Abstract. – OBJECTIVE: The present study is aimed to investigate the regulatory effect of microRNA (miRNA or miR)-503 on endothelial functions, as well as the mechanism by which high glucose leads to injury of endothelial cells.

MATERIALS AND METHODS: When reaching 80% confluency, human umbilical vein endothelial cells (HUVECs) were subjected to non-serum synchronization for 12 h, and medium of cells in high-glucose (HG) group was replaced by normal medium supplemented with 25 mmol/L D-glucose. HUVECs cultured in normal glucose (NG) medium were used as control. To overexpress miR-503, HUVECs were transfected with miR-503 mimics. To silence insulin-like growth factor-1 receptor (IGF-1R) mRNA, HUVECs were transfected with small interfering RNA (siRNA). To predict whether miR-503 targets IGF-1R, bioinformatics was performed. Quantitative Real-time polymerase chain reaction was used to determine miR-503 and IGF-1R mRNA expression, and Western blotting was employed to measure IGF-1R protein expression. Cell-Counting Kit 8 assay was used to determine HUVECs proliferation, while wound-healing assay was used to evaluate HUVECs migration. HUVECs apoptosis was investigated by measuring caspase 3 activity.

RESULTS: Expression of IGF-1R in HUVECs in high glucose was decreased compared to that in normal glucose. miR-503 was predicted to target IGF-1R mRNA, and miR-503 expression in HUVECs in high glucose was higher than that in normal glucose. Overexpression of miR-503 inhibited the transcription and the translation of IGF-1R gene reducing migration, suppressed proliferation and promoted apoptosis. Transfection with IGF-1R siRNA decreased IGF-1R protein expression in HUVECs. Down-regulated IGF-1R expression reduced migration and proliferation, but promoted apoptosis of HUVECs.

CONCLUSIONS: The present study demonstrates that miR-503 expression in HUVECs is elevated in high glucose condition. Also, miR-503 reduces migration and proliferation, but promotes apoptosis of HUVECs by inhibiting IGF-1R expression.

Key Words: microRNA-503, High glucose, Human umbilical vein endothelial cells, Insulin-like growth factor-1 receptor.

Introduction

Diabetic chronic vascular complications, such as atherosclerosis, coronary heart disease, cerebral thrombosis, and diabetic foot, are the main causes of death or disability in patients with diabetes. Endothelial dysfunction is characterized by: a barrier function impairment, a diminished vascular contractile function, an enhanced endothelial cell apoptosis, a mononuclear cell adhesion and an abnormal angiogenesis; moreover it is the initiating factor and the early manifestation of diabetic angiopathies. It has been discovered that hyperglycemia, advanced glycation end products (AGEs), oxidative stress, insulin resistance, and platelet activation participate in the vascular endothelial dysfunction. However, the exact mechanisms are still unclear. High blood sugar is the most important feature in patients with diabetes. Endothelial cells stimulated by high glucose in vitro produce excessive extracellular matrix components, such as collagen and fibrinogen, as well as coagulation factors such as...
von Willebrand factor (vWF) and tissue factors. Furthermore, proliferation, migration and fibrinolytic capacity are decreased, leading to enhanced apoptosis\textsuperscript{7-9}. High glucose causes sugar toxicity, which may result in the production of AGEs, sustained activation of protein kinase C (PKC) and increased reactive oxygen species (ROS). As a result, vascular oxidative stress, inflammatory responses, cell apoptosis and atherosclerosis occur, impairing endothelial functions\textsuperscript{10,11}. It is reported that insulin-like growth factor-1 receptor (IGF-1R) is a kind of vascular protective factor that is expressed in vascular endothelial cells\textsuperscript{12,13}. IGF-1R is a member of the tyrosine protein kinase receptor superfamily, and it participates in the proliferation, differentiation and viability of cells\textsuperscript{14-16}. IGF-1R may play an important role in the maintenance of the function of the endothelial barrier, and it protects vascular endothelial cells from oxidative stress and apoptosis\textsuperscript{7}. In the meantime, increased expression of IGF-1R may improve endothelial function and promote the regeneration of endothelial cells\textsuperscript{17,18}, whereas IGF-1R gene knockout increases diabetes-induced myocardial fibrosis\textsuperscript{19,20}. MicroRNA (miRNA or miR) is a kind of non-coding single-stranded small RNA molecule with 18-25 nucleotides. miRNA regulates the translation of mRNA by binding with the 3'-untranslated region (UTR) of its target gene\textsuperscript{21,22}. It is reported that miR-503 participates in the regulation of endothelial cell functions. For example, overexpression of miR-503 arrests the cell cycle of human umbilical vein endothelial cells (HUVECs) at G1/S transition phase, leading to inhibited cell proliferation\textsuperscript{23}. However, whether miR-503 affects or regulates other endothelial cell functions and its underlying mechanisms of action are still unclear. In the present study, we investigate the regulatory effect of miR-503 on endothelial functions, as well as the mechanism by which high glucose leads to injury of endothelial cells.

Materials and Methods

Cells
HUVECs (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in ECM medium (ScienCell Research Laboratories, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo-Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO\textsubscript{2}. When reaching 80% confluency, the cells in all groups were subjected to non-serum synchronization for 12 h, and the medium of cells in high-glucose (HG) group was replaced by normal medium supplemented with 25 mmol/L D-glucose, followed by incubation for 24 h. HUVECs cultured in medium with normal glucose (NG) were used as control group. For the silencing of IGF-1R mRNA (GenBank ID: NM_000875), HUVECs were transfected with small interfering RNA (siRNA) of IGF-1R (siRNA sequences: sense, 5'-AACGACTATCAGCAGCTGAAG-3'; anti-sense, 5'-AACAGCTGGAAACATGGTGGAT-3') or negative control (NC) siRNA (NC siRNA sequences: sense, 5'-UUCUCCGAACGUGUACGU-3'; anti-sense, 5'-ACGUGACACGUUCGAGA-3') (GenePharma, Shanghai, China). HUVECs were seeded in 6-well plates and cultured at 37°C and 5% CO\textsubscript{2}. When the cells reached 70% confluency, the medium was discarded and washed with phosphate-buffered saline (PBS) for three times, before addition of 2 ml Opti-MEM medium to each well. Then, siRNA and 5 μl Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were dissolved in 250 μl Opti-MEM in two individual Eppendorf tubes, respectively. After standing still at room temperature for 5 min, the two tubes were combined before incubation at room temperature for another 20 min. Subsequently, the mixture was added to each well. After incubation at 37°C and 5% CO\textsubscript{2} for 6 h, the medium was changed to normal medium. HUVECs with successful transfection were used in the following tests.

Bioinformatics
Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs\textsuperscript{24}. To understand whether IGF-1R was a target gene of miR-503, we used TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/home.do) for analysis.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)
HUVECs (2×10\textsuperscript{5}) were mixed with 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. Then, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription using Reverse Transcription System (TaKaRa, Dalian, China) from 1 μg RNA and stored at -20°C. Reverse transcription of miRNA was carried out using SYBR PrimeScript miRNA RT-PCR Kit.
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(TaKaRa, Dalian, China). The expression of miR-503 was determined by SYBR Green qRT-PCR kit (TaKaRa, Dalian, China), using U6 as internal reference. The reaction system (25 μl) contained 12.5 μl SYBR Premix Ex Taq, 1 μl PCR forward primer (miR-503, 5'-GTGCTGTAGACGCGG-GAACGTCTCTG-3'), 1 μl PCR reverse primer (provided by the kit), 2 μl template and 8.5 μl ddH2O. The reaction protocol was: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 20 s (iQ5; Bio-Rad, Hercules, CA, USA). The 2−ΔΔCt method was used to calculate the relative expression of miR-503 against U6. Each sample was tested in triplicate.

SYBR Green qRT-PCR kit (TaKaRa, Dalian, China) was used to detect mRNA expression of IGF-1R, using GADPH as internal reference. The reaction system (20 μl) was composed of 10 μl SYBR EX Taq-Mix, 0.5 μl upstream primer (IGF-1R, 5'-GCCCGAAGGTCTGTGAGGAAGAA-3'; GADPH, 5'-CCACCCATGGCAAATTCCATGGA-3'), 0.5 μl downstream primer (IGF-1R, 5'-GG-TACCGGTGCCAGGTTATGA-3'; GADPH, 5'-TCTAGACGGCAGGTCCAGTCCCACC-3'), 1 μl cDNA and 8 μl ddH2O. PCR condition was: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 40 s and elongation at 72°C for 30 s; final elongation at 72°C for 1 min (iQ5; Bio-Rad, Hercules, CA, USA). The 2−ΔΔCt method was used to calculate the relative expression of IGF-1R mRNA against GADPH. Each sample was tested in triplicate.

Western Blot

Cells were seeded into 6-well plates at a density of 5×10^4/well. At 48 h after transfection, the cells were trypsinized, collected and mixed with 600 μl precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, and 1% sodium deoxycholate) for lysis of 10 min on ice. Then, the mixture was centrifuged at 12,000 g/min and 4°C for 15 min. The supernatant was used to determine PCR condition was: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 40 s and elongation at 72°C for 30 s; final elongation at 72°C for 1 min (iQ5; Bio-Rad, Hercules, CA, USA). The 2−ΔΔCt method was used to calculate the relative expression of IGF-1R mRNA against GADPH. Each sample was tested in triplicate.

Cell-Counting Kit 8 (CCK-8) Assay

Cells were seeded at 2,000/well in 96-well plates for transfection. At 48 h after transfection, the cells were subjected to CCK-8 assay for the detection of proliferation. At 24, 48 and 72 h, the medium was discarded, and the cells were washed with phosphate-buffered saline PBS twice, followed by addition of 10% CCK-8 reaction reagent diluted in medium. After incubation at 37°C for 30 min, the absorbance of each well was measured at 450 nm for plotting cell proliferation curves. Each group was tested in 3 replicate wells and the values were averaged.

Wound Healing Assay

After transfection with miR-503 mimics or siRNA, the cells were cultured in normal medium supplemented with 10% fetal bovine serum FBS for 48 h. In each well of the culture plate, single-layer cells were scratched along a straight line crossing the center of the well by a pipetting tip. Afterward, the medium was replaced with normal medium without fetal bovine serum (FBS) to prevent cell proliferation. At 0 h and 24 h after scratching, the cells were imaged under a microscope and wound healing rate of each group was calculated. Each sample was tested in 3 replicate wells and repeated for 3 times.

Caspase 3 Activity Assay

To test the apoptosis of cells, caspase 3 activity assay was carried out following the manufactur-
er’s manual (Beyotime Biotechnology, Shanghai, China). After transfection with miRNA mimics or siRNA, HUVECs were cultured at 37°C for 48 or 96 h. After collecting the cells by trypsinization, 2 × 10^6 cells were lysed in 100 μl lysis buffer. Then, the deposited cells were resuspended, followed by lysis on ice for 15 min. After centrifugation at 4°C and 16,000 × g for 15 min, the supernatant was transferred to precooled tubes. Then, the supernatants were mixed with detection buffer and Ac-DEVD-pNA. After the mixture was incubated overnight at 37°C, absorbance at 405 nm was measured.

**Statistical Analysis**

Statistical analysis was performed using SPSS16.0 (IBM, Armonk, NY, USA). Measurement data were expressed as means ± standard deviations. Two groups of data were compared using t-test. Tukey’s test was used as post-hoc test. Differences with \( p < 0.05 \) were considered statistically significant.

**Results**

**Expression of IGF-1R mRNA and Protein in HUVECs Under High Glucose Condition is Decreased Compared with That Under Normal Glucose Condition**

To measure the expression of IGF-1R mRNA and protein in HUVECs, qRT-PCR and Western blotting were employed, respectively. The qRT-PCR data showed that the level of IGF-1R mRNA in HUVECs was significantly lower than that of NG group (\( p < 0.05 \)) (Figure 1A). Consistently, Western blotting showed that the protein expression of IGF-1R in HUVECs was significantly reduced compared with that in NG group (\( p < 0.05 \)) (Figure 1B). The results suggest that the expression of IGF-1R mRNA and protein in HUVECs under high glucose condition is decreased compared to that under normal glucose condition.

**miR-503 is Predicted to Directly Target IGF-1R mRNA and the Expression of miR-503 in HUVECs Under High Glucose Condition is Higher Than That Under Normal Glucose Condition**

To predict whether miR-503 can target IGF-1R, TargetScan and Miranda were used. The data showed that miR-503 was able to bind with the 3'-untranslated region (UTR) of IGF-1R mRNA (Figure 2A). To determine the expression of miR-503 in HUVECs, qRT-PCR was carried out. The data showed that the level of miR-503 in HUVECs under high glucose condition was significantly higher than that under normal glucose condition (\( p < 0.05 \)) (Figure 2B). The result indicates that miR-503 is predicted to directly target IGF-1R mRNA and the expression of miR-503 in HUVECs under high glucose condition is higher than that under normal glucose condition.

![Figure 1](image_url). Expression of (A) mRNA and (B) protein of IGF-1R in HUVECs. Quantitative Real-time polymerase chain reaction was used to determine the expression of mRNA, and Western blotting was employed to measure protein expression. *, \( p < 0.05 \) compared with NG group.
Overexpression of miR-503 Inhibits the Transcription and Translation of IGF-1R Gene

To test whether miR-503 regulates the expression of IGF-1R, HUVECs were transfected with miR-503 mimics. The data showed that HUVECs transfected with miR-503 mimics had a significantly higher miR-503 level than NC group ($p < 0.05$) (Figure 3A). In addition, the expression of IGF-1R mRNA and protein in HUVECs transfected with miR-503 mimics was significantly lower than that in NC group ($p < 0.05$) (Figure 3B-D). The results suggest that the overexpression of miR-503 inhibits the transcription and the translation of IGF-1R gene.

Overexpression of miR-503 Reduces the Migration Ability of HUVECs

To examine the effect of miR-503 on the migration ability of HUVECs, wound-healing assay was performed. The data showed that the wound-healing rate of HUVECs transfected with miR-503 mimics was significantly reduced than that in NC group at 24 h ($p < 0.05$) (Figure 4A).
and B). The result suggests that overexpression of miR-503 reduces the migration ability of HUVECs.

**Overexpression of miR-503 Inhibits the Proliferation of HUVECs**

To determine how miR-503 affects the proliferation of HUVECs, CCK-8 assay was carried out. The data showed that the absorbance of HUVECs transfected with miR-503 mimics was significantly lower than that of NC group at 48 h or 72 h ($p < 0.05$) (Figure 5). The result indicates that overexpression of miR-503 inhibits the proliferation of HUVECs.

**Overexpression of miR-503 Promotes the Apoptosis of HUVECs**

To test whether miR-503 affects the apoptosis of HUVECs, caspase 3 activity assay was carried out. The data showed that caspase 3 activity of HUVECs transfected with miR-503 mimics was significantly higher than that of NC group at 96 h ($p < 0.05$) (Figure 6). The result indicates that overexpression of miR-503 promotes the apoptosis of HUVECs.

**Transfection with IGF-1R siRNA Decreases the Expression of IGF-1R Protein in HUVECs**

To measure the expression of IGF-1R in HUVECs transfected with the siRNA of IGF-
miR-503 and IGF-1R in HUVECs under high glucose

1R, Western blotting was carried out. The data showed that IGF-1R protein expression in HUVECs transfected with IGF-1R siRNA was significantly lower than that in NC group ($p < 0.05$) (Figure 7A and B). The results indicate that transfection with IGF-1R siRNA decreases the expression of IGF-1R protein in HUVECs.

**Down-Regulated IGF-1R Expression Reduces the Migration and Proliferation, But Promotes the Apoptosis of HUVECs**

To determine the effect of silencing of IGF-1R on the biological functions of HUVECs, wound healing assay, CCK-8 assay and caspase 3 activity assay were carried out. The data showed that reduced IGF-1R expression decreased the wound healing rate of HUVECs compared to the NC group (Figure 8A-B), and significantly reduced the absorbance of HUVECs in CCK-8 assay (Figure 8C). In addition, caspase 3 activity in HUVECs with reduced IGF-1R expression was not significantly different from that in NC group at 48 h, but was significantly enhanced than that in NC group at 96 h (Figure 8D). The results suggest

![Figure 7. Effect of siRNA of IGF-1R on the protein expression of IGF-1R in HUVECs.](image)

- **A** Western blots of IGF-1R protein in cells transfected with negative control (NC) or siRNA of IGF-1R.
- **B** Relative expression of IGF-1R protein in HUVECs transfected with NC or siRNA of IGF-1R. Western blotting was employed to measure IGF-1R protein expression. *, $p < 0.05$ compared with NC group.

**Figure 8. Effect of siRNA of IGF-1R on the biological functions of HUVECs.**
- **A** Images of cells in wound healing assay at 0 h and 24 h after scratching.
- **B** Wound healing rates of HUVECs in NC and siRNA groups at 0 h and 24 h after scratching. *, $p < 0.05$ compared with NC group.
- **C** Proliferation of HUVECs at 24 h, 48 h and 72 h after transfection with negative control (NC) or siRNA of IGF-1R. CCK-8 assay was used to determine the proliferation of the cells. Absorbance of each well was measured at 450 nm with a microplate reader and cell proliferation curves were plotted. *, $p < 0.05$ compared with NC group.
- **D** Relative activity of caspase 3, a marker of apoptosis, was measured at 48 h and 96 h after transfection. *, $p < 0.05$ compared with NC group.
that down-regulated IGF-1R expression reduces the migration and proliferation, but promotes the apoptosis of HUVECs.

**Discussion**

Endothelial dysfunction is an important pathological change at the early stage of diabetic vascular complications. miRNA molecules widely participate in a variety of physiological and pathological processes. A large number of studies have shown that a variety of miRNAs is involved in the regulation of endothelial function and angiogenesis. However, there are few studies on miRNAs that participate in the pathological process of diabetic endothelial cell injury in diabetes. For example, miR-221 regulates endothelial damages induced by high glucose via c-kit protein. In addition, Loyer et al. discovered that inhibition of miR-92a expression in mice alleviates endothelial dysfunction and reduces atherosclerosis. It is also reported that the expression of miR-503 is reduced in tumor cells, being related to the invasion and metastasis of tumors. Because the invasion and metastasis of tumors are related to angiogenesis, miR-503 affects the regulatory process in angiogenesis. In the present study, we found that high glucose elevates the expression of miR-503 in HUVECs. Furthermore, up-regulated miR-503 expression inhibits the migration and proliferation of HUVECs, but promotes the apoptosis of HUVECs, suggesting that miR-503 plays a role in the mechanism of action of high glucose on endothelial function.

To identify target proteins of miR-503 in the regulation of endothelial functions, miRNA target gene/protein prediction algorithm was used. IGF-1R has been identified to be related with endothelial functions. In addition, the present study discovers that mRNA and protein expression of IGF-1R in cells under high glucose condition is significantly reduced. In addition, Zhang et al. report that miR-503 inhibits the protein expression of IGF-1R by directly interacting with IGF-1R mRNA, suggesting that miR-503 targets IGF-1R. Another work shows that miR-503 expression is significantly increased in ischemic muscle in lower limbs of STZ-induced type 2 diabetic mice, and CCNE1 and cdc25A are target genes of miR-503 in diabetic endothelial dysfunctions. This suggests that the target genes of miR-503 in diabetes are not unique. Our further researches show that exogenous up-regulation of miR-503 expression inhibits the expression of IGF-1R mRNA and protein in endothelial cells. This demonstrates that miR-503 interacts with IGF-1R. Then, silenced expression of IGF-1R by siRNA inhibits the migration and proliferation of HUVECs, and even promotes the apoptosis of the cells at 96 h.

**Conclusions**

We showed that expression of miR-503 in HUVECs was elevated under high glucose condition. In addition, miR-503 reduced the migration and proliferation, but promoted the apoptosis of HUVECs by inhibiting the expression of IGF-1R. Therefore, up-regulated expression of miR-503 by high glucose may be a key factor that impedes the repair and regeneration of endothelial functions.

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**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

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