

miR-539 Inhibits Inflammation in Renal Transplant Ischemia-Reperfusion Injury via Blocking the MyD88/NF- κ B Pathway

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Abstract

Objective: This study was conducted in order to investigate the anti-inflammatory effects of miR-539 on renal transplant ischemia-reperfusion (I/R) injury.

Methods: A mouse model replicating renal transplant I/R injury and a cellular model of oxygen-glucose deprivation (OGD) treatment were established. The blood urea nitrogen (BUN) levels were determined for all models. The miR-539 expressions were detected in the kidney tissues and HK-2 cells following the different transfections using qRT-PCR. Western blotting was used to analyze myeloid differentiation factor 88 (MyD88), as well as the unphosphorylated and phosphorylated Nuclear factor- κ B (NF- κ B) protein expressions. The interactions between miR-539 and MyD88 were examined through a luciferase reporter assay. Moreover, pro-inflammatory cytokines levels, including the tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-8, were measured using an enzyme-linked immunosorbent assay (ELISA). A renal tubular necrosis score (TNS) was employed as a means of assessing the renal function of mouse model.

Results: miR-539 was downregulated during renal I/R injury. In vitro, miR-539 relieved the secretion of pro-inflammatory cytokines. A luciferase reporter assay demonstrated that MyD88 was a direct target of miR-539. Further investigation revealed that miR-539 inhibited I/R injury-induced inflammation by downregulating the MyD88/NF- κ B pathway. It was shown that miR-539 exerted anti-inflammatory effects in the mice that underwent renal transplant I/R injury.

Conclusion: MiR-539 alleviated inflammation in renal transplant I/R injury through the MyD88/NF- κ B pathway.

Keywords: renal transplant; ischemia-reperfusion injury; anti-inflammatory; miR-539; MyD88/NF- κ B pathway

INTRODUCTION

For patients with end-stage renal disease such as acute renal failure, renal transplantation has been the optimal choice [1]. Over the past decades, advances in diagnosis, surgical technique, and immunosuppressive medications have greatly improved postoperative renal transplant outcomes, increasing the survival rate and quality of life for renal transplant patients [2]. However, the occurrence and development of inflammation harmfully impacts renal function, the prognosis of transplant surgery, and long-term postoperative outcomes [3]. Preventing perspective inflammation remains a major global challenge. Ischemia-reperfusion (I/R) injury is characterized as an early, non-specific inflammatory

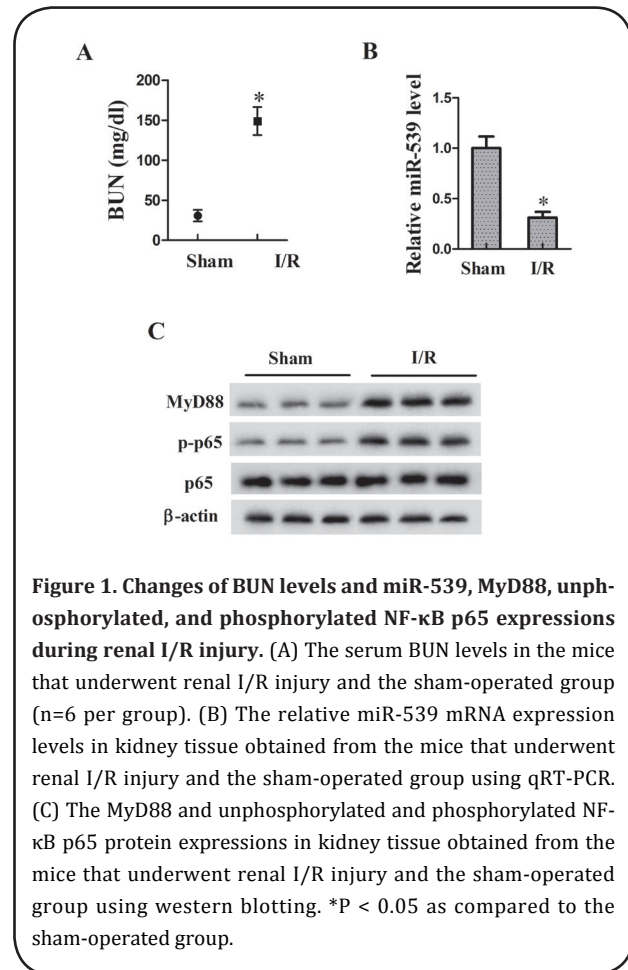
response, and its role in the pathophysiological process of various clinical diseases and surgical interventions is significant and well documented. These interventions include shock, organ transplantation, coronary thrombosis, and cardiopulmonary bypass [4]. I/R injury during renal transplant mainly consists of warm ischemia following the nephrectomy of the living kidney donor, cold ischemia during the cold preservation of the graft, and reperfusion following vascular anastomosis. Subsequently, a series of inflammatory events is triggered such as the release of pro-inflammatory cytokines, which increases the risk for short- and long-term renal allograft dysfunction, acute kidney injury, and ischemic acute renal failure [5,6]. Thus, an examination of preventative and therapeutic strategies relevant to I/R injury is of great significance to the medical field. Several studies have suggested that the primary pathophysiological changes of renal I/R injury are caused by energy depletion induced by ischemia and oxidative stress damage as a result of reperfusion, inflammatory response, and tubular cell apoptosis [7]. In the past

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years, evidence has strongly implied that NF- κ B is a central pathway that activates inflammatory response during renal I/R injury [8]. Jia et al. reported that renal I/R injury initiates the displacement and activation of the NF- κ B signaling pathway in renal tubular cells. This results in an inflammatory response and the release of pro-inflammatory cytokines, thereby promoting the apoptosis of tubular cells [9]. Multiple studies regarding the importance of pro-inflammatory cytokines (such as TNF- α , IL-6, and IL-8) for inflammatory response following renal I/R injury have also been conducted [10]. Furthermore, current evidence supports the assertion that MyD88 acts as a Toll-like receptor (TLR) adapter protein crucial for innate inflammation responses via the activation of downstream signaling pathways. Some of these pathways include NF- κ B and mitogen-activated protein kinase signaling pathways, driving robust synthesis, and the gene expression of cytokines and pro-inflammatory mediators [11,12].

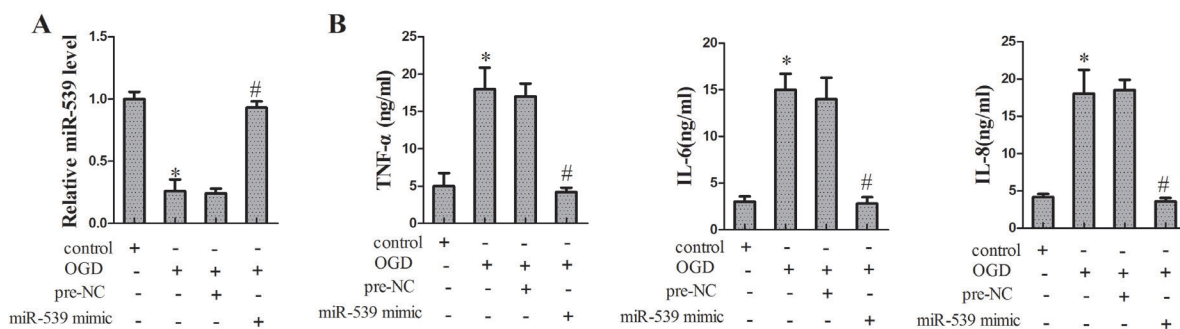
MicroRNAs (miRNAs), which are small noncoding RNAs consisting of endogenous 21-23 nucleotides, were bound to 3'- untranslated regions (UTR) of the target gene in order to post-transcriptionally regulate the eukaryotic gene expression. Increasing evidence has revealed that the dysregulation of miRNAs have contributed to the development of pathophysiologic progression, including renal I/R injury [13]. Bao et al. observed aberrant miR-539 expressions in the human embryonic kidney (HEK)-293 cell line [14]. As a result, miR-539 was identified as a vital tumor suppressor that plays a significant role in carcinogenesis and progression [15]. This study investigates the underlying molecular mechanism and biological function of miR-539 in I/R injury after kidney transplantation.

MATERIALS AND METHODS



Renal transplant I/R injury mouse model

This study used male C57BL/6J mice aged 12 weeks, wherein each specimen weighed 20~25g. The mice were obtained from Soochow University, and were fed a standard rodent diet under pathogen-free specific conditions. All mice were randomly divided into two equal groups: a sham-operated group and an I/R group. The



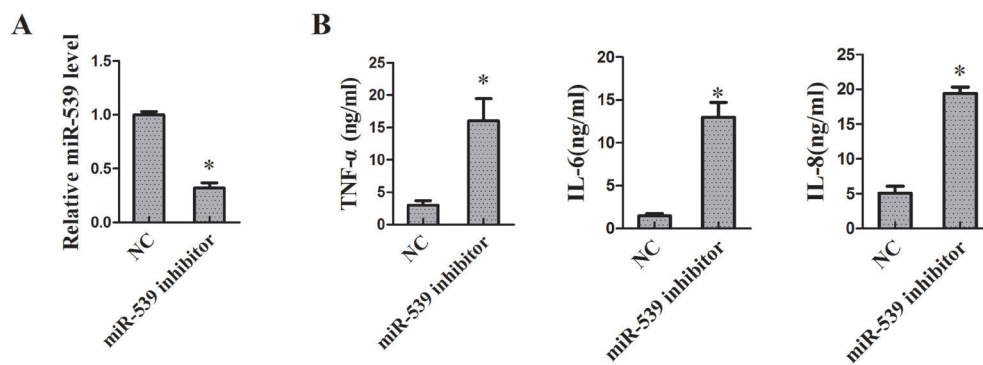


Figure 3 Effect of miR-539 knockdown on pro-inflammatory cytokines expression. (A) The relative miR-539 mRNA expression levels in HK-2 cells transfected with miR-539 and NC using qRT-PCR. (B) The pro-inflammatory cytokines levels containing TNF- α , IL-6, and IL-8 in HK-2 cells transfected with miR-539 and NC using ELISA. *P < 0.05 as compared to the NC-transfected group.

mice placed in the I/R group underwent renal transplant I/R injury [16]. Before the surgery, the mice were anesthetized with an inhalation of 2% isoflurane (Abbott GmbH, Vienna, Austria) and a standard midline abdominal incision was performed. The mice in the I/R injury group were subjected to acute I/R injury through the clamping of the left renal pedicle for 45 minutes, followed by reperfusion before the removal of the right kidney. The sham-operated mice were only subjected to the removal of their right kidney. Blood and renal tissue samples were harvested after 24 hours of renal removal. This was done in order to determine the serum BUN level, miR-539 mRNA expression using qRT-PCR, and the MyD88, p-p65, and p65 protein expression levels through the use of western blotting. All experimental procedures performed within this study were approved by the Animal Care and Use Committee of The First Affiliated Hospital of Soochow University.

Hypoxia treatment and cell grouping

To examine the role of miR-539 in regulating inflammatory cytokines in I/R injury, an oxygen-glucose deprivation (OGD) experiment was used to establish a cellular in vitro I/R injury model. Human proximal tubular epithelial cell line HK-2, purchased from the European Collection of Authenticated Cell Cultures (ECACC, Porton Down, UK), were randomly placed into four groups: control, OGD, OGD+pre-NC, and OGD+miR-539 mimic. Briefly, the HK-2 cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere using Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12; Life Technologies, Carlsbad, CA, USA). This solution was supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mM of glutamine (Life Technologies), and 100 μ g/mL of penicillin/streptomycin (Life Technologies). The cells were then washed with PBS and incubated for one hour in an OGD solu-

tion with a pH of 7.0. The solution contained 137 mM of NaCl, 4 mM of KCl, 1 mM of MgCl₂, 1.5 mM of CaCl₂, and 5 mM of HEPES in a hypoxic/ischemic incubator with a 37 °C humidified atmosphere of 5% CO₂ and 95% N₂. For reoxygenation, the cells were later incubated in a culture medium containing 5.5 mM of glucose at 37 °C with 5% CO₂ for an additional 24 hours instead of the OGD solution. The same procedure was performed in the other groups, except for the control group using Lipofectamine 2000 reagent (Invitrogen) where the manufacturer's instructions were followed. To confirm the biological functions of miR-539, the cultured HK-2 cells were transfected with NC or miR-539 inhibitors. After 48 hours of transfection, cells in each group were collected in order to determine the miR-539 expression and pro-inflammatory cytokines levels (including TNF- α , IL-6, and IL-8) using ELISA.

Bioinformatics and luciferase reporter assay

Targetscan bioinformatics software was used to predict the potential binding sites of miR-539 and MyD88 mRNA. An association between miR-539 and MyD88 was verified through the use of dual-luciferase reporter assays. In short, the 3'-UTR fragments of MyD88 and the predicted target sequences of miR-539 were amplified and sub-cloned into pISO plasmids (Promega, Madison, WI, USA). When confluence reached approximately 70%, 50 ng of pISO-MyD88-WT or pISO-MyD88-WUTHK-2 were co-transfected with either a miR-539 mimic or inhibitor into the HK-2 cells using Lipofectamine 2000 (Invitrogen), along with their corresponding empty vectors (pre-NC/NC) as negative controls. The relative luciferase activity was measured 24 hours post-transfection using a Dual Luciferase Assay Kit (Promega). To further evaluate the effects of miR-539 on MyD88 expression, the HE-2 cells were transfected with a miR-539 mimic or inhibitor, as well as a pre-NC

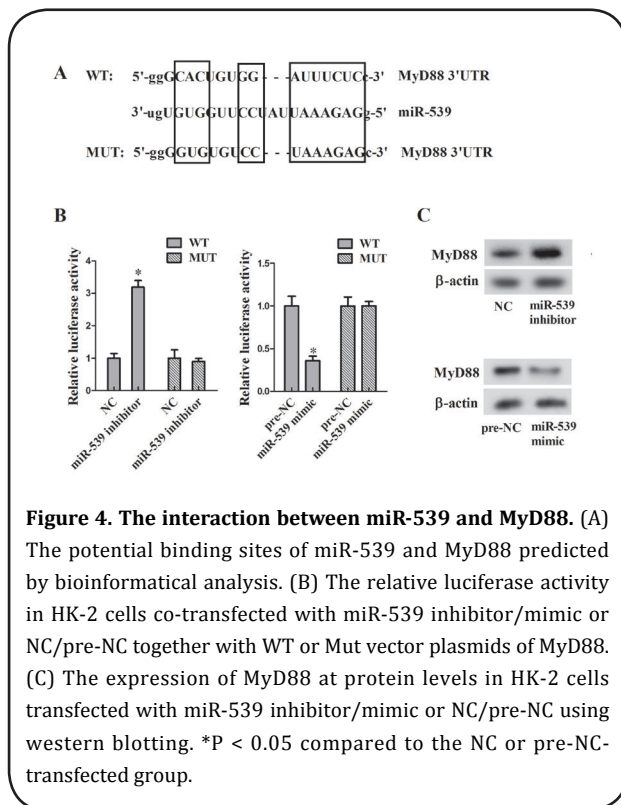


Figure 4. The interaction between miR-539 and MyD88. (A) The potential binding sites of miR-539 and MyD88 predicted by bioinformatical analysis. (B) The relative luciferase activity in HK-2 cells co-transfected with miR-539 inhibitor/mimic or NC/pre-NC together with WT or Mut vector plasmids of MyD88. (C) The expression of MyD88 at protein levels in HK-2 cells transfected with miR-539 inhibitor/mimic or NC/pre-NC using western blotting. * $P < 0.05$ compared to the NC or pre-NC-transfected group.

or NC in order to measure the MyD88 expression at each protein level.

Cell transfection

The molecular functions and biological roles of miR-539 within the in vitro I/R injury were then investigated. The HK-2 cells were randomly divided into six groups: control, OGD, OGD+pre-NC, OGD+miR-539 mimic, OGD+miR-539 mimic+pcDNA, and OGD+miR-539 mimic+pcDNA-MyD88. Prior to transfection, the cells were passaged three times, were seeded in 24-well plates, and were underwent OGD experimentation. Following this process, the cells were incubated in the culture medium for an additional 24 hours for reoxygenation. After the confluence reached 30-50%, cell transfection was performed using the Lipofectamine 2000 reagent (Invitrogen). The MyD88, p-p65, and p65 protein levels and the TNF- α , IL-6, and IL-8 levels were tested after 48 hours of transfection.

In vivo experiment

Following renal transplant I/R injury, a total of 12 male C57BL/6J mice were intravenously injected with 10 mg/kg miR-539 mimic-locked nucleic acid (LNA)-modified oligonucleotide probes, or its control scrambled sequence LNA (pre-NC-LNA). This was done after 12 hours of reperfusion. Forty-eight hours later, the mice were euthanized by cervical dislocation so that blood samples and ischemic renal tissues could be obtained in order to detect serum BUN levels, tubular necrosis

score, miR-539 expression, and the MyD88, p-p65, and p65 protein expressions.

Serum BUN measurement

Renal function was assessed by measuring the serum BUN levels of the mice. Blood samples were collected in centrifugal tubes, and were centrifuged at 3600 rpm for 10 minutes at 4 °C. The BUN levels were analyzed by using the HITACHI 7060 automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan), and calculated by multiplying the urea level by 0.467.

QRT-PCR

Total RNA was extracted from the tissue samples and the HK-2 cells by using the Trizol reagent (Invitrogen), and reversely transcribed into cDNA by using a MiRcute miRNA First-strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China). The relative quantification of the miRNA-539 expression levels were determined by using a MiRcute miRNA qPCR Detection Kit (Tiangen Biotech) on the ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA), and they were calculated by the $2^{-\Delta\Delta Ct}$ method. Within this study, U6 genes served as the internal controls.

Western blotting

Total protein was separated from the renal tissues and HK-2 cells by using a Radio-Immunoprecipitation Assay (RIPA) buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein was then centrifuged at 14000 rpm for 15 minutes at 4 °C. The total protein extraction was incubated with a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and was then transferred to the polyvinylidene fluoride membranes and blocked with tris buffered saline tween (TBST) containing 5% skim milk at room temperature for one hour. The membranes were probed with primary antibodies against MyD88, p-p65 (Ser 536), and p65 (1:1000; Santa Cruz Biotechnology) at 4 °C overnight. Following this, they were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:200; Abcam, Cambridge, UK) at room temperature for one hour. Band intensities were standardized against β -actin (Sigma, Scott, CA, USA), and relative density was analyzed on a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) using an enhanced chemiluminescence reagent (Thermo Scientific, Shanghai, China).

Assessment of pro-inflammatory cytokines levels

The levels of various pro-inflammatory cytokines, including TNF- α , IL-6, and IL-8, were measured using a Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Each blood sample was tested twice.

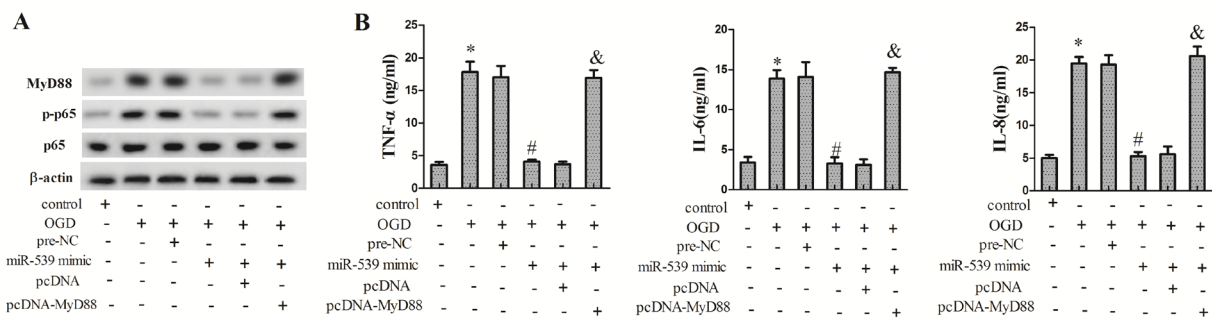


Figure 5. Effects of miR-539 on inflammatory response during I/R injury. (A) The MyD88 and unphosphorylated and phosphorylated NF-κB p65 protein expressions in the HK-2 cell subgroups consisting of control, OGD, OGD+pre-NC, OGD+miR-539 mimic, OGD+miR-539 mimic+pcDNA, and OGD+miR-539 mimic+pcDNA-MyD88 using western blotting. (B) The pro-inflammatory cytokines levels containing TNF-α, IL-6, and IL-8 in the HK-2 cell subgroups consisting of control, OGD, OGD+pre-NC, OGD+miR-539 mimic, OGD+miR-539 mimic+pcDNA, and OGD+miR-539 mimic+pcDNA-MyD88 using ELISA. *P < 0.05 as compared to the control group; #P < 0.05 as compared to OGD+pre-NC treated group; &P < 0.05 as compared to the OGD+miR-539 mimic+pcDNA treated group.

Renal TNS

Renal tissue samples were fixed in formalin, embedded in paraffin, and cut into 4 mm sections. Histological sections were stained with hematoxylin and eosin. The degree of tubular necrosis was scored by a renal pathologist using the following 4-point quantitative scale: score 0, normal tubule; score 1, < 5% tubular necrosis; score 2, 5~25% of tubular necrosis; score 3, 25~75% tubular necrosis; and score 4, total destruction of all epithelial cells with naked basement membranes [17].

Statistical analysis

All data was presented as the mean ± standard deviation and analyzed by SPSS version 22.0 (SPSS, Chicago, IL, USA). Comparisons between the groups were performed using a two-sided Student's t test. Within these comparisons, a P-value of < 0.05 was considered statistically significant.

RESULTS

miR-539 was downregulated, while MyD88/NF-κB were upregulated the mice with renal transplant I/R injury

In contrast to the sham-operated mice model, the mice who underwent renal transplant I/R injury had a dramatic increase in serum BUN levels (Figure 1A, P < 0.05), a significant downregulation of miR-539 (Figure 1B, P < 0.05), and a remarkable increase in the MyD88 protein expression and phosphorylated expressions of NF-κB p65 (Figure 1C, P < 0.05). These results suggest that miR-539, MyD88, and NF-κB were involved in the pathological process of renal I/R injury.

miR-539 overexpression suppressed the secretion of

pro-inflammatory cytokines under hypoxic conditions

Through this study, we discovered the role of miR-539 in regulating inflammatory response in I/R injury. Exposure to OGD for mimicking I/R-like conditions in vitro led to a decrease in miR-539 expression levels (Figure 2A, P < 0.05), and an elevated pro-inflammatory cytokines containing TNF-α, IL-6, and IL-8 relative to those in the control group (Figure 2B, P < 0.05). The overexpression of miR-539 was upregulated significantly (Figure 2A, P < 0.05), reducing the TNF-α, IL-6, and IL-8 expressions following exposure to OGD (Figure 2B, P < 0.05). Our data indicates that the overexpression of miR-539 alleviated inflammatory response following I/R injury.

miR-539 knockdown upregulated pro-inflammatory cytokines

In order to confirm the anti-inflammatory effects of miR-539, the expression of the miR-539, TNF-α, IL-6, and IL-8 in HK-2 cells transfected with NC or a miR-539 inhibitor were measured. The results demonstrated that miR-539 knockdown observably diminished the expression levels of miR-539 (Figure 3A, P < 0.05), enhancing TNF-α, IL-6, and IL-8 levels and revealing an anti-inflammatory effect exerted by miR-539 in vitro (Figure 3B, P < 0.05).

MyD88 was a direct target of miR-539

The bioinformatics database predicted that miR-539 harbored one putative binding site for MyD88 (Figure 4A). A luciferase reporter assay was used to verify potential binding interactions between miR-539 and MyD88. As expected, the co-transfected miR-539 inhibitor increased luciferase activity when compared

with the NC group, while the miR-539 mimic inhibited luciferase activity when compared with the pre-NC in pISO-MyD88-WT. However, MyD88-MUT scarcely responded to the miR-539 mimic or inhibitor (Figure 4B, $P < 0.05$). To further validate these results, the miR-539 inhibitor and mimic were transfected into normal cultured HK-2 cells. The results showed a conspicuous increase in MyD88 expressions in the HK-2 cells transfected with the miR-539 inhibitor when compared to the NC control. Conversely, the overexpression of miR-539 downregulated MYD88 protein levels, suggesting a direct binding between miR-539 and MyD88 (Figure 4C, $P < 0.05$).

miR-539 inhibited I/R injury-induced inflammation by downregulating MyD88 and NF- κ B *in vitro*

The effect of miR-539 on I/R injury-induced changes in MyD88, unphosphorylated, and phosphorylated NF- κ B p65 protein expressions, and pro-inflammatory cytokines levels were then examined. The results of this investigation reveal that OGD treatment increased the levels of MyD88 and phosphorylated NF- κ B p65 protein expressions (Figure 5A, $P < 0.05$), as well as TNF- α , IL-6, and IL-8 (Figure 5B, $P < 0.05$). In contrast, upregulation was prevented in the HK-2 cells transfected with miR-539 mimic for MyD88 and phosphorylated NF- κ B p65 expressions (Figure 5A, $P < 0.05$) and pro-inflammatory cytokines (Figure 5B, $P < 0.05$) produced by OGD. Nevertheless, the co-overexpression of miR-539 and MyD88 restored the elevated MyD88 and phosphorylated NF- κ B p65 protein expressions (Figure 5A, $P < 0.05$), along with the TNF- α , IL-6, and IL-8 (Figure 5B, $P < 0.05$) in OGD treated HK-2 cells. Thus, MiR-539 mitigated I/R injury-induced inflammatory response via mediating the MyD88/NF- κ B pathway.

miR-539 exerted anti-inflammatory effects for mice with renal transplant I/R injury by downregulating the MyD88/NF- κ B pathway

To investigate the *in vivo* role of miR-539 in renal I/R injury, we used a systemic injection of miR-539 mimic-LNA oligonucleotides and its control scrambled LNA. The group of mice that underwent renal I/R injury and received miR-539 mimic showed a decrease in serum BUN levels and renal tubular necrosis (Figure 6A, $P < 0.05$), a significant increase in miR-539 mRNA expression (Figure 6B, $P < 0.05$), and a decrease in MyD88 and phosphorylated NF- κ B p65 protein expressions (Figure 6C, $P < 0.05$) when compared to the mice that received the control sequence-scrambled oligonucleotide. Jointly, these *in vitro* and *in vivo* findings suggest that miR-539 suppresses the MyD88/NF- κ B pathway, thereby inhibiting inflammatory response during renal I/R injury.

DISCUSSION

Renal transplant-induced I/R injury is a clinical and experimental syndrome. The features of this syndrome include renal dysfunction, tubular cell necrosis, and inflammatory cell infiltration [18]. Inflammatory response is a major consequence of I/R injury, and is responsible for organ transplant failure [19,20]. In the present study, we established a mouse model of renal transplant I/R injury in order to examine the altered expressions of relevant miRNAs and proteins. Our data indicates that as the serum BUN levels increased in mice with renal I/R injury, miR-539 was downregulated and MyD88 and phosphorylated NF- κ B p65 protein expressions were upregulated when compared to the group of sham-operated mice.

Recent evidence suggests that miR-539 functions as a tumor suppressor gene in a series of cancers, including thyroid, prostate, nasopharyngeal carcinoma, non-small cell lung, and human colorectal cancer [15]. As such, it has become an early diagnostic indicator and disease therapy target. Because of this, the protective effects of miR-539 in renal I/R injury were examined

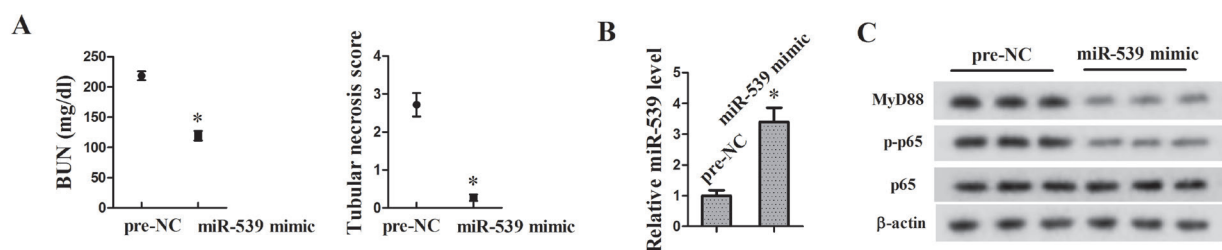


Figure 6. Effects of miR-539 on inflammation in the renal transplant I/R injury mouse model. (A) The serum BUN levels and renal tubular necrosis score (TNS) in mice receiving miR-539 mimic or pre-NC. (B) The relative miR-539 mRNA expression levels in mice receiving miR-539 mimic or pre-NC using qRT-PCR. (C) The MyD88 and unphosphorylated and phosphorylated NF- κ B p65 protein expressions in mice receiving miR-539 mimic or pre-NC using western blotting.

* $P < 0.05$ as compared to the pre-NC injected group.

in this study. We found that the overexpression of miR-539 in HK-2 cells following I/R injury induced by OGD treatment suppressed the expression of pro-inflammatory cytokines, TNF- α , IL-6, and IL-8. In contrast, the knockdown of miR-539 enhanced pro-inflammatory cytokines expressions in the cultured HK-2 cells. Consequently, our data illustrates the first case where miR-539 has exerted anti-inflammatory effects on HK-2 cells in vitro.

It is generally recognized that the MyD88/NF- κ B signaling pathway participated in the lipopolysaccharide (LPS)-induced inflammatory response [21,22]. A novel document reported that the MyD88/NF- κ B signaling pathway was associated with a pyrin domain containing 3 (NLRP3) inflammasome activities and an IL-1 β secretion, which were believed to be important for innate immunity [12]. Furthermore, Jang et al. found that the MyD88/NF- κ B signaling pathway was closely related to the occurrence and development of inflammatory diseases [23]. Thus, an exploration of its potential as a biomarker or therapeutic target gene would provide significant insight into renal I/R injury treatment options.

By using a luciferase reporter assay, this study verifies that MyD88 is a downstream target gene of miR-539. This conclusion had not been confirmed prior to the completion of this study. The research also shows that miR-539 knockdown increases the MyD88 protein expression, while the overexpression of miR-539 suppresses the protein expression of MyD88. As a result of further cell transfection experiments verifying the molecular functions and biological roles of miR-539 in renal I/R injury, we confirmed that the overexpression of miR-539 in OGD-treated HK-2 cells downregulated MyD88 and phosphorylated NF- κ B p65 protein expressions and TNF- α , IL-6, and IL-8 levels. Contrastingly, the co-overexpression of miR-539 and MyD88 led to the opposite effect. These findings indicate that miR-539 blocks the MyD88/NF- κ B signaling pathway by directly targeting MyD88. In turn, inflammatory response induced by renal I/R injury is inhibited.

We then investigated the anti-inflammatory effects of miR-539 on mice that underwent renal transplant I/R injury. The injection of miR-539 mimic in the mice not only decreased their serum BUN levels, but also markedly reduced renal tubular necrosis. This suggests that miR-539 effectively alleviates renal tubular injury and restores renal function if used for treatment. Additionally, miR-539 mimic injection upregulated miR-539 expression, while it suppressed MyD88 and phosphorylated NF- κ B p65 protein expressions. This was consistent with the in vitro results. This study has investigated the anti-inflammatory effects of miR-539 in renal transplant I/R injury via suppressing MyD88/

NF- κ B signaling activation for the first time. Hence, the results of this study have the ability to provide novel preventive and therapeutic options for treating renal transplant I/R injury.

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