Knockdown of lncRNA SNHG14 alleviates LPS-induced inflammation and apoptosis of PC12 cells by regulating miR-181b-5p

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Abstract. Spinal cord injury (SCI) is a traumatic central nervous system disorder that leads to permanent functional loss, and unavailable treatment of this disease results in poor quality of life. However, the specific role of long non-coding RNA small nucleolar RNA host gene 14 (lncRNA SNHG14) in SCI has not been fully studied. The aim of the current study was to investigate the role of SNHG14 and its regulatory mechanism in lipopolysaccharide (LPS)-induced PC-12 cells. LPS was used to stimulate PC-12 cells to simulate inflammatory injury following SCI in vitro. Cell viability and apoptosis were respectively assessed by Cell Counting Kit-8 assay and TUNEL assay. Western blotting was performed to detect the expressions of apoptosis-related proteins. The mRNA levels of SNHG14 and microRNA (miR)-181b-5p were detected by reverse transcription-quantitative PCR. The target of SNHG14 was predicted by bioinformatics analysis and subsequently validated by a luciferase reporter assay. ELISA was then used to detect the levels of inflammatory factors. The results indicated that LPS induced inflammation, decreased cell viability and increased the apoptosis of PC-12 cells. Interference of SNHG14 alleviated this type of injury of PC-12 cells. Bioinformatics prediction and luciferase reporter assay demonstrated that miR-181b-5p could directly bind to SNHG14. Moreover, mechanistic investigations revealed that the miR-181b-5p inhibitor could reverse the inhibitory effects of SNHG14 silencing on cell viability, inflammation and apoptosis of PC-12 cells. To conclude, the present results showed that SNHG14 knockdown alleviated PC-12 cell inflammation and apoptosis induced by LPS via regulating miR-181b-5p, which might provide a novel insight into the treatment of SCI.

Introduction

The spinal cord, which functions as a transporter to relay messages from the brain to the body and vice versa, transmits messages from the autonomic nervous system, which regulates numerous physiological functions, including heartbeat, blood pressure and temperature regulation. However, damage to the spinal cord can perturb all these functions. Spinal cord injury (SCI) is a severe central nervous system disorder that has been increasingly observed in the clinic over the past few years in China (1). Due to lack of successful treatment for SCI, 50-80% of patients with SCI suffer with long-term moderate to severe traumatic pain, which significantly impacts the families of the patients both financially and in providing care (2). Since significant damage is produced by secondary injury alone, the inhibition of processes involved in secondary injury may be one of the most effective approaches to prevent the deterioration of SCI and promote functional recovery (3,4). The secretion of stimulating cytokines, which can facilitate neuronal damage and cell apoptosis, triggers the stimulus response and secondary injury. Previous studies have demonstrated that the levels of inflammatory factors, including IL-1β, TNF-α and IL-6, were elevated at the early SCI stage (5-7). Interestingly, inhibiting the secretion of these inflammatory factors was reported to serve a central role in protecting nerve cells (6). Thus, investigating the molecular mechanism by which inflammatory cytokines could be inhibited may be a novel and effective strategy for the treatment of SCI.

Long non-coding RNAs (lncRNAs) are non-coding RNAs of >200 nucleotides in length that lack protein-coding ability (8). LncRNAs were reported to be associated with the initiation and progression of numerous types of diseases, including Parkinson's disease, leukemia and periodontitis (9-13). Knockdown of small nucleolar RNA host gene 14

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(SNHG14) protected nerve cells from damage by alleviating inflammation (14). In addition, IncRNA SNHG14 was revealed to promote neuronal impairment and inflammatory response induced by cerebral ischemia/reperfusion injury by regulating the microRNA (miRNA/miR)-136-5p/Rho-associated protein kinase 1 (ROCK1) signaling pathway, while SNHG14 knockdown could improve neurological function in vivo (15). SNHG14 knockdown was also discovered to alleviate lipo-polysaccharide (LPS)-induced acute lung injury through the miR-34c-3p-dependent inhibition of Wnt inducible signaling pathway protein 1 (16).

miRNAs are a class of non-coding RNAs that serve as important gene expression regulators (17). miRNAs negatively regulate target gene expression at the post-transcriptional level, which in turn regulates numerous cellular processes, such as cell differentiation, aging and apoptosis (17,18). Previous studies have reported that miR-181a/b-5p, which was found to modulate vascular inflammation and endotoxin tolerance, alleviated inflammation in monocrotaline-induced pulmonary arterial hypertension by targeting endocan (19,20).

To the best of our knowledge, no previous studies have reported the role of SNHG14 in SCI. Therefore, the present study aimed to investigate whether SNHG14 could participate in the neuronal protection and alleviate inflammation by regulating miR-181a/b-5p in SCI, in addition to determining the underlying mechanism.

Materials and methods

Cell culture and treatment. The PC-12 cell line was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured at a density of 1x10^6 cells/ml in DMEM-high glucose (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C and the medium was replaced every 2-3 days.

To stimulate inflammatory cell injury following SCI, PC-12 cells were treated with 1, 2, 5 or 10 µg/ml LPS (Sigma-Aldrich; Merck KGaA) for 12 h. In addition, the 12-h LPS treatment of cells were treated with 1, 2, 5 or 10 µg/ml LPS (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Bio, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from PC-12 cells was extracted and purified using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Bio, Inc.) using the following protocol: 65˚C for 10 min, 25˚C for 10 min, 37˚C for 60 min and 70˚C for 10 min. qPCR was subsequently performed on an ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR Green Real-Time PCR kit (Takara Bio, Inc.). The PCR reaction mixture (20 µl) consisted of 10 µl Real Time PCR Master mix, 2 µl primer mix (0.2 µM) and 20 ng cDNA diluted in RNase-free water. The primer sequences were as follows: SNHG14 forward, 5'-CGT TGT CGA AAG CTA AAA GAA-3' and reverse, 5'-TGT TTT CCA TCT CAC ACA AAT GC-3'; GAPDH forward, 5'-GCACCTCAAGGCTAGA-3' and reverse, 5'-TGGTTGAAGACGCCAGTGA-3'; miR-181-5p forward, 5'-ACACTCCAGCTGGAGACCTGTTAGC-3' and reverse, 5'-TGGTGTCGGAGCTGAC-3'; and U6 forward, 5'-CTCGCTTCCGCGAGCACAGGCA-3' and reverse, 5'-AAGCTTACGCAGGCGCATCGTCA-3'. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, then 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, for a total of 40 cycles. GAPDH and U6 served as the internal controls. Expression levels were quantified using the 2^-ΔΔCq method (21).
Western blotting. Following treatment, whole cell lysates were acquired using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was quantified using a bicinchoninic acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 20 μg protein was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto nitrocellulose membranes and blocked in 5% non-fat milk for 2 h at room temperature. The membranes were then incubated with the following primary antibodies at 4°C overnight: Anti-Bcl-2 (cat. no. ab32124; 1:1,000; Abcam), anti-Bax (cat. no. ab32503; 1:1,000; Abcam), anti-cleaved caspase-9 (cat. no. ab2324; 1:1,000; Abcam), anti-cleaved caspase-3 (cat. no. ab32042; 1:1,000; Abcam), anti-caspase 3 (cat. no. ab13847; 1:1,000; Abcam), anti-caspase-9 (cat. no. ab32539; 1:1,000; Abcam) and anti-GAPDH (cat. no. ab8245; 1:1,000; Abcam). Following primary antibody incubation, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) at room temperature for 2 h in the dark. Protein bands were visualized using an ECL Western Blotting Detection reagent (GE Healthcare) and densitometric analysis was performed using Image J software (v.1.52; National Institutes of Health).

ELISA. The cell supernatant was collected by centrifugation at 12,000 x g for 10 min at 4°C and the concentrations of inflammatory cytokines were measured using interleukin (IL)-1β ELISA kit (cat. no. PI305; Beyotime Institute of Biotechnology), IL-6 ELISA kit (cat. no. PI330; Beyotime Institute of Biotechnology) and tumor necrosis factor (TNF)-α kit (cat. no. PT518; Beyotime Institute of Biotechnology) according to the manufacturers’ protocols. All experiments were repeated in triplicate.

Dual luciferase reporter assay. The combination between SNHG14 and miR-181b-5p were predicted using StarBase 3.0 (https://starbase.sysu.edu.cn/index.php). Cells were seeded into 24-well plates at a density of 5x10^4 cells/well and cultured at 37°C for 24 h. The fragment of SNHG14 containing the predicted wild-type (WT) or mutant (MUT) miR-181b-5p-binding sequences were amplified by Shanghai GenePharma Co., Ltd., and inserted into the pmirGLO luciferase reporter gene vector (Promega Corporation) to produce the reporter plasmids SNHG14-WT and SNHG14-MUT, respectively. Subsequently, the cells were co-transfected with SNHG14-WT/SNHG14-MUT and miR-181b-5p mimic/mimic-NC using Lipofectamine 2000 reagent for 24 h. The culture medium was then removed, and the cells were rinsed twice with PBS. The cells were added to the cell lysates, which were swirled for 10 min and centrifuged at 12,000 x g for 10 min at 4°C, and the supernatant was subsequently transferred to a new Eppendorf tube. A dual luciferase kit (cat. no. D0010; Beijing Solarbio Science & Technology Co., Ltd.) was used to measure luciferase activity according to the manufacturer’s protocol. Firefly luminescence and Renilla luminescence were detected by enzyme markers, and Renilla luminescence was used as internal reference.

Statistical analysis. Each experiment was performed three times and data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc.). Statistical differences between groups were analyzed using one-way ANOVA with Tukey's test or Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

LPS induces PC-12 cell inflammatory injury. CCK-8 assay was performed to determine the dose of LPS to use for further experiments. As presented in Fig. 1A, PC-12 cell viability was barely affected following treatment with 1 μg/ml LPS, whilst treatment with 5 μg/ml LPS significantly decreased the cell viability compared with the control group. Therefore, 5 μg/ml LPS was used for subsequent experiments. TUNEL assay results revealed a significant increase in cell apoptosis in LPS-treated cells compared with controls (Fig. 1B). These findings were validated using western blotting, which revealed that compared with controls, the expression levels of Bcl-2 were significantly downregulated, while the expression levels of Bax, cleaved caspase-9 and cleaved caspase-3 were significantly upregulated following treatment with 5 μg/ml LPS (Fig. 1C and D). As presented in Fig. 1E, the secretory levels of pro-inflammatory factors, including IL-1β, IL-6 and TNF-α, were significantly increased in the 5 μg/ml LPS group compared with controls. These results suggested that LPS could induce PC-12 cell inflammatory injury.

Knockdown of SNHG14 alleviates LPS-induced inflammation and apoptosis of PC-12 cells. RT-qPCR analysis revealed that the expression levels of SNHG14 in PC-12 cells were significantly upregulated following treatment with 5 μg/ml LPS compared with controls (Fig. 2A). After constructing the interference plasmids and transfecting them into PC-12 cells, shRNA-SNHG14-1 demonstrated greater transfection efficiency compared with shRNA-SNHG14-2. Therefore, shRNA-SNHG14-1 was selected for use in subsequent experiments. As presented in Fig. 2B, PC-12 cells treated with LPS and transfected with shRNA-SNHG14 had significantly higher cell viability (Fig. 2C) and significantly lower cell apoptosis levels (Fig. 2D) compared with those treated with LPS alone. In addition, western blotting results revealed that the expression levels of Bcl-2 were significantly upregulated, while the expression levels of Bax, cleaved caspase-9 and cleaved caspase-3 were significantly downregulated in cells treated with 5 μg/ml LPS and transfected with shRNA-SNHG14 compared with the 5 μg/ml LPS group (Fig. 2E and F). ELISA results illustrated that the secretory levels of IL-1β, IL-6 and TNF-α in PC-12 cells following LPS treatment were significantly downregulated when the cells were also transfected with shRNA-SNHG14 (Fig. 2G). These results suggested SNHG14 knockdown may alleviate LPS-induced inflammation and apoptosis of PC-12 cells.

SNHG14 negatively regulates miR-181b-5p expression levels in PC-12 cells. To investigate the regulatory mechanism of SNHG14 in PC-12 cells, RT-qPCR analysis was conducted to determine the interaction between SNHG14 and miR-181b-5p. As presented in Fig. 3A, compared with the control, the mRNA expression levels of miR-181b-5p were significantly
downregulated following treatment with 5 µg/ml LPS. However, the expression levels of miR-181b-5p in PC-12 cells were significantly upregulated following SNHG14 knockdown compared with the control group (Fig. 3B). Subsequently, a miR-181b-5p mimic was constructed and successfully transfected into PC-12 cells to overexpress miR-181b-5p, as presented in Fig. 3C. The expression levels of SNHG14 were significantly downregulated in PC-12 cells transfected with miR-181b-5p mimic compared with the control (Fig. 3D). The binding sites between SNHG14 and miR-181b-5p were subsequently predicted (Fig. 3E). Dual luciferase reporter assay results revealed that the relative luciferase activities of PC-12 cells did not change following co-transfection with mimic-NC + SNHG14-MUT or miR-181b-5p mimic + SNHG14-MUT; however, the relative luciferase activity was significantly decreased following co-transfection with miR-181b-5p mimic + SNHG14-WT (Fig. 3F). These findings indicated that SNHG14 may negatively regulate miR-181b-5p in PC-12 cells. Knockdown of SNHG14 alleviates LPS-induced inflammation and apoptosis of PC-12 cells via upregulating miR-181b-5p expression levels. To confirm whether SNHG14 could reverse inflammation and apoptosis in PC-12 cells through the newly identified regulatory mechanism, a CCK-8 assay was performed. The results revealed that the decreased cell viability induced by LPS stimulation was rescued following transfection with shRNA-SNHG14, while the addition of the miR-181b-5p inhibitor significantly decreased cell viability (Fig. 4A). In addition, as presented in Fig. 4B, cell apoptosis was
Figure 2. SNHG14 knockdown alleviates inflammation and apoptosis of PC-12 cells induced by LPS. (A) SNHG14 expression in PC-12 cells after LPS induction was detected by RT-qPCR. ***P<0.001 vs. Control group. (B) The transfection efficiency of shRNA-SNHG14-1/2 was verified by RT-qPCR. ***P<0.001 vs. Control group. #P<0.05 vs. shRNA-NC group. †††P<0.001 vs. shRNA-SNHG14-1 group. (C) LPS-induced PC12 cell viability after transfection was detected by Cell Counting Kit-8 assay. *P<0.05 and ***P<0.001 vs. Control group. ††P<0.01 vs. 5 µg/ml LPS group. †††P<0.001 vs. 5 µg/ml LPS + shRNA-NC group. (D) LPS-induced PC12 cell apoptosis after transfection was detected by TUNEL assay. Magnification, x200. ***P<0.001 vs. Control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. 5 µg/ml LPS. †P<0.05, ††P<0.01 and †††P<0.001 vs. 5 µg/ml LPS + shRNA-NC group. (E) The expression of apoptosis-related proteins was analyzed by western blot analysis and (F) quantified. *P<0.05 and ***P<0.001 vs. Control group. †P<0.05, ††P<0.01 and †††P<0.001 vs. 5 µg/ml LPS group. **P<0.01 vs. 5 µg/ml LPS + shRNA-NC group. (G) The levels of TNF-α, IL-1β and IL-6 in the supernatant of LPS-induced PC12 cells after transfection were detected by ELISA. *P<0.05 and ***P<0.001 vs. Control group. †††P<0.001 vs. 5 µg/ml LPS group. **P<0.01 vs. 5 µg/ml LPS + shRNA-NC group. SNHG14, small nucleolar host gene 14; LPS, lipopolysaccharide; shRNA, short hairpin RNA; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.
significantly decreased following stimulation with LPS and transfection with shRNA-SNHG14, while co-transfection with shRNA-SNHG14 and miR-181b-5p inhibitor into LPS-treated cells attenuated cell apoptosis. The downregulated expression levels of Bcl-2 and upregulated expression levels of Bax, cleaved caspase-9 and cleaved caspase-3 induced by 5 µg/ml LPS were reversed by SNHG14 knockdown (Fig. 4C and D). However, miR-181b-5p inhibition suppressed the effects of SNHG14 knockdown in LPS-induced cells. As presented in Fig. 4E, the secretory levels of proinflammatory factors IL-1β, IL-6 and TNF-α were downregulated following stimulation with LPS and transfection with shRNA-SNHG14. However, following LPS stimulation, the secretory levels of these inflammatory factors were increased when the cells were co-transfected with shRNA-SNHG14 and miR-181b-5p inhibitor compared with those transfected with shRNA-SNHG14 alone. These results suggested that SNHG14 knockdown may upregulate the expression levels of miR-181b-5p and subsequently attenuate LPS-induced inflammation and apoptosis of PC-12 cells.

**Discussion**

Despite progress being made in understanding the basic neurobiology of SCI, strategies available for the treatment of SCI remain limited (22). Current clinical treatments, including surgical procedures and high-dose methylprednisolone, can improve the overall survival rate of patients, but they cannot repair nerve function that has been damaged (23). In the present study, the role of IncRNA SNHG14 in LPS-induced PC-12 cells was investigated, in addition to its potential mechanism, with the aim to provide a novel strategy for the treatment of SCI.

IncRNAs serve a role in various fundamental biochemical and cellular processes and have emerged as pivotal regulators that can enhance neural regeneration in the development of SCI (24). Moreover, the expression levels of certain IncRNAs, such as SNHG5 and tectonic family member 2, have been discovered to be upregulated in SCI (25,26). Therefore, therapies that target IncRNAs may hold promise for the treatment of SCI (27). SNHG14 is located within the Prader-Willi critical region and extends into the region of the ubiquitin protein ligase E3A gene, whose deficiency in brain cells in children was revealed to contribute to neurogenetic disorders (28). A previous study has verified that SNHG14 may aggravate inflammation in cerebral ischemia/reperfusion injury by damaging the miR-136-5p-dependent inhibition of ROCK1 (15). It is well known that inflammation is one of the most common features in the occurrence of the SCI (29). Therefore, it was hypothesized that SNHG14 may also induce the development of SCI. In the present study, inflammatory injury following SCI was stimulated in cells by LPS treatment, and the results revealed that the expression levels of SNHG14 were upregulated following LPS stimulation compared with the control group. Following the subsequent construction of interference plasmids targeting SNHG14 and transfection into PC-12 cells,
Figure 4. SNHG14 knockdown alleviates inflammation and apoptosis of PC-12 cells induced by LPS via upregulating miR-181b-5p. (A) LPS-induced PC12 cell viability after transfection was detected by Cell Counting Kit-8 assay. ***P<0.001 vs. Control group. ##P<0.01 vs. 5 µg/ml LPS group. △△△P<0.001 vs. 5 µg/ml LPS + shRNA-NC + inhibitor-NC group. **P<0.01 vs. 5 µg/ml LPS + shRNA-SNHG14-1 + inhibitor-NC group. (B) LPS-induced PC12 cell apoptosis after transfection was detected by TUNEL assay. Magnification, x200. ***P<0.001 vs. Control group. #P<0.05 and ###P<0.001 vs. 5 µg/ml LPS group. △△△P<0.001 vs. 5 µg/ml LPS + shRNA-NC + inhibitor-NC group. $$P<0.001 vs. 5 µg/ml LPS + shRNA-SNHG14-1 + inhibitor-NC group. (C) The expression of apoptosis-related proteins was analyzed by western blot analysis and (D) quantified. **P<0.01 and ***P<0.001 vs. Control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. 5 µg/ml LPS group. △P<0.01 and △△P<0.001 vs. 5 µg/ml LPS + shRNA-NC + inhibitor-NC group. P<0.05 and ###P<0.001 vs. 5 µg/ml LPS + shRNA-SNHG14-1 + inhibitor-NC group. (E) The levels of TNF-α, IL-1β and IL-6 in the supernatant of LPS-induced PC12 cells after transfection were detected by ELISA. **P<0.01 and ***P<0.001 vs. Control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. 5 µg/ml LPS group. △△△P<0.001 vs. 5 µg/ml LPS + shRNA-NC + inhibitor-NC group. SNHG14, small nucleolar host gene 14; LPS, lipopolysaccharide; shRNA, short hairpin RNA; NC, negative control; miR, microRNA; WT, wild-type; MUT, mutant; luc, luciferase; lncRNA, long non-coding RNA.
it was observed that SNHG14 knockdown could rescue cell viability under an LPS-induced inflammatory environment. Since the sub-acute phase of secondary SCI is involved in apoptosis, the expression levels of apoptosis-related proteins in PC-12 cells were analyzed (30). The results revealed that the genetic silencing of SNHG14 could reduce cell apoptosis and alleviate inflammation. These findings suggested that SNHG14 may facilitate the progression of SCI, which was consistent with the findings of Jiao et al (31), who reported that SNHG14 participated in the deterioration of non-small cell lung cancer.

Using advanced high-throughput sequencing technologies, a large number of differentially expressed genes (lncRNA LINRIS, lncRNA HOXA-AS2 and lncRNA ZNF667-AS1), which participated in the development of various types of disease, have been identified (32-34). Previous research has also noted the role of miRNAs following SCI and suggested that they could be novel biomarkers for the diagnosis, treatment and prognosis of such injuries (35). Accumulating evidence has verified that miRNAs can regulate their expression and subsequent biological functions by interacting with lncRNAs (36). LncRNAs have been found to exert their effects by targeting or sponging miRNAs in various types of cancer (37-40). However, whether lncRNAs could sponge or target a certain miRNA in SCI remain to be determined. The present study used StarBase 3.0 software to predict that miR-181b-5p, a member of the miR-181 family, was a target of SNHG14. Indeed, the genetic knockdown of SNHG14 upregulated miR-181b-5p expression levels, while SNHG14 expression levels in PC-12 cells transfected with a miR-181b-5p mimic were downregulated, indicating the negative regulatory role of SNHG14 on miR-181b-5p expression levels. Furthermore, miR-181b-5p was discovered to promote cell viability and inhibit the apoptosis of PC-12 cells.

In conclusion, the results of the present study suggested that SNHG14 may serve as a pathogenic lncRNA in PC-12 cells, as the genetic knockdown of SNHG14 alleviated LPS-induced inflammation and apoptosis of PC-12 cells by negatively regulating miR-181b-5p expression levels. These results may provide a novel perspective for future therapeutic strategies for SCI. However, the present study is that only performed in vitro experiments. Future studies will involve in vivo experiments and assess other underlying mechanisms.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YX conceived and designed the experiments. HJ and JN performed all the experiments and YZ helped HJ and JN to collect experimental data. HJ and JN authenticated the raw data in this study. YZ performed the statistical analysis. HJ and JN wrote the paper together which was revised and polished by YX. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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