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MicroRNA-494-3p alleviates inflammatory response in sepsis by targeting TLR6

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Abstract. – OBJECTIVE: To clarify whether microRNA-494-3p could exert an anti-inflammation effect by suppressing the expression of toll-like receptor 6 (TLR6), thus inhibiting the development of sepsis.

PATIENTS AND METHODS: Plasma levels of microRNA-494-3p and TLR6 in sepsis patients and healthy controls were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Diagnostic potential of microRNA-494-3p in sepsis was evaluated by receiver operating characteristic (ROC) curve. *In vitro* macrophage inflammation model was established by lipopolysaccharides (LPS) induction in RAW264.7 cells. Expression levels of microRNA-494-3p, TLR6 and tumor necrosis factor-α (TNF-α) in LPS-induced RAW264.7 cells were observed. After transfection of microRNA-494-3p mimics in LPS-induced RAW264.7 cells, mRNA and protein levels of TNF-α were determined by qRT-PCR and Western blot, respectively. Meanwhile, cytoplasmic and nuclear fractions of nuclear factor-kappa B (NF-κB) p65 were respectively extracted for evaluating nuclear translocation of NF-κB p65 by Western blot analysis. Dual-luciferase reporter gene assay was performed to verify the binding between microRNA-494-3p and TLR6. Finally, rescue experiments were carried out to elucidate whether microRNA-494-3p attenuated sepsis-induced inflammation through degrading TLR6.

RESULTS: Plasma level of microRNA-494-3p in sepsis patients was markedly lower than healthy controls, while plasma level of TLR6 was conversely higher in sepsis patients. With the prolongation of LPS induction in RAW264.7 cells, expression levels of TLR6 and TNF-α gradually increased, whereas microRNA-494-3p expression decreased. Transfection of microRNA-494-3p mimics in RAW264.7 cells reduced TNF-α level, and inhibited nuclear translocation of NF-κB p65. TLR6 was found to be a target gene of microRNA-494-3p, and its expression was markedly downregulated by microRNA-494-3p overexpression. Finally, we proved that the inhibitory effects of microRNA-494-3p on TNF-α level and nuclear translocation of NF-κB p65 were reversed by TLR6.

CONCLUSIONS: High expression of microRNA-494-3p attenuated sepsis-induced inflammatory response by degrading TLR6.

Key Words:
Sepsis, MicroRNA-494-3p, TLR6, NF-κB p65.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by infectious factors. It is a common complication in severe trauma, shock, infection, and critically ill patients. Sepsis is one of the leading causes of death in Intensive Care Units (ICU)[1]. It is reported that sepsis causes about 6 million deaths each year[2]. The occurrence of sepsis is associated with infection factors, cascade of hypersensitivity after severe tissue damage, and excessive inflammatory mediators. Responses of neutrophils and macrophages against cytokines and chemokines eventually exacerbate the inflammatory response, finally leading to sepsis shock or even death[3,4]. Early diagnosis, accurate evaluation of disease condition, and prognosis will prevent the aggravation of sepsis, thus reducing its mortality. MicroRNA (miRNA) is an endogenous, non-coding RNA with regulatory functions, containing about 20-25 nucleotides in length. It recognizes target mRNA by complementary base pairing, thus degrading or inhibiting translation of mRNA. Recent studies have confirmed the crucial role of miRNAs in various infectious diseases. Inhibition of certain miRNAs could stimulate intracellular infection. Through controlling virus transcription and replication, miRNAs allow the long-term survival of virus in cells[5-7]. An innate immune-related research indicated that host cells can rapidly regulate miRNA expression under the stimulation of pathogenic microorganisms[8]. As a consequence, immediate release of inflammatory factors lead to hyperthyroidism. Meanwhile, inflammatory factors could also be degraded at the same time to induce

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cell apoptosis and immunosuppression[9]. Evidence10 proved the crucial effects of miRNAs on the pathogenesis of sepsis. In this study, plasma level of microRNA-494-3p remained low in sepsis patients. Through bioinformatics analysis, toll-like receptor 6 (TLR6) was verified as a potential target gene of microRNA-494-3p and remained a high plasma level in sepsis patients. We specifically investigated the roles of microRNA-494-3p and TLR6 in lipopolysaccharides (LPS)-induced macrophages, so as to reveal their potential functions in sepsis-induced inflammatory response.

Patients and Methods

Sample Collection

6 mL of peripheral venous blood was extracted from all subjects and preserved in anticoagulation tubes. Blood samples were centrifuged at 2500 r/min for 10 min within 2 h of collection. 600 μL of plasma were harvested and stored in a 1.5 mL of RNA-free Eppendorf (EP) tube at -80°C. Subjects volunteered to participate in the study signed written informed consent before sample collection. This study has been approved by the Ethics Committee of Tianjin First Center Hospital (Tianjin, China).

Cell Culture

RAW264.7 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO2 incubator.

Cell Transfection

Transfection was performed when the cell density reached 70-80%. Cells were transfected with microRNA-494-3p mimics, pcDNA-TLR6 or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After cell culture for 4 h, fresh medium was replaced and incubated for 24 h.

Preparation of LPS Solution

1 mg of solid LPS was completely dissolved in a sterile phosphate-buffered solution (PBS), prepared for 1 mg/mL stock solution, and stored at -20°C. LPS solution was diluted 10 times aseptically for preparing the working solution of 100 μg/mL at the time of usage.

RNA Extraction

Total RNA was extracted according to the instructions of the American ABI miRVana PARIS kit (Applied Biosystems, Foster City, CA, USA). All processes were carried out on a clean bench. The extracted RNA was finally dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime Biotechnology, Shanghai, China). RNA concentration and purity were determined by a U-2800 ultraviolet spectrophotometer. RNA samples were placed in a refrigerator at -20°C for use.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Extracted RNA was quantified, purified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). U6 was used as an internal reference. PCR system including SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), template, upstream/downstream primer and DEPC was subjected to qRT-PCR using a Real-time PCR machine. Primers used were as follows: MicroRNA-494-3p, F:5'-ACACTCCAGCTGGGTGAAACATACACGGGA-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAGGTTTC-3'; U6, F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'; TLR6, F: 5'-ACTCACCAGAGGTCCAA-3', R: 5'-GGATGAATGGCGTGTC-3'; TNF-α, F: 5'-GCCACCACGCTCTTCTG-3', R: 5'-GCAGCCTTGTCCCTTGA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH),F:5'-GAAGAGAGAGACCCTCACGCTG-3', R: 5'-ACTGTGAGGAGGGGAGATTCAGT-3'.

Dual-Luciferase Reporter Gene Assay

The transcript 3'Untranslated Region (3'UTR) sequence of TLR6 was cloned into the vector pGL3 containing the luciferase reporter gene, which was the TLR6 WT. TLR6 MUT was constructed by mutating the core binding sequences using a site-directed mutagenesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were co-transfected with microRNA-494-3p mimics or negative control and TLR6 WT or TLR6 MUT, respectively. At 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 μL of the supernatant were collected for determining the luciferase activity.

Extraction of Nuclear and Cytoplasmic Fractions

Cell suspension was centrifuged at 1000 rpm for 5-7 min, washed with phosphate-buffered saline (PBS) and centrifuged again. The precipitate was harvested for extracting cytoplasmic and nu-

clear proteins using the Nuclear and Cytoplasmic Extraction Reagents (NE-PER) reagents (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot

Total protein was extracted using the cell lysate for determining protein expression. Protein sample was quantified by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The t-test was used for comparing differences between the two groups. Receiver operating characteristic (ROC) curve was introduced to evaluate the specificity and sensitivity of microRNA-494-3p in sepsis diagnosis. $p<0.05$ was considered statistically significant.

Results

MicroRNA-494-3p was Lowly Expressed in Sepsis Patients

We first examined the plasma level of microRNA-494-3p in sepsis patients and healthy controls. As qRT-PCR data revealed, microRNA-494-3p was lowly expressed in sepsis patients (Figure 1A). ROC curve was plotted based on the clinical data of enrolled sepsis patients, indicating the potential diagnostic value of microRNA-494-3p in sepsis (AUC=0.8375, cut-off value=1.785, Figure 1B). To further explore the role of microRNA-494-3p in regulating sepsis, an in vitro macrophage inflammation model was established by LPS induction in RAW264.7 cells. Expression level of the inflammatory factor TNF-α was detected after LPS induction for 0 h, 3 h, 6 h, and 12 h, respectively. Both mRNA and protein levels of TNF-α increased with the prolongation of LPS induction, suggesting the successful construction of in vitro sepsis model (Figure 1C and 1D). In the following experiments, LPS induction was set for 12 h.

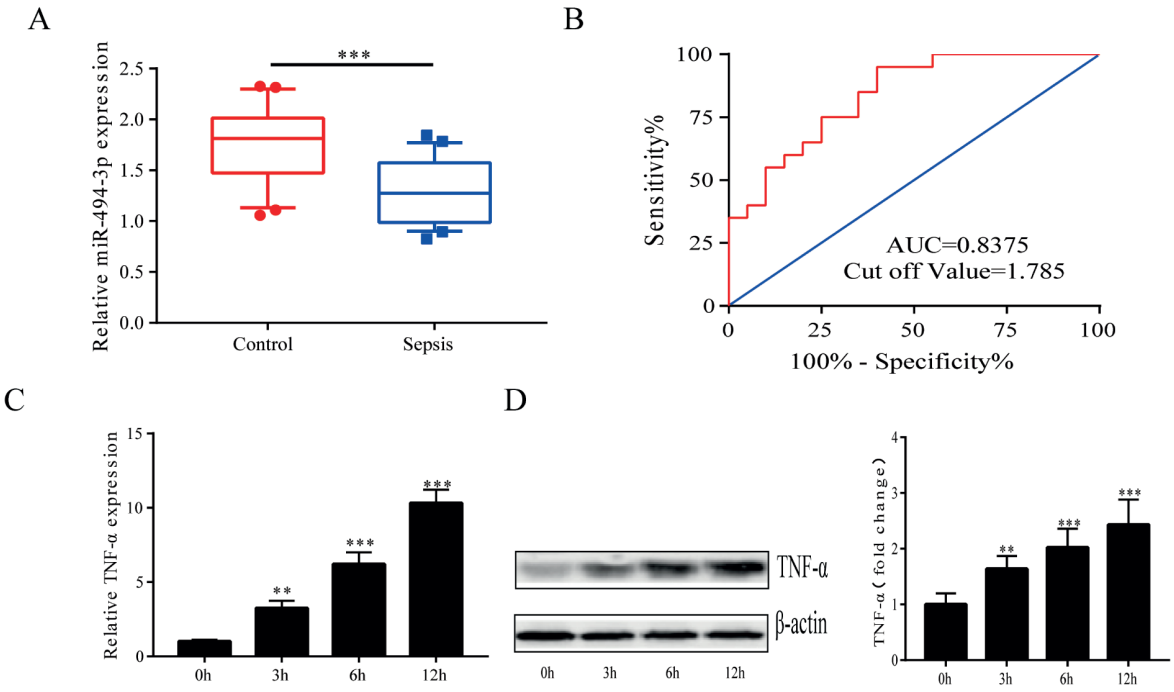

Figure 1. MiR-494-3p was lowly expressed in sepsis patients. ***A,*** Plasma level of miR-494-3p was lower in sepsis patients than healthy controls. ***B,*** ROC curve plotted based on miR-494-3p expression in sepsis patients (AUC=0.8375, cut-off value=1.785). ***C,*** The mRNA level of TNF-α gradually increased with the prolongation of LPS induction in RAW264.7 cells. ***D,*** The protein level of TNF-α gradually increased with the prolongation of LPS induction in RAW264.7 cells. $^{**}p<0.01$, $^{***}p<0.001$.

MicroRNA-494-3p Inhibited LPS-Induced Inflammation

We, thereafter, detected microRNA-494-3p expression in LPS-induced RAW264.7 cells at different time points. QRT-PCR results demonstrated a gradual decrease in microRNA-494-3p expression with the prolongation of LPS induction (Figure 2A). We speculated that microRNA-494-3p could have a potential role in regulating the development of sepsis. Subsequently, transfection efficacy of microRNA-494-3p mimics was verified in RAW264.7 cells (Figure 2B). MicroRNA-494-3p overexpression downregulated both mRNA and protein levels of TNF-αin LPS-induced RAW264.7 cells (Figure 2C). Since TNF-α is a key factor in TLRs/NF-κB pathway and TNF-α production relies on NF-κB, we presumed that microRNA-494-3p may affect NF-κB activation. Cytoplasmic and nuclear fractions of NF-κB p65 were extracted from RAW264.7 cells overexpressing microRNA-494-3p, respectively. Western blot results indicated that microRNA-494-3p overexpression markedly inhibited nuclear translocation of NF-κB p65 (Figure 2D and 2E). Therefore, we speculated the anti-inflammatory effect of microRNA-494-3p on sepsis was dependent on NF-κB inhibition.

TLR6 was the Target Gene of microRNA-494-3p

To further explore the regulatory mechanism of microRNA-494-3p in the development of sepsis, TLR6 was predicted to be a potential target gene of microRNA-494-3p through online websites. We further performed dual-luciferase reporter gene assay and verified their binding (Figure 3A). Transfection of microRNA-494-3p mimics in RAW264.7 cell downregulated both mRNA and protein levels of TLR6, indicating the negative regulation of microRNA-494-3p on TLR6 expression (Figure 3B). Subsequently, we determined plasma level of TLR6 in sepsis patients and healthy controls. As the data indicated, sepsis patients had a higher plasma level of TLR6 than controls (Figure 3C). During the process of LPS induction, TLR6 expression gradually increased in RAW264.7 cells (Figure 3D). Therefore, we speculated that TLR6 was greatly involved in the process of inflammatory response of sepsis.

MicroRNA-494-3p Exerted its Function in Sepsis Through Degrading TLR6

A series of rescue experiments were carried out to clarify whether microRNA-494-3p attenuated inflammatory response through inhibi-

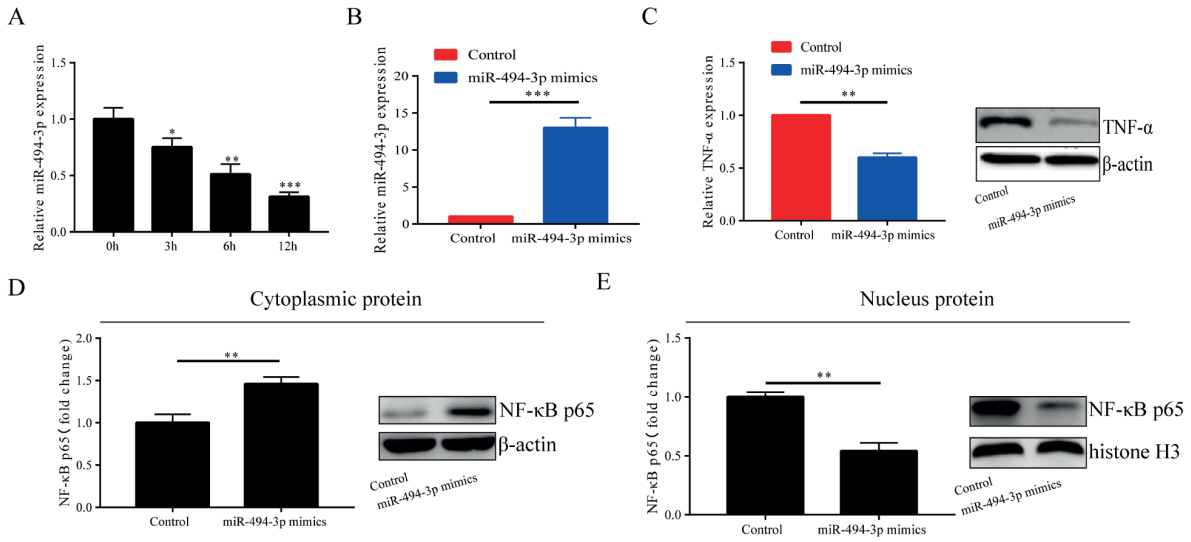

Figure 2. MiR-494-3p inhibited LPS-induced inflammation. ***A,*** MiR-494-3p expression gradually decreased with the prolongation of LPS induction. ***B,*** Transfection efficacy of miR-494-3p mimics in RAW264.7 cells. ***C,*** Overexpression of miR-494-3p inhibited expression level of TNF-α. ***D-E***, Overexpression of miR-494-3p inhibited nuclear translocation of NF-κB p65. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

ting TLR6. Overexpression plasmid of TLR6 was first constructed and its transfection efficacy in RAW264.7 cells was verified at both mRNA and protein levels (Figure 4A). Cells were co-overexpressed with microRNA-494-3p and TLR6 or only overexpressed with microRNA-494-3p. The mRNA and protein level of TNF-α increased in co-overexpressed cells than those only overexpressing microRNA-494-3p (Figure 4B). It is suggested that the effect of microRNA-494-3p on inhibiting TNF-α level was reversed by TLR6 overexpression. Moreover, the inhibitory role of microRNA-494-3p in nuclear translocation of NF-κB p65 was partially reversed by TLR6 overexpression (Figure 4C and 4D). To sum up, microRNA-494-3p exerted its anti-inflammation function in sepsis through target degrading TLR6.

Discussion

Toll-like receptors (TLRs) are a class of transmembrane proteins that recognize pathogen-as-

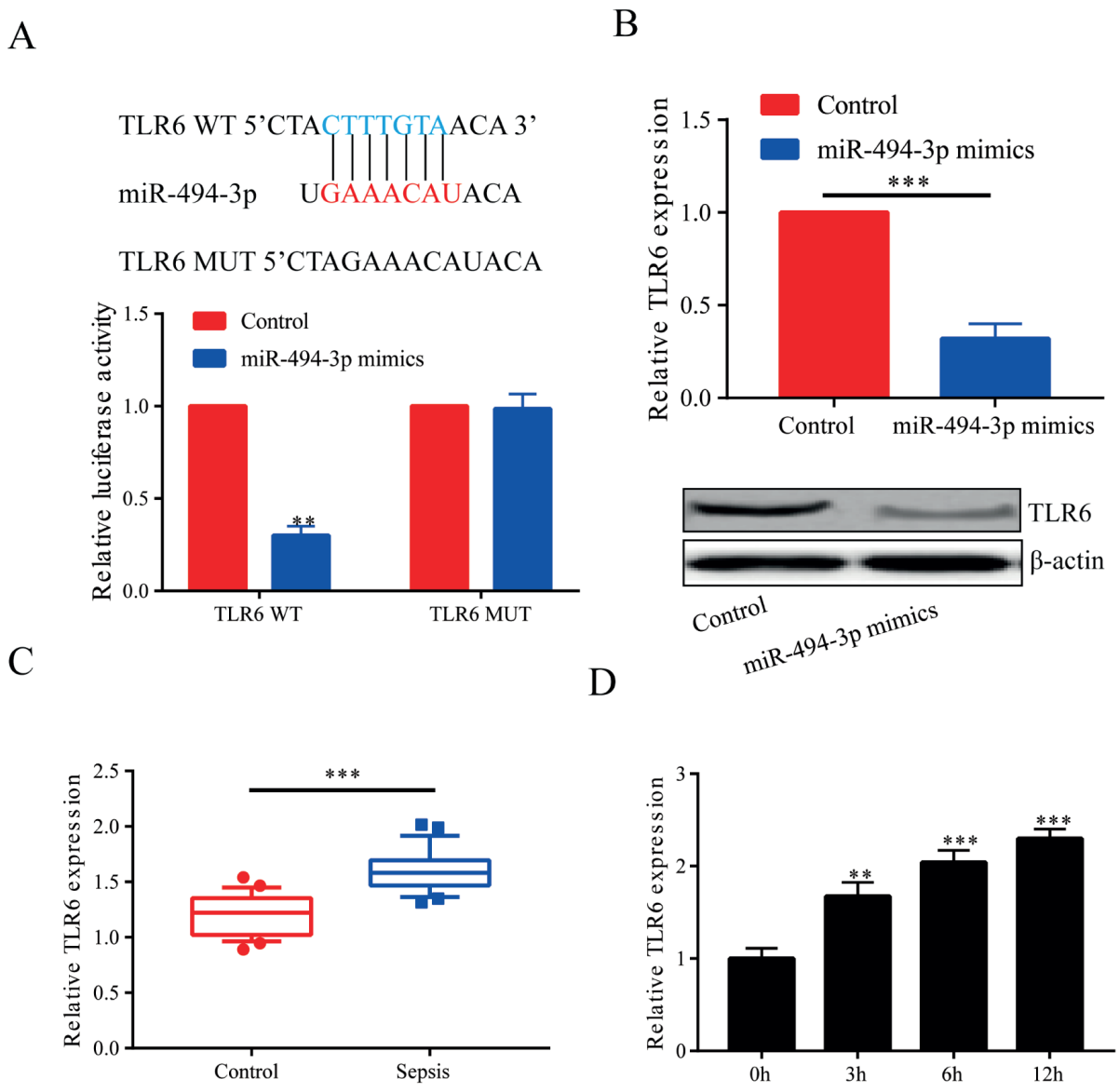

Figure 3. TLR6 was the target gene of miR-494-3p. ***A,*** Potential binding sites between miR-494-3p and TLR6. Dual-luciferase reporter gene assay verified their binding. B, Overexpression of miR-494-3p in RAW264.7 cells inhibited mRNA and protein levels of TLR6. ***C,*** Plasma level of TLR6 was higher in sepsis patients than healthy controls. D, TLR6 expression gradually increased with the prolongation of LPS induction in RAW264.7 cells. $^{**}p<0.01$, $^{***}p<0.001$.

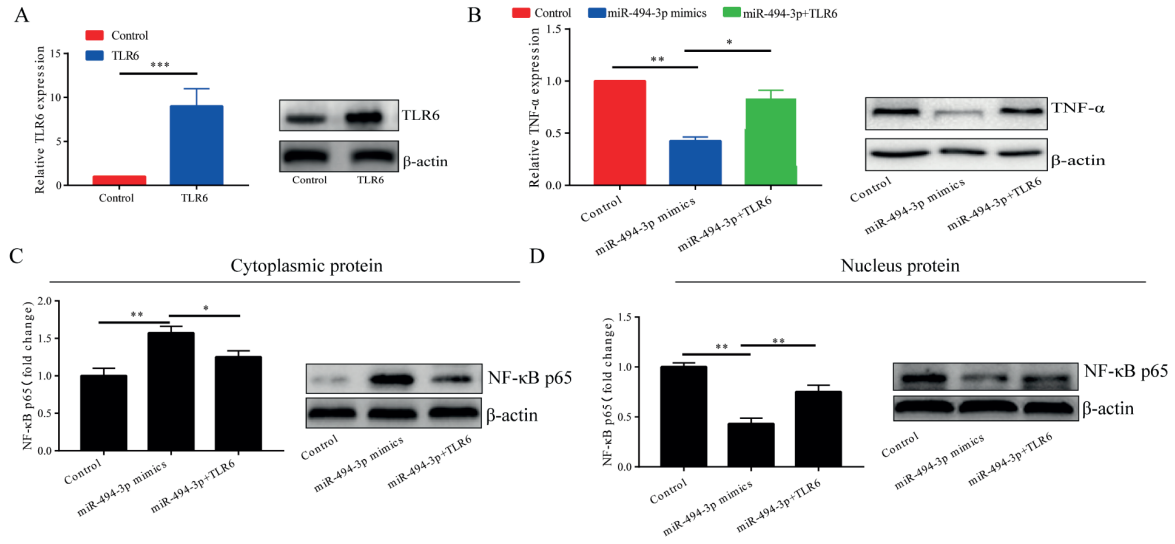

Figure 4. MiR-494-3p exerted its function through degrading TLR6. ***A,*** Transfection efficacy of overexpression plasmid of TLR6 in RAW264.7 cells. ***B,*** Overexpression of miR-494-3p inhibited expression level of TNF-α, which was partially reversed by TLR6 overexpression. ***C-D***, Overexpression of miR-494-3p inhibited nuclear translocation of NF-κB p65, which was partially reversed by TLR6 overexpression. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

sociated molecular patterns (PAMPs). The TLRs/NF-κB pathway is closely related to the development of sepsis. Expression levels of TLRs and pro-inflammatory cytokines increase with the severity of sepsis. TLR4 is a key recognition receptor for identifying LPS. LPS releases into the blood and rapidly binds to LBP (LPS-binding protein). Subsequently, it binds to TLR4 through anchoring cell membrane with glycosyl-phosphatidyl inositol, further activating the intracellular cascade in a MyD88-dependent or independent pathway. The produced inflammatory mediators eventually lead to sepsis and may even cause the multiple organ dysfunction disease at the final stage12. MiRNAs not only directly participate in the regulation of anti-inflammatory/pro-inflammatory homeostasis, but also indirectly regulates the homeostasis through activating TLRs pathway13. MiR-146a is one of the earliest discovered miRNAs relative to inflammation. It activates inflammatory mediators in LPS-induced monocytes via NF-κB pathway[14,15]. Li et al[16] pointed out that miR-155 and miR-146 are upregulated after LPS induction in monocytes. MiR-146a inhibits inflammatory response through targeting IRAK1 kinase and TRAF6 ligase via activating TLRs pathway. MiR-155 is involved in the development of sepsis by targeting on FADD, IKKI3 and IKKs in TLRs pathway[17]. In a study of neonatal sepsis, miR-15a and miR-16 markedly are upregulated in sepsis newborns than healthy controls. Overexpression of miR-15a and miR-16 could suppress the LPS-induced inflammatory pathways, suggesting the potentials of miR-15a and miR-16 to be the diagnostic and prognostic markers for neonatal sepsis18. In this study, qRT-PCR data first revealed a lower plasma level of microRNA-494-3p in sepsis patients relative to healthy controls. Further experiments showed that microRNA-494-3p exerted its anti-inflammation effect by suppressing the nuclear translocation of NF-κB p65 to inactivate TNF-α. Through online prediction, TLR6 was identified to be a potential target gene of microRNA-494-3p. TLR6 belongs to the TLRs family, which is capable of activating the NF-κB pathway19. An *in vivo* experiment pointed out that inflammatory response is attenuated in TLR6-deficient mice through inhibition of NF-κB pathway[20]. Here, we verified the binding between microRNA-494-3p with TLR6 through dual-luciferase reporter gene assay. Plasma level of TLR6 was higher in sepsis patients in comparison with healthy controls. More importantly, TLR6 upregulation partially reversed the inhibitory effect of microRNA-494-3p on inflammatory response of sepsis. We postulated that the anti-inflammatory effect of microRNA-494-3p on sepsis was dependent on TLR6 degradation.

Conclusions

We found that high expression of microRNA-494-3p attenuated inflammatory response of sepsis by degrading TLR6. Hence, microRNA-494-3p may be utilized as a new target for early prevention and treatment of sepsis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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