Original Article
Up-regulation of microRNA-17 aggravates vascular endothelial damages in patients with sepsis possibly by down-regulating the expression of ATG16L gene

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Received February 5, 2016; Accepted August 9, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Aims: The present study is to determine the expression of microRNA-17 (miR-17) in the peripheral blood of patients with sepsis, and to investigate its biological functions and mechanisms of action. Methods: Peripheral blood (5 ml) was collected from 35 patients with sepsis and 20 healthy subjects for RNA extraction. Expression of miR-17 was measured using quantitative real-time polymerase chain reaction. Lipopolysaccharide was used to stimulate human umbilical vein endothelial cells (HUVEC). Transfection of HUVEC cells were performed by using Lipofectamine 2000. Cell-Counting Kit 8 assay, transwell assay, flow cytometry, and laser confocal microscopy were carried out to determine HUVEC cell proliferation, migration, apoptosis, and autophagy, respectively. Western blotting was employed to determine protein expression. Interference of ATG16L was achieved by using small interfering RNA. Dual luciferase reporter assay was used to test whether ATG16L is a target gene of miR-17. Results: Elevated expression of miR-17 in peripheral blood was closely related with the occurrence and development of sepsis. Lipopolysaccharide stimulation enhanced the expression of miR-17 in HUVEC cells. Expression of miR-17 inhibited the proliferation of HUVEC cells. Elevated expression of miR-17 reduced the migration ability of HUVEC cells. Increased miR-17 expression promoted the apoptosis of HUVEC cells possibly by regulating the expression of its target genes. Mir-17 expression inhibited the activation of autophagy-related signaling pathways in HUVEC cells, and reduced the autophagy activity of HUVEC cells. Mir-17 exerted its biological effects by regulating its predicted target gene ATG16L1. Mir-17 was able to bind with 3’-UTR of the mRNA of its target gene ATG16L1. Conclusions: The present study demonstrates that up-regulation of miR-17 aggravates vascular endothelial damages by inhibiting cell proliferation, migration and autophagy, and facilitating apoptosis. The mechanism of action is possibly the down-regulation of the expression of miR-17 target gene ATG16L.

Keywords: MicroRNA-17, ATG16L, sepsis, vascular endothelial damage, proliferation, migration, autophagy, apoptosis

Introduction
Sepsis is a clinically common systemic inflammatory response syndrome (SIRS) that is mainly induced by infection and usually accompanied by bacterial existence or highly suspicious infection foci, and is also a precursor of multiorgan dysfunction syndrome (MODS) [1-3]. It has been reported that over 750,000 people in USA are affected by sepsis every year, with a fatality rate of more than 50% [4]. During the occurrence of sepsis, bacteria release a large amount of endotoxin into the blood, and endotoxin binds with vascular endothelial cells, induces vascular endothelial injury, and stimulates inflammatory response and cytokine release, finally leading to proinflammatory - anti-inflammatory system imbalance and coagulation disorders [5-7]. This process suggests that vascular endothelial cells play an important role in the occurrence and development of sepsis.

MicroRNA (miRNA or miR) is a class of highly conserved non-encoding small RNA molecules (18-23 nt) that inhibit protein translation by binding with the 3’-untranslated regions (UTR) of target gene mRNA [8]. It has been reported that, as an important gene transcription level
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regulation factor, miRNA plays an important role in the occurrence and development of sepsis [9]. Song et al. find that miR-29a plays a role in sepsis by targeting STAT3 signaling pathway and facilitating mononuclear cell apoptosis [10]. Liu et al. demonstrate that up-regulation of miR-155 expression in the peripheral blood of patients with sepsis induces the differentiation of CD39+ Treg cells and immunosuppression, and facilitates the development of sepsis [11]. Wang et al. show that up-regulation of miR-15a/16 expression in the peripheral blood of sepsis patients inhibits inflammatory responses induced by LPS [12]. In addition, miRNA can regulate the function of vascular endothelial cells in sepsis [13], but its molecular mechanism of action is still unclear. miR-17 is an miRNA molecule that is recently identified to be important in the occurrence and development of tumors. It regulates the proliferation, migration, and apoptosis of multiple tumor cells, but its expression and regulatory mechanism in sepsis patients are still unclear [14]. A study shows that vascular endothelial growth factor regulates angiogenesis by stimulating the up-regulation of miR-17 [15]. Zhai et al. find that miR-106, a member of miR-17 family, regulates lung cancer cell autophagy activity, and affects the survival of these cells [16]. In the present study, we measure the expression of miR-17 in sepsis, and investigate the mechanism of action of miR-17 in the occurrence and development of sepsis.

Materials and methods

Patients

A total of 35 patients with sepsis and 20 healthy subjects (control group) admitted at our hospital between December 2013 and October 2014 were included in the present study. Peripheral blood (5 ml) was obtained from each subject. Clinical information and pathological data were collected from all patients. All procedures were approved by the Ethics Committee of Qingdao University. Written informed consents were obtained from all patients or their families.

Cells

Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI-1640 complete medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. The medium was refreshed every two days. When reaching a confluency of 80-90%, the cells were passaged. Cells with passage numbers of 3, 4, and 5 were used for experiments. HUVEC cells were seeded onto 24 well plates, and incubated with 2 μg/ml lipopolysaccharide (LPS) 24 h to simulate injuries of endothelial cells induced by endotoxin in peripheral blood of patients with sepsis.

After seeding HUVEC into 24 well plates, the cells were divided into negative control group (miR-NC) and miR-17 mimics group. Before transfection, 1.25 μl miR-17 mimics (20 μM) and 2 μl Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were added into two individual vials containing 50 μl OptiMem medium, respectively. Five minutes later, the liquids in the two vials were mixed before standing still for another 15 min. Then, the mixture was added onto cells with a confluency of 70-90% in miR-17 mimics group for an incubation of 6 h. After changing fresh medium, the cells were cultured under normal condition for 24 h before incubation with 2 μg/ml LPS for another 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extract from 250 μl serum using TRizol reagent following the manufacturer's manual (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and quality of RNA was measured using ultraviolet spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA, USA). Then, 0.5 μg total RNA was subjected to reverse transcription by the addition of PolyA tail. Afterwards, cDNA was stored at -20°C. Expression of mRNA was quantified using StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Expression of glyceraldehyde-3-phosphatedehydrogenase was used as reference. Quantitative measurements were performed using the 2^-ΔΔCt method. All samples were measured in triplicate, and mean values were considered for comparative analysis.

Western blotting

At 48 h after transfection, total protein was extracted using radioimmunoprecipitation assay lysis buffer containing 1% phenylmethylsulfonyl fluoride. The resulted protein (20 μg) was loaded onto sodium dodecyl sulfate polyacrylamide gel (100 g/L) for electrophoresis and then transferred onto polyvinylidene fluoride
membrane. After blocking with skimmed milk (50 g/L) for 2 h, rabbit anti-human primary antibodies (1:1,000; Abcam, Cambridge, UK) were added for incubation at 4°C overnight. On the next day, goat anti-rabbit secondary antibody (1:1,000; Abcam, Cambridge, UK) was added for incubation at room temperature for 1.5 h. The membrane was washed with Tris-buffered saline with Tween 20. Immunoreactive bands were detected using electrochemiluminescence reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). The relative band densities were analyzed using Image-Pro Plus v7.0 (Media Cybernetics, Bethesda, MD, USA).

Cell-Counting Kit 8 (CCK-8) assay

For growth curve assay, HUVEC cells were seeded into 96-well plates at a density of 2,000 transwell assay was performed by seeding 2 × 10^5 HUVEC cells into the upper chamber (8 μm, and 24 wells; Corning Inc., Corning, NY, USA) containing 200 μl serum-free RPMI-1640 medium. In addition, 600 μl RPMI-1640 medium supplemented with 10% fetal bovine serum was added into the lower chamber. After 24 h, the chamber was removed and the cells in the upper chamber were wiped off. After being fixed with 4% formaldehyde for 10 min, the membrane was stained using Giemsa method for microscopic observation of 5 random fields (200×). The number of transwell cells was calculated for the evaluation of cell migration ability. All procedures were carried out on ice with pipetting tips being cooled at 4°C.

Flow cytometry

At 24 h after transfection with miR-17 mimics, 1×10^6 cells were washed with pre-cooled phosphate-buffered saline twice before being subjected to the detection of apoptosis using ANXV FITC APOPTOSIS DTEC KIT I following the manufacturer’s manual (BD Biosciences, Franklin Lakes, NJ, USA). Cells with positive Annexin-V staining only were early apoptotic cells, those only positively stained with propidium iodide were necrotic cells, while those with double staining by Annexin-V and propidium iodide were late apoptotic cells. Apoptotic index was obtained from the percentage of cells double-stained by Annexin-V/propidium iodide. Each test was performed in triplicate.

Transwell assay

For growth curve assay, HUVEC cells were seeded into 96-well plates at a density of 2,000
After transfection with miR-NC or miR-17 mimics, HUVEC cells were cultured for another 24 h. Then, the cells were infected by mRFP-GFP-LC3 adenovirus at a ratio of 20 viruses per cell. After normal culture for 48 h, the medium was discarded and the cells were washed with pre-cooled phosphate-buffered saline twice. After fixation with 4% formaldehyde for 15 min, the cells were washed again with phosphate-buffered saline twice. Then, autophagy of HUVEC cells was observed under a laser confocal microscope (SP8; Leica, Wetzlar, Germany). Under the excitation by laser, autophagosomes showed green and red fluorescence. After fusion of autophagosomes with lysosomes, green fluorescent protein was quenched in acidic environment, and only red fluorescence remained. The autophagy of cells was evaluated by counting the numbers of autophagosomes and lysosomes.

**Bioinformatics**

The potential target genes of miR-17 were predicted using bioinformatics. miR-17 was identified to be capable of binding with 3'-UTR of ATG16L mRNA. ATG16L gene expression was interfered by small interfering RNA.

**Dual luciferase reporter assay**

According to bioinformatics results, wild-type (WT) and mutant seed regions of miR-17 in the 3'-UTR of ATG16L1 gene were synthesized in vitro, added with Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.5 μg) with WT or mutant 3'-UTR DNA sequences were co-transfected with miR-17 mimics into HEK293T cells. After cultivation for 24 h, the cells were lysed using dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer’s manual, and fluorescence intensity was measured using GloMax 20/20 luminometer (Promega, Fitchburg, WI, USA). Using renilla fluorescence activity as internal reference, the fluorescence values of each group of cells were measured.

**Statistical analysis**

The results were analyzed using SPSS 17.0 statistical software (IBM, Armonk, NY, USA). All data were expressed as means ± standard deviation. Group comparison was performed using group t-test. Differences with P < 0.05 were considered statistically significant.
Results

Elevated expression of miR-17 in peripheral blood is closely related with the occurrence and development of sepsis

To measure the level of miR-17 in peripheral blood, qRT-PCR was carried out. The data showed that miR-17 expression in patients with sepsis was significantly higher than in normal subjects ($P < 0.05$) (Figure 1A). The result suggests that elevated expression of miR-17 in peripheral blood is closely related with the occurrence and development of sepsis.

LPS stimulation enhances the expression of miR-17 in HUVEC cells

To test the effect of LPS stimulation on the expression of miR-17 in HUVEC cells, the cells were incubated with 2 μg/ml LPS for 24 h. The qRT-PCR data showed that the level of miR-17 in HUVEC cells treated with LPS was more than four folds of that in control ($P < 0.05$) (Figure 1B). The result indicates that LPS stimulation enhances the expression of miR-17 in HUVEC cells.

Expression of miR-17 inhibits the proliferation of HUVEC cells

To study HUVEC proliferation, CCK-8 assay was performed. The data showed that the proliferation of HUVEC in miR-17 mimics group was significantly decreased compared with those in control and miR-NC groups at 48 h or 72 h ($P < 0.05$) (Figure 2). The result suggests that expression of miR-17 inhibits the proliferation of HUVEC cells.

Elevated expression of miR-17 reduces the migration ability of HUVEC cells

To determine the migration ability of HUVEC, transwell assay was used. The data showed that the number of transwell HUVEC cells in miR-17 mimics group was significantly lower than that in miR-NC group ($P < 0.05$) (Figure 3). The results indicate that elevated expression of miR-17 reduces the migration ability of HUVEC cells.

Increased miR-17 expression promotes the apoptosis of HUVEC cells possibly by regulating the expression of its target genes

To detect HUVEC apoptosis, flow cytometry was employed. The data showed that the apoptotic rate of HUVEC cells in miR-17 mimics group was significantly lower than that in miR-NC group ($P < 0.05$) (Figure 4). The result suggests that increased miR-17 expression promotes the apoptosis of HUVEC cells possibly by regulating the expression of its target genes.
miR-17 expression inhibits the activation of autophagy-related signaling pathways in HUVEC cells, and reduces the autophagy activity of HUVEC cells

To observe autophagy of HUVEC cells, laser confocal microscopy and Western blotting were adopted. Laser confocal microscopy showed that the numbers of autophagosomes and lysosomes in miR-17 mimics group were lower than those in miR-NC group, respectively (P < 0.05) (Figure 5A). Western blotting analysis showed that the ratio of type I LC3 protein to type II LC3 protein in miR-17 mimics group was significantly greater than that in miR-NC group (P < 0.05) (Figure 5B). These results indicate that miR-17 expression inhibits the activation of autophagy-related signaling pathways in HUVEC cells, and reduces the autophagy activity of HUVEC cells.

miR-17 exerts its biological effects by regulating its predicted target gene ATG16L1

To test whether miR-17 exerts its effect by regulating its predicted target gene ATG16L1, we performed Western blotting, CCK-8 assay, transwell assay, flow cytometry, and laser confocal microscopy. Western blotting showed that transfection with miR-17 mimics reduced ATG-16L1 protein expression compared with miR-NC (Figure 6A). In addition, interference of ATG16L gene expression by small interfering RNA led to lower ATG16L1 protein expression (Figure 6B), and as a result, HUVEC cell proliferation was significantly inhibited (P < 0.05) (Figure 6C). Transwell assay showed that the number of cells crossing the membrane in siR-ATG16L group was significantly reduced compared with NC group (P < 0.05) (Figure 6D). Flow cytometry showed that down-regulation of ATG16L promoted the apoptosis of HUVEC cells (Figure 6E). Western blotting showed that the ratio of type I LC3 protein over type II LC3 protein in siR-ATG16L group was higher than that in miR-NC group (Figure 6A), and the numbers of autophagosomes and lysosomes in siR-ATG16L were significantly lower than those in NC group, respectively (P < 0.05) (Figure 6F). These results suggest that the miR-17 exerts its biological effects by regulating its predicted target gene ATG16L1.

miR-17 is able to bind with 3’-UTR of the mRNA of its target gene ATG16L1

To confirm interactions between miR-17 and 3’-UTR of ATG16L1 gene, dual luciferase report-
er assay was carried out. The data showed that fluorescence values of cells co-transfected with miR-17 mimics and pMIR-REPORT-WT plasmids were significantly lower than control (P < 0.05), while those of cells co-transfected with miR-17 mimics and pMIR-REPORT-mutant plasmids were not different from control (P > 0.05) (Figure 7). The result indicates that miR-17 is able to bind with 3'-UTR of the mRNA of its target gene ATG16L1.

Discussion

It has been reported that the occurrence and development of sepsis is closely associated with the immunity, inflammation, and coagulation of human bodies, and many types of cells are participating in these complicated patho-
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Vascular endothelial cells are important barrier cells that play important roles in maintaining hemodynamic and vascular functions and regulating inflammation, being a key factor in the occurrence and development of sepsis [20]. LPS in the peripheral blood of patients with sepsis leads to vascular endothelial cell barrier dysfunctions, increases vascular endothelial cell gaps, and enhances permeability [21]. In addition, damaged endothelial cells facilitate the release of inflammatory factors and the expression of inflammation-related genes [23]. These findings demonstrate that vascular endothelial cells have important biological functions in sepsis.

The present study shows that the expression of miR-17 is elevated in the peripheral blood of patients with sepsis, suggesting that miR-17 is associated with the occurrence and development of sepsis. In vitro studies demonstrate that miR-17 inhibits HUVEC cell proliferation, migration and autophagy, and promotes its apoptosis by down-regulating ATG16L gene expression. A previous study shows that overexpression of miR-17 promotes the invasiveness of liver cancer cells [24]. Using bioinformatics, we discover that miR-17 inhibits autophagy by down-regulating ATG16L expression. Furthermore, the direct binding between miR-17 and ATG16L mRNA was demonstrated by dual luciferase reporter assay. These results indicate that miR-17 plays important roles in aggravating vascular endothelial damages in the occurrence and development of sepsis. To summarize, elevated miR-17 expression in the peripheral blood of sepsis patients inhibits proliferation, migration and autophagy, and promotes the apoptosis of vascular endothelial cells, by directly targeting transcriptional factor ATG16L.

Acknowledgements

This work was supported by the Qingdao University and Laiwu City People’s Hospital.

Disclosure of conflict of interest

None.

References


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