miR-489-3p overexpression inhibits lipopolysaccharide-induced nucleus pulposus cell apoptosis, inflammation and extracellular matrix degradation via targeting Toll-like receptor 4

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Abstract. Intervertebral disc degeneration (IDD) is a common disease with a high morbidity rate, which results in a significant deterioration in the quality of life of patients. MicroRNAs (miRNAs/miRs) are a class of endogenous small non-coding RNAs that influence target genes and serve critical roles in numerous biological processes. However, the role of miR-489-3p in lumbar disc degeneration is yet to be elucidated. In the present study, human NP cells were treated with 10 ng/ml lipopolysaccharide (LPS) for 24 h to investigate the role of miR-489-3p in IDD in an *in vitro* model. Reverse transcription-quantitative (RT-q)PCR was performed to determine the expression levels of miR-489-3p. Then, the TargetScan database was used to predict the potential binding sites between miR-489-3p and Toll-like receptor (TLR)4, and a dual-luciferase reporter assay was performed to verify the findings. Subsequently, RT-qPCR and western blotting were used to analyze the expression levels of TLR4. In addition, human nucleus pulposus (NP) cells were transfected with a miR-489-3p mimic and TLR4 overexpression plasmid to study the effects of miR-489-3p on LPS-induced human NP cells. Cell apoptosis and cell viability were also determined using flow cytometry and MTT assays, respectively. Finally, ELISAs were performed to analyze the levels of inflammatory factors. The expression levels of miR-489-3p were discovered to be downregulated in LPS-treated human NP cells. In addition, TLR4 was revealed to be a direct target gene of miR-489-3p, and its expression levels were upregulated in LPS-treated human NP cells. miR-489-3p was found to inhibit the LPS-induced decreases in cell viability and increases in apoptosis, and the concentration of inflammatory cytokines. Furthermore, miR-489-3p suppressed the LPS-induced decreases in extracellular matrix deposition via decreasing the expression levels of aggrecan and collagen type II in human NP cells. Finally, the results revealed that miR-489-3p inhibited the LPS-induced activation of the NF-κB signaling pathway in human NP cells. Conversely, all of the effects of miR-489-3p on LPS-induced human NP cells were reversed by the TLR4 overexpression plasmid. These findings suggested that miR-489-3p may represent a novel therapeutic target for the treatment of IDD.

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Introduction

Intervertebral disc degeneration (IDD) is a degenerative disease of the spine originating from the intervertebral disc, which results in instability of the spine, disc herniation, spinal stenosis and cervical spondylosis (1). IDD is one of the most significant causes of musculoskeletal disability and it is a contributor to the increased motor dysfunction in the population (2). The incidence of lumbar disc degeneration is associated with age; an increase in age results in a higher incidence of lumbar disc degeneration (3). In addition, there are multiple other factors that are associated with IDD, such as obesity, bone density, smoking, diabetes, occupation and exercise (4-8). Lumbar disc degeneration significantly and adversely impacts the quality of life of patients (9). The combination of basic treatment, biomechanical adjustment and active exercise rehabilitation is a novel concept and represents a novel trend in the rehabilitative treatment of degenerative disc disease (10). Notably, previous studies have revealed that microRNAs (miRs/miRNAs) influence the occurrence and development of IDD (11).

At present, increasing evidence has indicated that miRNAs serve a crucial role in various types of disease (12), such as cancer (13), atherosclerosis (14) and cardiovascular diseases (15). miRNAs are a class of small non-coding RNAs that are ~20-22 nucleotides in length (in contrast with mRNA-transcribed proteins) which inhibit the expression of multiple target genes by binding to their 3'untranslated region (3'UTR) (16-18). Previous studies have demonstrated that

miR-25-3p regulates the proliferation and apoptosis of cancer cells in a variety of types of cancer. In addition, miR-25-3p was reported to be associated with the degradation of human nucleus pulposus (NP) cells (19); Lv *et al* (20) revealed that miR-146a may represent a novel target for IDD treatment; and Liu *et al* (21) demonstrated that miR-132 accelerated extracellular matrix (ECM) degradation in human NP cells via the targeting of the expression levels of growth differentiation factor 5. Moreover, miR-21 has been reported to promote ECM degradation by inhibiting autophagy via the PTEN/AKT/mTOR signaling pathway in human degenerated NP cells (22).

Previous research has also revealed that miR-489-3p serves an important role in secondary spinal cord injury (23). Other studies have also indicated the important roles of miR-489-3p in cell apoptosis, ECM and inflammation regulation (23-25). However, the role of miR-489-3p in IDD remains poorly understood.

Human NP cells secrete type II collagen, aggrecan and other components of the ECM, which serve a crucial role in maintaining intervertebral disc integrity (26,27). During the progression of IDD, the excessive apoptosis of IVD cells and excessive degradation of ECM are observed (28). In addition, proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, also serve important roles in IDD (29). Currently, lipopolysaccharide (LPS)-stimulated NP cells are widely used as an *in vitro* model for research into disc degeneration (20-22). It was hypothesized that miR-489-3p may be involved in IDD via regulating human NP cell apoptosis and ECM deposition. Thus, the present study aimed to investigate whether miR-489-3p influences IDD via the regulation of human NP cells.

Materials and methods

Cell culture and treatment. Human NP cells were obtained from Procell Life Science & Technology Co., Ltd. (cat. no. CP-H097). The cells were cultured in DMEM/F12 medium (Invitrogen; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% (v/v) penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified atmosphere at 5% CO_2 and 37˚C. When cells reached 80% confluency, the cells were subsequently treated with 10 ng/ml LPS (Sigma-Aldrich; Merck KGaA) at 37˚C for 24 h to establish the IDD cell model *in vitro*. Cells without any treatment were used as the control.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from human NP cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All processes were carried out on ice. After extracting the RNA, the concentration of each sample was determined using an ultraviolet spectrophotometer. Total RNA was reverse transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's protocol. The reverse transcription reaction conditions were: 70˚C for 5 min, 37˚C for 5 min and 42˚C for 60 min. qPCR was subsequently performed using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.), according to the manufacturer's protocol. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 3 min; followed by 40 cycles of 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 30 sec. The following primer sequences were used for the qPCR: miR-489-3p forward, 5'-GTGACATCACATATACGG-3' and reverse, 5'-GAACATGTCTGCGTATCTC-3'; TLR4 forward, 5'-CCTGACACCAGGAAGCTTGAA-3' and reverse, 5'-TCTGATCCATGCATTGGTAGGT-3'; aggrecan forward, 5'-CTACCAGTGGATCGGCCTGAA-3' and reverse, 5'-CGTGCCAGATCATCACCACA-3'; collagen type II forward, 5'-GGCAATAGCAGGTTCACGTACA-3' and reverse, 5'-CGATAACAGTCTTGCCCCACTT-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; and GAPDH forward, 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse, 5'-GTAGAGGCAGGGATGATGTTCT-3'. Expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (30) and GAPDH or U6 served as the internal control for normalization.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Total protein was quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 30 μg protein/lane was separated via 15% SDS-PAGE. The separated proteins were subsequently transferred onto a PVDF membrane (EMD Millipore) and blocked at room temperature using 5% fat-free powdered milk dissolved in TBS-0.1% Tween for 1.5 h. The membranes were incubated with the following primary antibodies at 4˚C overnight: Anti-TLR4 antibody (cat. no. ab13556; 1:1,000; Abcam), anti-GAPDH (cat. no. ab181602; 1:1,000; Abcam), anti-aggrecan (cat. no. ab3778; 1:1,000; Abcam), anti-collagen type II (cat. no. ab34712; 1:1,000; Abcam), anti-p65 (cat. no. ab16502; 1:1,000; Abcam) and anti-phosphorylated (p)-p65 (cat. no. ab86299; 1:1,000; Abcam). Following the primary antibody incubation, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. ab7090; 1:2,000; Abcam) at room temperature for 2 h. Protein bands were visualized using an enhanced chemiluminescence substrate (EMD Millipore) and analyzed using ImageJ version 2.0 software (National Institutes of Health). The expression levels were normalized to GAPDH.

Dual-luciferase reporter assay. Bioinformatics analysis using TargetScan 7.2 (http://www.targetscan.org/vert_72/) was performed to determine the binding sites between miR-489-3p and TLR4. The wild-type (WT) or mutant (MUT) 3'UTR of TLR4 was cloned into the pmiRGLO vector (Promega Corporation) and the recombinant plasmids were acquired using an EndoFree Plasmid Maxi kit (Vazyme Biotech Co., Ltd.). To point-mutate the miR-489-3p binding domain in the 3'UTR of TLR4, a QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc.) was used according to the manufacturer's instructions. Cells were seeded into 24-well plates at a density of $5x10^4$ cells/well and co-transfected with a miR-489-3p mimic or mimic control and the MUT or WT 3'UTR of TLR4 using Fugene transfection reagent (Promega Corporation) according to the manufacturer's protocol, together with the *Renilla* luciferase pRL-TK vector (Promega

Corporation) as a control. Following transfection at 37˚C for 48 h, firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Cell transfection. Human NP cells ($5x10^4$ cells per well) were transiently transfected with a 100 nM mimic control (5'-UUG UCCGAACGUGUCACGUTT-3'; Suzhou GenePharma Co., Ltd.), 100 nM miR-489-3p mimic (5'-GUGACAUCACAU AUACGGCAGC-3'; Suzhou GenePharma Co., Ltd.), 1 μg Control CRISPR Activation Plasmid (cat. no. sc-437275; Santa Cruz Biotechnology, Inc.), 1 μg TLR4 CRISPR Activation Plasmid (cat. no. sc-400068-ACT; Santa Cruz Biotechnology, Inc.), 100 nM miR-489-3p mimic + 1 μg control-plasmid or 100 nM miR-489-3p mimic + 1 μg TLR4-plasmid at 37˚C for 24 h using Fugene transfection reagent (Promega Corporation) according to the manufacturer's protocol. The transfection efficiency was detected using a RT-qPCR assay. Following 24 h of cell transfection, the cells were treated with 10 ng/ml LPS at 37˚C for 24 h and then the cells were subjected to subsequent experiments (Fig. S1).

MTT assay. Cell viability was determined via an MTT assay. Briefly, transfected human NP cells were treated with 10 ng/ml LPS at 37˚C for 24 h and then cells were plated in 96-well plates at a density of $5x10^3$ cells/well. Following 48 h of incubation at 37˚C, 20 μl MTT reagent (Beyotime Institute of Biotechnology) was added into each well and incubated for 4 h at 37˚C. Subsequently, 150 μl DMSO was added into each well and the solution was agitated at 37˚C for 15 min. The optical density values were measured at 570 nm using a microplate reader.

Flow cytometric analysis of apoptosis. Cell apoptosis was analyzed using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, the cells ($1x10^6$) were washed with 1X PBS three times, centrifuged at 4˚C for 5 min at 1,000 x g, and trypsinized into single cell suspensions with 500 μl buffer (Beyotime). The cells were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide at room temperature for 15 min. Apoptotic cells were subsequently analyzed using BD FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.1 software (FlowJo LLC).

ELISAs. Following 24 h of cell transfection, NP cells were treated with 10 ng/ml LPS, and the supernatants were subsequently harvested through centrifugation at 500 x g at 4˚C for 5 min. Subsequently, specific ELISA kits (Beyotime Institute of Biotechnology) were used, according to the manufacturers' protocol, to determine the concentrations of TNF-α (cat. no. PT518), IL-1β (cat. no. PI305) and IL-6 (cat. no. PI330) in the cell culture supernatant from the different groups.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc.); each experiment was performed in triplicate and all data are presented as the mean ± SD. Statistical differences between two groups were determined using a unpaired Student's t-test, whereas an one-way ANOVA followed by Tukey's post hoc test was used to analyze the statistical differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

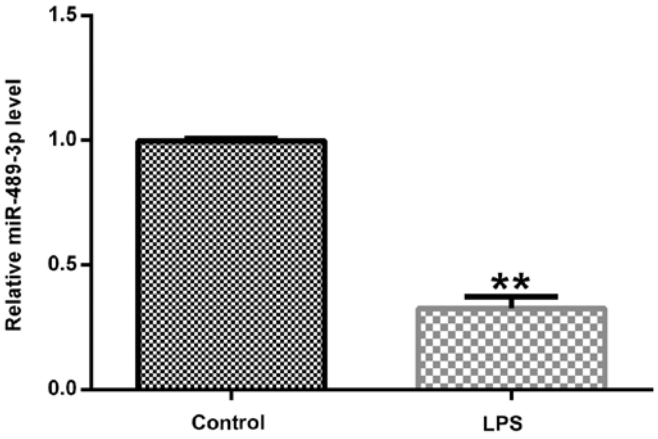

Figure 1. miR-489-3p expression levels are downregulated in an intervertebral disc degeneration *in vitro* cell model. Reverse transcription-quantitative PCR was used to analyze the expression levels of miR-489-3p in human NP cells treated with 10 ng/ml LPS for 24 h. miR, microRNA; LPS, lipopolysaccharide. $^{**}P<0.01$ vs. control.

Results

Expression levels of miR-489-3p in human NP cells. To investigate the effects of miR-489-3p on an IDD model *in vitro*, RT-qPCR was performed to detect the expression levels of miR-489-3p. The expression levels of miR-489-3p were significantly downregulated in the LPS-treated group compared with the control group (Fig. 1).

TLR4 is a direct target gene of miR-489-3p. Subsequently, to determine the interaction between miRNA and its target genes, the TargetScan tool was used to predict the target genes of miR-489-3p; it was revealed that the TLR4 3'UTR contained a putative site that was partially complementary to miR-489-3p (Fig. 2A). Furthermore, the dual-luciferase reporter assay was used to determine whether miR-489-3p interacted directly with the target gene TLR4. The reporter containing the TLR4-WT 3'UTR exhibited significantly decreased relative luciferase activity in the human NP cells co-transfected with the miR-489-3p mimic compared with the mimic control (Fig. 2B). Taken together, the current results indicated that TLR4 may be a direct target gene of miR-489-3p.

Subsequently, RT-qPCR and western blotting assays were used to determine the expression levels of TLR4. The results of these assays revealed that TLR4 expression levels were upregulated in the LPS-treated group compared with control group at both the mRNA and protein level (Fig. 3A and B).

TLR4 is negatively regulated by miR-489-3p in human NP cells. Human NP cells were transfected with either a mimic control or miR-489-3p mimic. RT-qPCR revealed that compared with the mimic control group, the miR-489-3p mimic significantly increased the expression levels of miR-489-3p in human

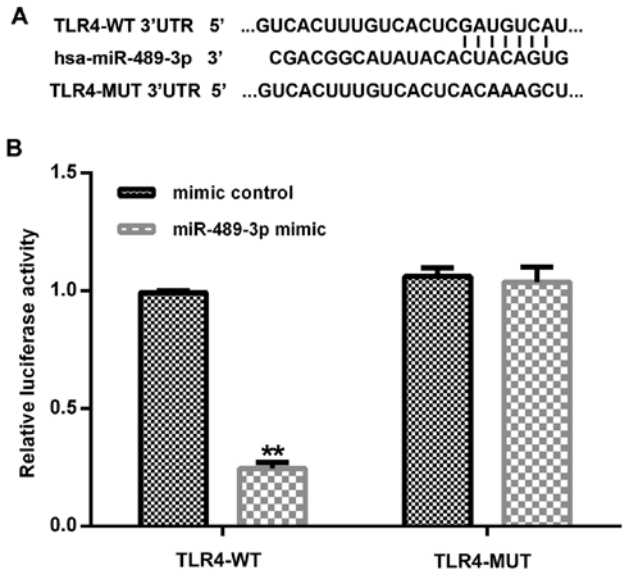

Figure 2. TLR4 is a direct target gene of miR-489-3p. (A) TargetScan was used to predict the TLR4 3'UTR binding site for miR-489-3p. (B) miR-489-3p mimic or mimic control and WT or MUT TLR4 3'UTR reporter were co-transfected into human nucleus pulposus cells. A dual-luciferase reporter activity was used to determine the relative luciferase activity. miR, microRNA; TLR4, Toll-like receptor 4; 3'UTR, 3'untranslated region; WT, wild-type; MUT, mutant. **P<0.01 vs. mimic control.

NP cells (Fig. 4A). Subsequently, the cells were transfected with either the control- or TLR4-plasmid; the results indicated that compared with the control-plasmid group, TLR4 mRNA expression levels were significantly upregulated in the TLR4-plasmid group (Fig. 4B). Then, the effect of miR-489-3p on TLR4 expression levels was examined. Human NP cells were transfected with either a miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid. It was demonstrated that compared with the mimic control group, the miR-489-3p mimic significantly decreased the expression levels of TLR4 in human NP cells, which was partially restored following the co-transfection with the TLR4-plasmid (Fig. 4C and D).

miR-489-3p promotes cell viability and inhibits apoptosis in LPS-induced human NP cells. The effect of miR-489-3p on cell viability and apoptosis in LPS-induced human NP cells was examined. Compared with the control group, LPS treatment significantly inhibited NP cell viability and induced apoptosis (Fig. 5A-C). However, compared with the LPS-treated group, the miR-489-3p mimic group exhibited significantly increased NP cell viability (Fig. 5A) and decreased cell apoptosis (Fig. 5B and C), which was partially reversed following the co-transfection with the TLR4-plasmid.

miR-489-3p inhibits the levels of inflammatory cytokines in LPS-induced human NP cells. To determine the effect of miR-489-3p on the levels of inflammatory factors in LPS-induced human NP cells, ELISAs were performed to analyze the expression levels of TNF-α, IL-1β and IL-6. The results indicated that compared with the control group, LPS treatment significantly increased the secretion of TNF-α, IL-1β and IL-6 in human NP cells (Fig. 6A-C). Notably, the transfection with the miR-489-3p mimic significantly reduced these LPS-induced increases in the expression levels of TNF-α, IL-1β and IL-6 (Fig. 6A-C), which were significantly restored following transfection with the TLR4-plasmid.

miR-489-3p inhibits ECM breakdown in LPS-induced human NP cells. RT-qPCR and western blot assays were conducted to analyze the expression levels of ECM-associated proteins. Compared with the control group, LPS treatment significantly reduced the expression levels of aggrecan and collagen type II in human NP cells at the protein (Fig. 7A-C) and mRNA level (Fig. 7D and E). Moreover, compared with the LPS-treated group, the miR-489-3p mimic significantly increased the expression levels of aggrecan and collagen type II in human NP cells, and this effect was partially reversed following the co-transfection with the TLR4-plasmid.

miR-489-3p inhibits the activation of the NF-κB signaling pathway in LPS-induced human NP cells. Finally, the specific mechanism underlying the influence of miR-489-3p in IDD was investigated. Western blotting revealed that LPS treatment significantly increased the protein expression levels of p-p65 and the ratio of p-p65/p65 in human NP cells compared with the control group (Fig. 8A and B). However, the miR-489-3p mimic decreased the protein expression levels of p-p65 (Fig. 8A) and the ratio of p-p65/p65 (Fig. 8B); this effect was significantly restored following co-transfection with the TLR4-plasmid.

Discussion

In the present study, human NP cells were treated with LPS to establish an IDD model *in vitro* and it was revealed that TLR4 is a direct target gene of miR-489-3p. Notably, miR-489-3p expression levels were discovered to be downregulated in LPS-treated human NP cells and TLR4 expression levels were observed to be negatively associated with miR-489-3p expression levels. Subsequently, NP cells were transfected with a miR-489-3p mimic and TLR4 overexpression plasmid to study the effect of miR-489-3p on human NP cells. The results indicated that miR-489-3p suppressed the LPS-induced decreases in cell viability and increases in cell apoptosis in human NP cells. Moreover, miR-489-3p decreased the expression levels of the inflammatory cytokines, TNF-α, IL-1β and IL-6, and inhibited the degradation of the ECM (evidenced by increased expression levels of the ECM proteins), in LPS-induced human NP cells. Finally, it was also revealed that miR-489-3p decreased the expression levels of p-p65, which is associated with the NF-κB signaling pathway (31).

IDD is the primary cause of lower back pain and it is a medical condition that places a heavy burden on the global medical system, resulting in significant socioeconomic consequences (32-34). At present, due to both professional and personal factors, the incidence of IDD is increasing, particularly in China (35). Currently, rehabilitation is important following lumbar spine fusion surgery for degenerative diseases and it is critical for the healing of degenerative diseases (36).

Previous studies have indicated that miRNAs are regulators of gene expression and serve important roles in the prevention and treatment of IDD (37,38). It has been reported

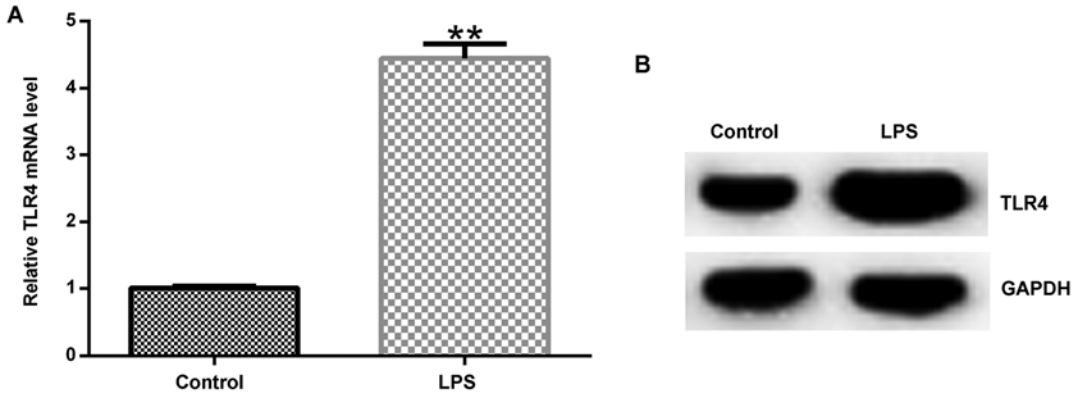

Figure 3. TLR4 expression levels are upregulated in an intervertebral disc degeneration *in vitro* cell model. (A) Reverse transcription-quantitative PCR and (B) western blotting were used to analyze the expression levels of TLR4 at the mRNA and protein level, respectively. TLR4, Toll-like receptor 4; LPS, lipopolysaccharide. **P<0.01 vs. Control.

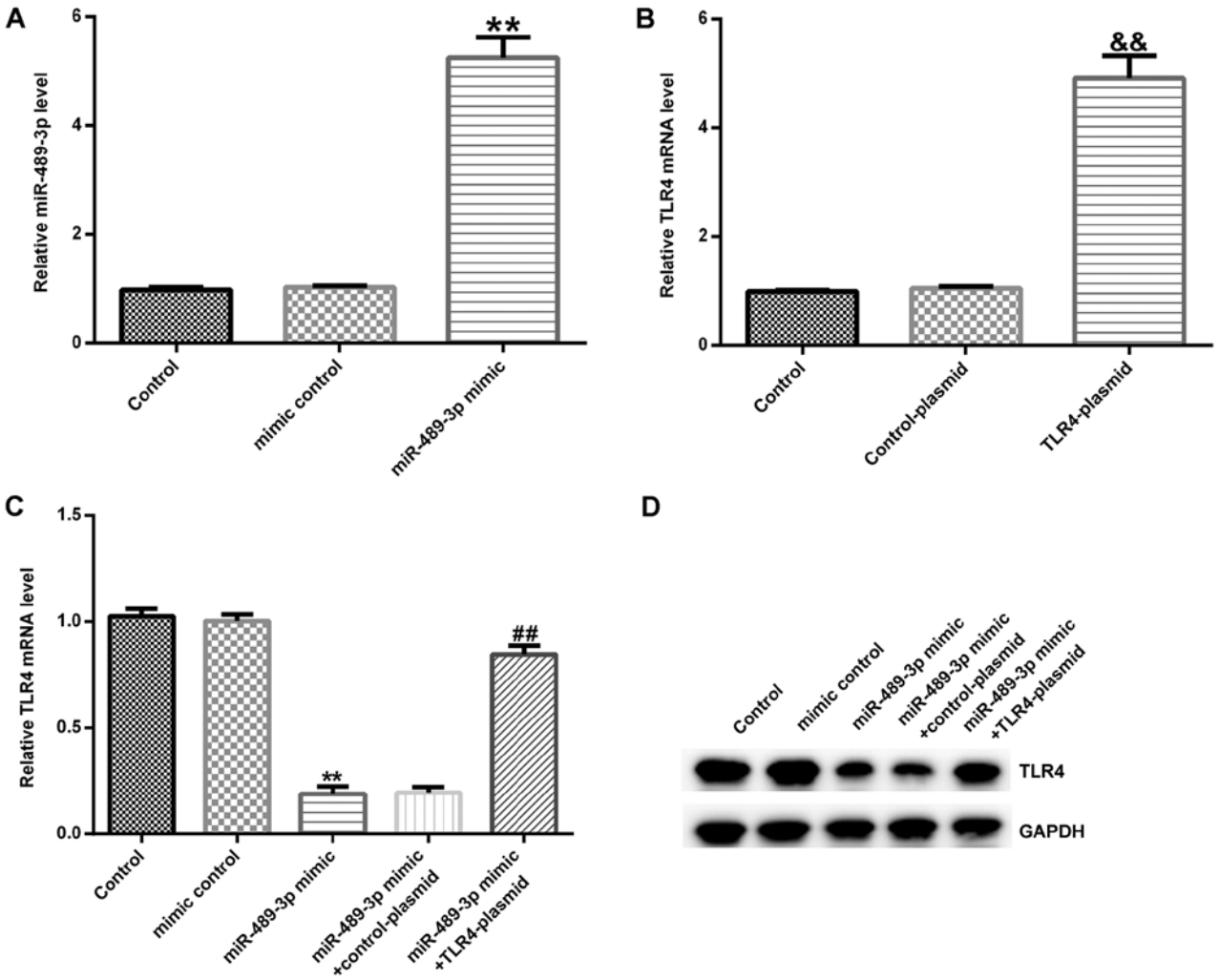

Figure 4. Transfection efficiency of TLR4 and miR-489-3p in human NP cells. RT-qPCR was used to determine the expression levels of (A) miR-489-3p and (B) TLR4 following transfection of the miR-489-3p mimic or TLR4-plasmid, respectively. (C) RT-qPCR and (D) western blotting was used to analyze the expression levels of TLR4 at the mRNA and protein level, respectively. TLR4, Toll-like receptor 4; NP, nucleus pulposus; RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA. **P<0.01 vs. mimic control; $^{\&\&}$P<0.01 vs. control-plasmid; $^{\#\#}$P<0.01 vs. miR-489-3p mimic+control-plasmid.

that several miRNAs were found to be dysregulated in IDD, including miR-21, -10b, -155 and -27 (37,39-41), whereas miR-200c was discovered to be upregulated in degenerative NP tissues (42). In the present study, miR-489-3p was demonstrated to be downregulated in LPS-treated human NP cells. miR-489 expression levels were decreased in numerous types of cancer tissue, such as gastric cancer (43) and breast cancer (44). In addition, miR-489 was revealed to influence

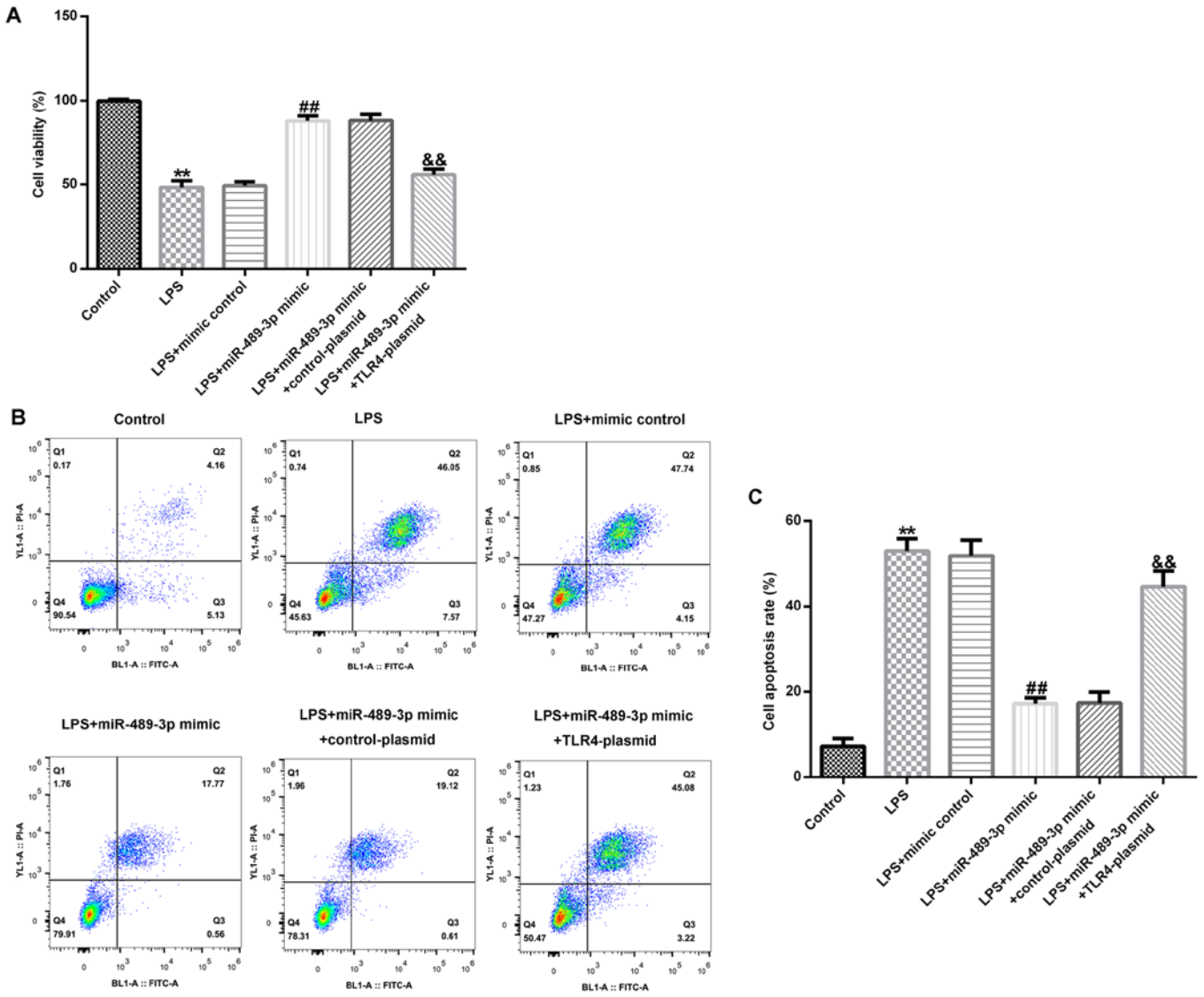

Figure 5. miR-489-3p enhances the cell viability and reduces the apoptotic rate in LPS-induced human NP cells. (A) MTT assay was used to determine the cell viability of human NP cells transfected with mimic control, miR-489-3p mimic, miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid for 24 h and treated with 10 ng/ml LPS for 24 h. (B) Flow cytometry was used to determine the apoptotic rate of cells transfected with mimic control, miR-489-3p mimic, miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid for 24 h and then treated with 10 ng/ml LPS for 24 h. (C) apoptotic rate of cells was calculated and presented. TLR4, Toll-like receptor 4; miR, microRNA; NP, nucleus pulposus; LPS, lipopolysaccharide; PI, propidium iodide. **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. *LPS*+mimic control; $^{\&\&}$P<0.01 vs. LPS+miR-489-3p mimic+control-plasmid.

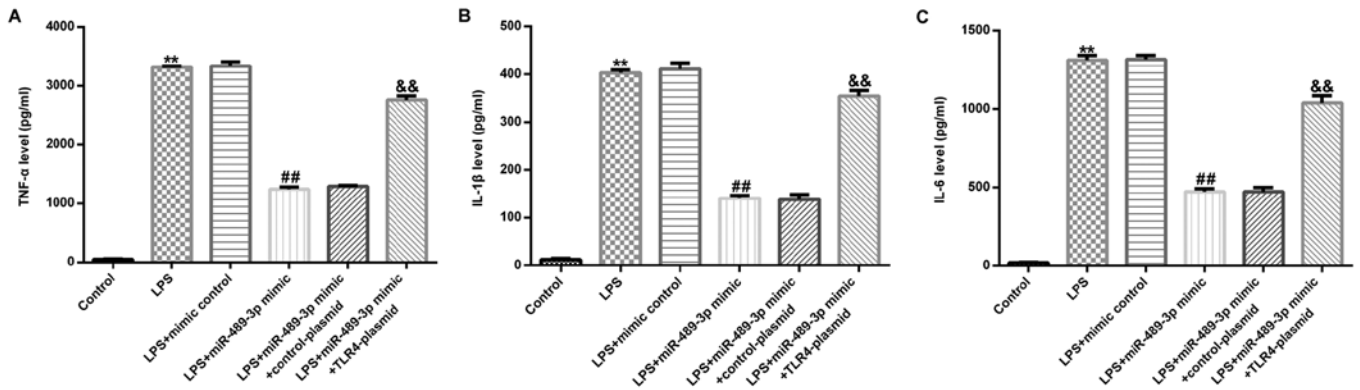

Figure 6. miR-489-3p suppresses the secretion of TNF-α, IL-1β and IL-6 in LPS-induced human NP cells. Human NP cells were transfected with the mimic control, miR-489-3p mimic, miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid for 24 h and treated with 10 ng/ml LPS for 24 h. ELISAs were used to analyze the expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 in human NP cells. TLR4, Toll-like receptor 4; miR, microRNA; NP, nucleus pulposus; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor. **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. *LPS*+mimic control; $^{\&\&}$P<0.01 vs. LPS+miR-489-3p mimic+control-plasmid.

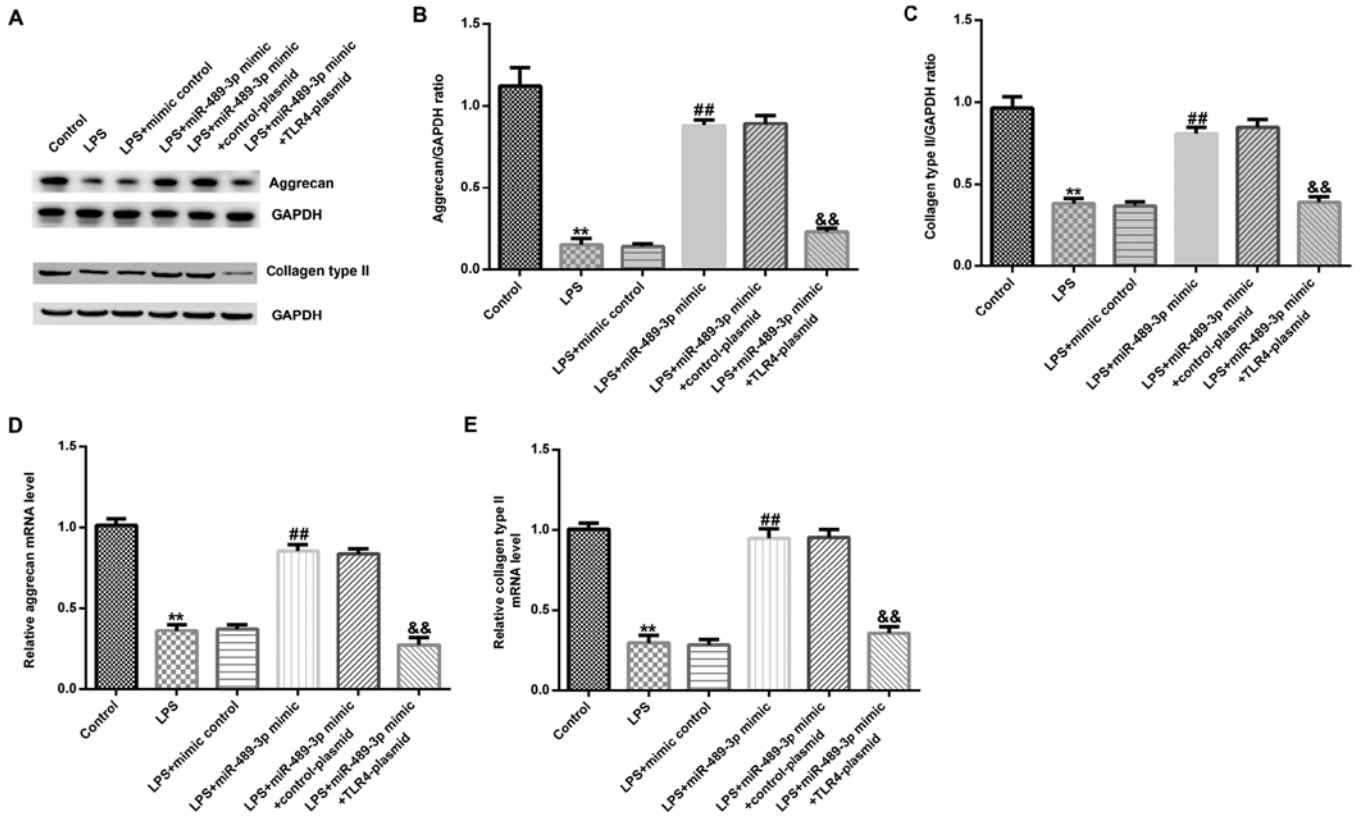

Figure 7. miR-489-3p inhibits extracellular matrix degradation in LPS-induced human NP cells. Human NP cells were transfected with mimic control, miR-489-3p mimic, miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid for 24 h and treated with 10 ng/ml LPS for 24 h. (A) Western blotting was used to analyze the expression levels of aggrecan and collagen type II at the protein level. The expression levels of (B) aggrecan and (C) collagen type II were semi-quantified from the blot presented in part A. Reverse transcription-quantitative PCR was used to analyze the expression levels of (D) aggrecan and (E) collagen type II at the mRNA level. TLR4, Toll-like receptor 4; miR, microRNA; NP, nucleus pulposus; LPS, lipopolysaccharide. **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. *LPS*+mimic control; $^{\&\&}$P<0.01 vs. LPS+miR-489-3p mimic+control-plasmid.

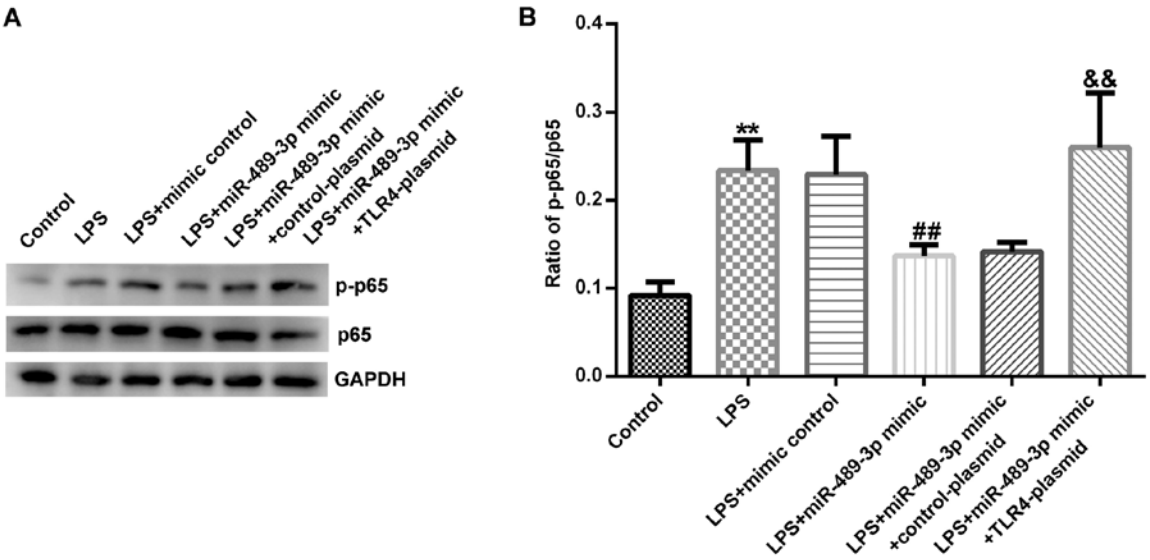

Figure 8. Association between miR-489-3p and NF-κB signaling pathway in LPS-induced human NP cells. Human NP cells were transfected with mimic control, miR-489-3p mimic, miR-489-3p mimic + control-plasmid or miR-489-3p mimic + TLR4-plasmid for 24 h and treated with 10 ng/ml LPS for 24 h. (A) Western blotting was used to analyze the expression levels of p-p65 and p65. (B) Ratio of p-p65/p65 was semi-quantified from the blot presented in part A. TLR4, Toll-like receptor 4; miR, microRNA; NP, nucleus pulposus; p-, phosphorylated; LPS, lipopolysaccharide. **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. *LPS*+mimic control; $^{\&\&}$P<0.01 vs. LPS+miR-489-3p mimic+control-plasmid.

multiple pathological processes, such as proliferation, apoptosis, invasiveness and the metastasis of tumor cells (45). It has also been reported that the miR-489-3p sequence shares high homology to the miR-489 sequence.

The majority of miRNAs serve their biological function by complementarily binding their target gene (46). In the current study, it was discovered that TLR4 was a target gene of miR-489-3p. TLR4 is an innate and adaptive immune cell receptor (47), which serves a vital role in the inflammatory response (48). Notably, Yang *et al* (47) also indicated that TLR4 may be a target gene of miR-760. In addition, TLR4 has been reported to be regulated by several miRNAs, including miR-708-5p (49), miR-106a (50) and miR-20a (51). Wu *et al* (52) reported that the primary pathological changes in IDD were NP apoptosis and the significant degradation of the ECM. miRNAs have been identified to regulate cell apoptosis and ECM protein expression (53-56). Notably, several studies have demonstrated that miRNAs influence the occurrence and development of IDD by regulating the apoptosis and ECM deposition of NP cells (20-22,57,58). For example, miR-132 was revealed to promote matrix degradation in IDD via increased ECM catabolic factors (matrix metalloproteinase 13 and A disintegrin and metalloproteinase with thrombospondin motifs 4), and decreased anabolic proteins (type II collagen and aggrecan) in NP cells (21). In other previous studies, miR-21 was also demonstrated to contribute to type II collagen and aggrecan catabolism in human NP cells (22); miR-145 suppressed apoptosis and promoted ECM synthesis in NP cells (57); and miR-499a-5p was revealed to suppress the apoptosis of human NP cells and inhibit the degradation of the ECM via targeting the transcription factor SOX-4 (58). In the present study it was determined that miR-489-3p suppressed the LPS-induced decreases in cell viability and the increase in cell apoptosis in human NP cells. However, the cell viability was analyzed following the treatment of human NP cells with 10 ng/ml LPS for 24 h; this was a limitation of the present study and the study protocol could be improved by subjecting the cells to longer term viability assays. In addition, the results indicated that miR-489-3p may inhibit the LPS-induced degradation of the ECM in human NP cells.

Altogether, the findings of the present study suggest that miR-489-3p may regulate the LPS-induced NP cell inflammatory response, apoptosis and ECM-related protein expression; however, their interaction and relationship with each other requires further investigation in the future. Furthermore, the present study did not use a miR-489-3p mimic control + TLR4-plasmid + LPS group, which is also represents a limitation. Thus, the targeting of miR-489-3p may represent a promising therapeutic strategy and the current study may have identified novel therapeutic targets for the development of treatments for IDD.

In conclusion, miR-489-3p was discovered to inhibit the LPS-induced decreases in cell viability and increases in apoptosis, the inflammatory response and ECM degradation in human NP cells via the suppression of the NF-κB signaling pathway via the targeting of TLR4. Therefore, miR-489-3p may represent a potential target for IDD treatment. However, the current study is only a preliminary study of the role of miR-489-3p in IDD. To clarify the mechanism underlying the role of miR-489-3p in IDD, numerous additional in-depth experiments are required. For example, the role of TLR4-plasmid in LPS-treated NP cells should be studied and investigations into the role of the miR-489-3p/TLR4 axis in IDD *in vivo* should be performed. In addition, the expression levels of miR-489-3p/TLR4 in patients with IDD and the association between miR-489-3p and TLR4 expression levels and the clinicopathological characteristics of patients with IDD should be investigated; our future studies aim to cover some of these investigations.

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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

LD contributed to the design of the study, data collection, statistical analysis, data interpretation and manuscript preparation. BD contributed to the data collection, statistical analysis and manuscript preparation. All authors read 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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