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Influences of up-regulation of miR-126 on septic inflammation and prognosis through AKT/Rac1 signaling pathway

H.-F. WANG, Y.-Q. WANG, L. DOU, H.-M. GAO, B. WANG, N. LUO, Y. LI

Department of Intensive Care Unit, Tianjin First Center Hospital, Tianjin, China

Abstract. – OBJECTIVE: To explore the influences of the up-regulation of micro ribonucleic acid (miR)-126 on septic inflammation and prognosis through the AKT/Rac1 signaling pathway.

MATERIALS AND METHODS: Human pulmonary microvascular endothelial cells (HMVECs) were cultured and transfected with miR-126 mimics. The HMVECs in the logarithmic growth phase in different groups were incubated with thrombin. The transmembrane resistivity of HMVECs was detected as the permeability via Electric Cell-substrate Impedance Sensing (ECIS) system. The endothelial cell space was observed via immunofluorescence. The mouse model of sepsis was then established and the serum was extracted to detect interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The survival curve was plotted based on the death time. The Statistical Product and Service Solutions (SPSS) 22.0 was used for statistical analysis, and $p<0.05$ suggested that the difference was statistically significant.

RESULTS: Thrombin could significantly increase the permeability of HMVECs, while the overexpression of miR-126 markedly inhibited the increased permeability. The overexpression of miR-126 also reduced the endothelial cell space induced by thrombin. In addition, the serum IL-6 and TNF-α levels of sepsis mice in miR-126 overexpression group were significantly decreased compared to those in the control group. Moreover, the death rate of mice exogenously expressing miR-126 was lower than that in the control group.

CONCLUSIONS: The up-regulation of miR-126 inhibited the septic inflammation and improved the prognosis of sepsis mice through the AKT/Rac1 signaling pathway.

Key Words:
MiR-126, AKT/Rac1, Septic inflammation, Prognosis.

Introduction

Sepsis is one of the leading causes of death in the intensive care unit (ICU) in the United States and affects more than 1,000,000 people every year[1-3]. Sepsis is characterized by the release of a large number of inflammatory cytokines, which promote the activation and transport of immune cells. In addition, sepsis often damages the microcirculation and leads to organ failure due to the endothelial dysfunction[4]. Therefore, inactivating the release of cytokines and chemokines, and alleviating the endothelial cell damage in sepsis possess have huge therapeutic benefits.

The therapy using the RNA interference strategy has displayed the potential in treating cardiovascular diseases, cancer and inflammatory diseases. Micro-ribonucleic acids (miRNAs) are non-coding RNAs with 21-25 nucleotides in length and post-transcriptionally regulate genes expression by causing gene instability and preventing translation of messenger RNA[5]. MiRNAs can effectively prevent the endothelial cell damage and improve the repair mechanisms of cell stress and inflammatory response in sepsis[6]. MiR-126 is the most abundantly expressed miRNA in endothelial cells and is extremely important for maintaining the vascular integrity and angiogenesis[7]. The genetic deletion of pre-miR-126 hinders the vascular development, prevents the angiogenesis and increases the vascular permeability[8,9]. The inhibition of miR-126 in the inflammatory microenvironment improves the expression of vascular endothelial growth factor (VEGF) and leads to increased vascular permeability.

It has been proved recently that the endothelial progenitor cells (EPCs) in sepsis mice showed increased circulation level of miR-126, reduced the vascular leakage and improved the survival rate of mice. In addition, it was observed that EPCs release abundant exosomes of miR-126[10]. It is known that miR-126 inhibited a variety of genes that affect the endothelial cell homeostasis. Specifically, miR-126-3p targeted the Sprouty-related EVH1 domain-containing protein 1, an angiogenesis inhibitor, and led

Corresponding Author: Yin Li, MM; e-mail: yinlilitj@163.com

to phosphorylated extracellular signal-regulated kinase (ERK) signal transduction and E-cadherin stabilization[11]. Moreover, miR-126-5p targeted the NOTCH1 inhibitor DLK-1 to enhance the endothelial cell proliferation[12]. Considering the potential beneficial effect of miR-126 on endothelial cell function, we speculated that the up-regulation of miR-126 might improve the therapeutic effect on sepsis.

Materials and Methods

Establishment of Mouse Model of Sepsis

Female C57BL/6J mice aged 6-8 weeks weighing 18-20 g were selected and randomly divided into 3 groups with 6 mice in each group. The mice were fasted for solids and had free access to water 12 h before the experiment. The mice in the control group were not injected with the lipopolysaccharide (LPS), while about 200 μL LPS was intraperitoneally injected into mice in the other two groups based on the weight (40 mg/kg). The mice in the three groups were stimulated for 4 h, 12 h, and 24 h, respectively. After that, the blood was taken from the orbit, placed in a refrigerator at 4°C for more than 6 h and centrifuged at 3000 rpm and 4°C for 10 min. Then the supernatant was transferred into a new Eppendorf (EP) tube and stored at -80°C. This study was approved by the Animal Ethics Committee of Tianjin First Center Hospital Animal Center.

Cell Culture and Transfection

Human pulmonary microvascular endothelial cells (HMVECs) were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in an incubator with 5% CO_2 at 37°C. The medium was replaced once every 48 h. The cells were passaged when cell fusion degree reached 70%, and cells in the 3rd-5th generation were used for follow-up experiments.

The miR-126 mimic/negative control (NC) mimic was synthesized by GenePharma (Shanghai, China). The cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's solution.

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

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After different treatment, the cells were collected to extract the total RNA according to the instructions. 1 μL RNA solution was taken on a microplate reader to detect the concentration and purity of total RNA. The RNA samples with absorbance $(A)_{260}$/A_{280} of 1.6-1.8 were considered to be of good quality. The remaining RNA solution was stored at -80°C for standby application.

Complementary deoxyribonucleic acid (cDNA) was synthesized using TaKaRa PrimeScript™ Kit (Otsu, Shiga, Japan) according to the instructions. First, a total of 2 μg RNA, 1 μL Oligo (dT) primer (50 μM) and 1 μL dNTP Mixture (10 mM) were mixed, and then the enzyme-free double distilled water was added till the total volume of 10 μL. The mixture was heated at 65°C for 5 min in the PCR instrument and quickly cooled on ice. The above reaction system was added with 4 μL 5×PrimeScript Buffer, 1 μL PrimeScript RTase, 0.5 μL RNase inhibitor and 4.5 μL enzyme-free water. The reaction system (a total of 20 μL) was mixed evenly and heated in the PCR instrument at 42°C for 45 min, and at 95°C for 5 min. Then the mixture was quickly cooled on ice to obtain the single-stranded cDNA. Finally, cDNA was stored at -20°C for PCR amplification. Primer sequences used in this study were as follows: miR-126, F: 5'-CGGCAGGAACCTCCTTACTC-3', R: 5'-TGTGCCCTAGGGACGAAGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Rac1 Activity Detection

The cells were inoculated into the plate and cultured in the incubator with 5% CO_2 at 37°C overnight according to the instructions of the kit. The medium was replaced with the serum-free complete medium for another 6 h. The cells were washed with pre-cooled phosphate-buffered saline (PBS) 3 times and added with the protein lysis buffer on ice. The supernatant was collected to determine the protein concentration using the DC protein assay kit. A small number of samples were taken as the total Rac1 protein sample, added with the loading buffer, boiled *via* boiling water bath for 5 min and stored in the refrigerator at -20°C. The remaining samples were diluted to 1 mL with lysis buffer based on the concentration. The activated Rac1-GTP was captured by 1 μL anti-Rac1 mouse monoclonal antibody and the antibody-binding activator protein was precipitated using 20 μL protein A/G agarose. The captured complex was then subjected to an ice bath at 4°C, rotation on a horizontal rotator at 400 rpm for 1

h and centrifugation at 5000 g for 1 min. The supernatant was carefully discarded, and the protein was washed with protein lysis buffer 3 times (centrifuged every time). Finally, the precipitate was added with the loading buffer, boiled *via* boiling water bath for 5 min and stored in the refrigerator at -20°C. Western blotting was performed and the anti-Rac1 rabbit polyclonal antibody was used as the primary antibody.

Western Blotting

The protein concentration was determined using the BCA protein concentration assay kit (Beyotime, Shanghai, China). A total of 40 μg total proteins were separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated with the primary antibody (1:2000, Abcam, Cambridge, MA, USA) at 4°C and incubated again with the corresponding horseradish peroxidase-labeled secondary antibody (1:1000, Beyotime, Shanghai, China) at room temperature for 1 h. 200 μL luminescence solution was added dropwise for image development using the chemiluminescence imaging analysis system. The gray value of the band on the image was calculated using the ImageJ software, followed by statistical analysis.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The *t*-test was adopted for the comparison between the two groups, and one-way analysis of variance followed by Post Hoc Test (Least Significant Difference) was adopted for the comparison among groups. The two-sided 95% confidence interval (CI) was used in all tests, and $p<0.05$ suggested that the difference was statistically significant.

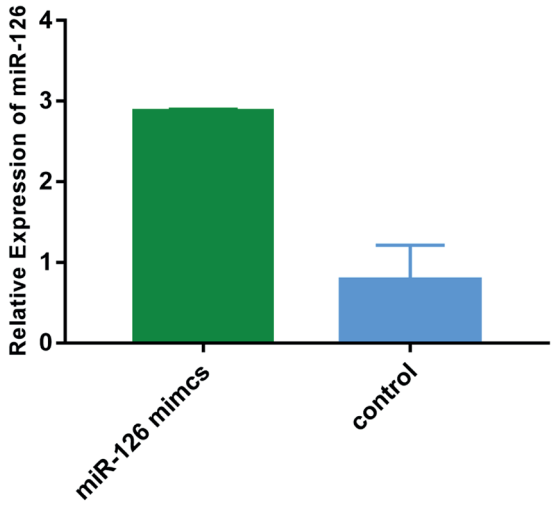

Figure 1. Cell transfection and validation. The miR-126 expression level after transfection is significantly higher than that in the control group ($p<0.01$).

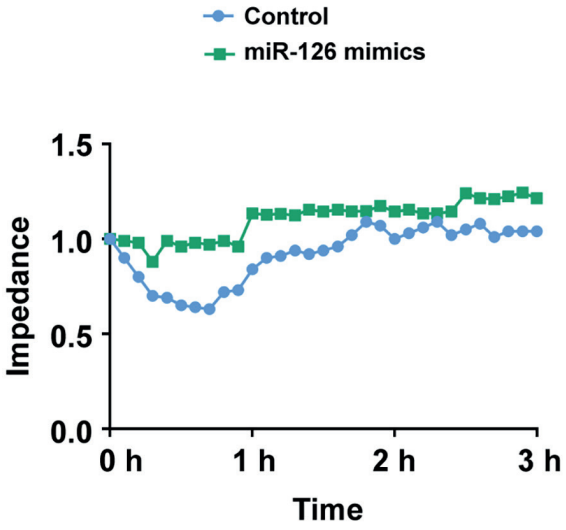

Figure 2. Transmembrane resistivity. The transmembrane resistivity of HMVECs in the control group declines rapidly after thrombin treatment, and then it begins to recover slowly. The decreasing rate of transmembrane resistivity in miR-126 overexpression group is significantly reduced after thrombin treatment ($p<0.01$).

Results

MiR-126 Overexpression Inhibited the Increased Cell Permeability Induced by Thrombin

The expression level of miR-126 after transfection was detected *via* qRT-PCR, and the results revealed that the expression level of miR-126 after transfection was significantly higher than that in the control group ($p<0.01$) (Figure 1).

The HMVECs in the logarithmic growth phase in different groups were incubated with thrombin. The transmembrane resistivity of HMVECs was detected as permeability by ECIS system. The higher transmembrane resistivity corresponds to the tighter cellular junction. The results showed that the transmembrane resistivity of HMVECs in the control group declined rapidly after thrombin treatment (the largest decline at 50 min), and then

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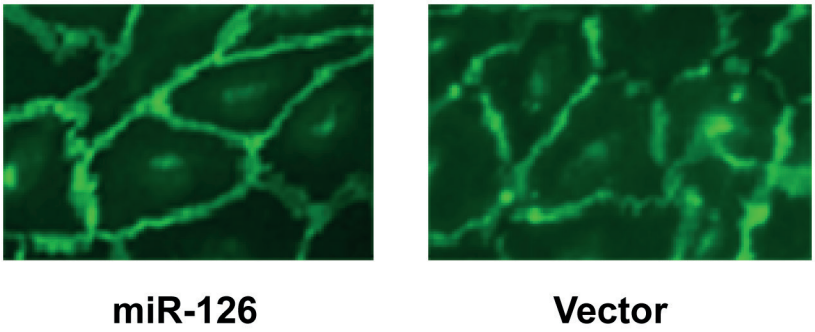

Figure 3. Immunofluorescence. After thrombin treatment, the cellular junction in miR-126 overexpression group is tighter than that in the control group (Magnification x 40).

it began to recover slowly. The transmembrane resistivity of cells treated with thrombin in miR-126 overexpression group also declined, but the decreasing rate was significantly reduced when comparing to the thrombin group (Figure 2). The above data indicated that thrombin significantly increased the permeability of HMVECs, while the overexpression of miR-126 showed an inhibitory effect on such increased permeability.

The influence of overexpression of miR-126 on changes in thrombin-induced intercellular tight junction was observed *via* immunofluorescence staining. It was found that after thrombin treatment, the cellular junction in miR-126 overexpression group was tighter than that in the control group, indicating that the overexpression of miR-126 reduced the thrombin-induced endothelial cell space (Figure 3).

MiR-126 Activated the AKT/Rac1 Pathway

The overexpression of miR-126 remarkably up-regulated the expression of phosphorylated AKT and activated Rac1 in cells (Figure 4, 5). After 28 h transfection with miR-126, the expression of phosphorylated AKT and activated Rac1 in different groups showed statistically significant differences. The overexpression of miR-126 significantly up-regulated the expression of phosphorylated AKT and activated Rac1 in cells compared with that in the control group.

In vivo Mouse Experiments

After miR-126 mimics and miR-126 Vector were injected into mice through the caudal vein, cecal ligation puncture (CLP) was then performed (Figure 6). The serum samples were collected 4 and 12 h after CLP. The levels of serum IL-6 (192±12.7) and TNF-α (2459.4±210.25) in miR-126 overexpression group were significantly lower than those in the control group [(206.9±34.4) and (4985.7±234.10)] (p<0.01) (Figure 7).

About 200 μL LPS was intraperitoneally injected into each mouse (40 mg/kg), and the number of deaths was observed within 24 h. The survival curve manifested that a large number of mice died in the control group within 24 h, and the death rate of mice was reduced in miR-126 overexpression group (p<0.05) (Figure 8).

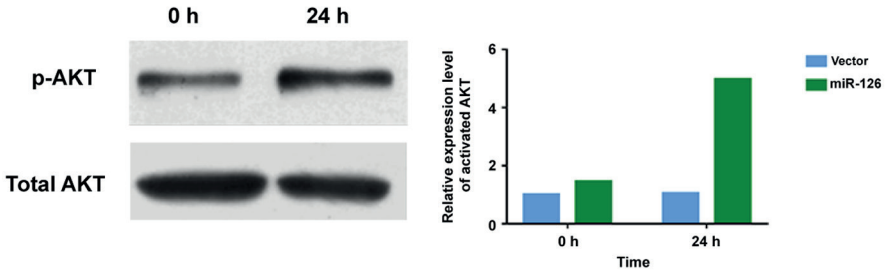

Figure 4. Expression level of AKT. The expression level of phosphorylated AKT in HMVECs increases to (5.9 ± 1.2) times compared to that in the control group at 24 h (p<0.05).

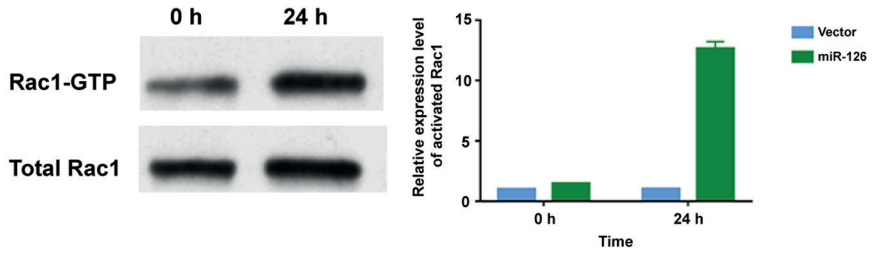

Figure 5. Expression level of Rac1. The expression level of activated Rac1 in HMVECs increases to (13.9 ± 2.2) times compared to that in the control group at 24 h ($p<0.05$).

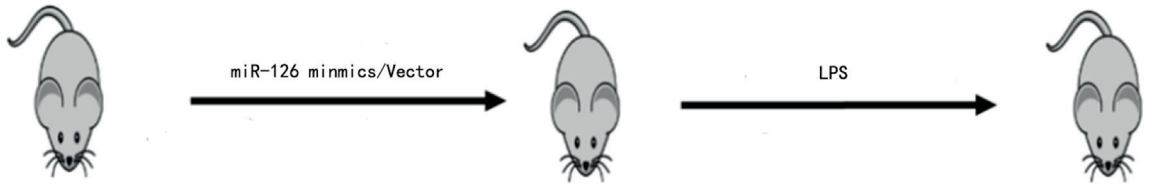

Figure 6. Injection procedure. After LPS is intraperitoneally injected into 7 mice in each group (40 mg/kg) for 12 h, the number of deaths is observed once every 2 h till 24 h, and the survival curve is plotted according to the death time.

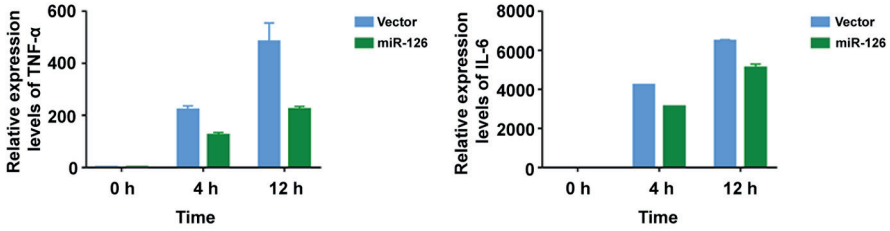

Figure 7. Expression levels of serum inflammatory factors. The serum samples are collected at 4 and 12 h after CLP, and the serum IL-6 and TNF-α levels in miR-126 overexpression group are significantly lower than those in the control group ($p<0.01$).

Discussion

MiR-126 is the most abundantly expressed miRNA during differentiation of endothelial cells, and the miR-126 knockout increased the vascular permeability[13-16]. On the contrary, miR-34a was reported to inhibit angiogenesis and promote inflammation-induced EPC senescence by inhibiting the silent information regulator 1 (SIRT1). In addition, both miR-155 and miR-125b are expressed in endothelial cells, and they have been proved to regulate the inflammation and TNF-α expression in sepsis model by targeting the regulatory factor for toll-like receptor signal transduction[17,18].

The results of this work showed that the overexpression of miR-126 remarkably improved the survival rate of mice receiving CLP compared with that of the control group. These findings, complementing previous studies, revealed that the enrichment of miR-126-5p significantly increased the survival rate of EPC after CLP. The circulating cytokines and oxidative stress led to endothelial dysfunction and increased vascular permeability in sepsis. It was observed that the treatment with miR-126 reduced the levels of cytokines, including IL-6 and IFN-α, in sepsis. Besides, the miR-126/VEGF axis was reported to regulate the angiogenesis and connexin stability in the body, and was also related to the increased vascular permeability in sepsis. MiR-126-3p promoted the activation of VEGF-mediated pathways, among which the phosphorylation of

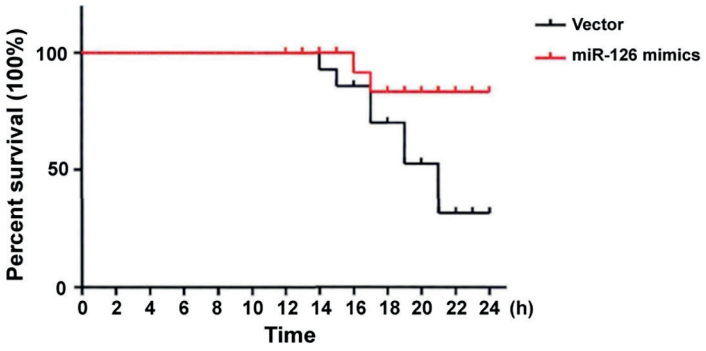

Figure 8. Survival curve of mice. At 12 h after LPS injection, a large number of mice die in control group within 24 h, and the death rate of mice is markedly reduced in miR-126 overexpression group ($p<0.05$).

ERK has been proved to be harmful to vascular integrity under pathophysiological conditions[19].

In this work, the role of miR-126 in the inflammatory process was determined. The effect of miR-126 in LPS-induced sepsis mice was confirmed *via* overexpression of miR-126. MiR-126 could inhibit the inflammatory response induced by intraperitoneal injection of LPS into the animal model. To better understand the underlying mechanisms, we identified AKT/Rac1 as a potential target for miR-126 by searching the target gene prediction database. The results of this study showed that miR-126 participated in the inflammatory process of sepsis by regulating the AKT/Rac1 pathway. Therefore, the overexpression of miR-126 might be a promising therapeutic strategy for sepsis. Sepsis often leads to severe tissue damage and organ dysfunction due to cytokine storm. LPS was used to treat mice to induce the acute inflammation *in vivo*. The inflammatory response in this model was similar to that in clinical sepsis, based on which the role of miR-126 in inflammation was studied in this paper. However, the immunosuppression was not contained in this model and our results need to be verified before the clinical test. The lungs, kidneys, liver and heart are the most commonly affected organs in sepsis. Due to severe inflammation, the lung tissues are manifested as the alveolar structural disorder, collapse and serious inflammatory cell infiltration. There are necrosis and edema of liver cells in sepsis in liver tissues. Macrophages are also involved in the sepsis and the properties of different subtypes of macrophages depend on the microenvironment. M1 macrophages produce severe cytokine storm, thus resulting in severe inflammatory response and tissue damage. M2 macrophages are thought to be correlated with immunosuppression. These two phenotypes are extreme states representing a series of subtypes, and the intrinsic changes in macrophages rely on a number of transcription factors and signaling pathways, such as the STAT1, NF-κB, STAT6, or the AKT pathway. MiRNAs have been proved to affect the macrophage polarization recently, which is consistent with the decline of mRNA levels of several inflammatory factors[20]. Previous studies have demonstrated that the AKT pathway was activated by miR-126 and also involved in macrophage polarization. In this work, the activation of the AKT pathway was increased and inflammatory cytokines were reduced, which further supported the viewpoint that AKT was involved in M2 macrophage activation. In addition, the protective effect of AKT in sepsis was also consistent in this work and recent studies.

Conclusions

We demonstrated that miR-126 may serve as a new therapeutic target for sepsis, providing a theoretical foundation and basis for subsequent studies.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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