5p expression and inflammatory reaction were also enhanced.

Further, the pleomorphic adenoma 1 (pleomorphic adenoma gene 1, PLAG1), a proto oncogene located in human chromosome 8q12 containing 5 exons, is a target gene of microRNA-21-5p. Its total length of cDNA is 7313bp, among which only the last end of exon 4 and the starting end of exon 5 are encoded, with a total coding box of 1503 bp. Its abnormal expression has been reported in hepatoblastoma, lymphocytic leukemia, and other tumors[20,21]. However, PLAG1 has not been reported in research on SCI. In this study, for the first time, PLAG1 expression in peripheral blood of SCI patients was examined, and it was found to be remarkably downregulated and negatively correlated with microRNA-21-5p. Furthermore, *in vitro* cell experiments showed that the downregulation of microRNA-21-5p or upregulation of PLAG1 could inhibit inflammatory responses and microRNA-21-5p might regulate inflammatory responses induced by SCI by modulating PLAG1. In this study, it was firstly reported that microRNA-21-5p was firstly reported to be involved in the regulation of inflammatory response caused by SCI, which may provide a new target for the future treatment.

Conclusions

MicroRNA-21-5p was firstly found to be involved in the regulation of inflammatory response caused by SCI, possibly by direct regulation of PLAG1 to change the levels of inflammation-related factors IL-1/IL-6/TNF-α. This result not only enriched the research content of SCI, but also provided a new therapeutic target for intervention for secondary SCI. However, the pathophysiological changes of SCI are very complex processes, involving a variety of cytokines, inflammatory mediators, and cells. Therefore, further studies are needed on the secondary SCI and effective intervention measures.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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