miR-195 suppresses abdominal aortic aneurysm through the TNF-α/NF-κB and VEGF/PI3K/Akt pathway

XIAOHUI MA1*, HAIRONG YAO2*, YUHONG YANG3, LONG JIN4, YU WANG5, LINAG WU5, SHENGLI YANG6 and KANG CHENG2

1Department of Vascular Surgery, General Hospital of People's Liberation Army, Beijing 100853; 2Department of Cardiology, Xi'an No. 3 Hospital, Xi'an, Shaanxi 710018; 3Department of Cardiology, People's Hospital of Yuncheng, Yuncheng, Shanxi 044000; 4Department of Cardiology, Xi'an Ninth Hospital, Xi'an, Shaanxi 710004; 5Department of Nursing, Xi'an No. 3 Hospital, Xi'an, Shaanxi 710018; 6Department of Cardiology, Xi Jing Hospital, Xi'an, Shaanxi 710032, P.R. China

Received January 23, 2017; Accepted January 18, 2018

DOI: 10.3892/ijmm.2018.3426

Abstract. In the present study, the function of microRNA (miR)-195 on abdominal aortic aneurysm (AAA) and its possible mechanism were investigated. Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the expression of miR-195 in patients with AAA. The expression levels of miR-195 in patients with AAA were effectively increased. The present study also used miR-195 mimics to increase the expression of miR-195, and ELISA kits and western blot analysis were used to analyze the levels of interleukin (IL)-1β and IL-6, and the protein expression levels of matrix metalloproteinase (MMP)-2, MMP-9, tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB, vascular endothelial growth factor (VEGF), phosphoinositide 3-kinase (PI3K) and phosphorylated (p-)Akt. The overexpression of miR-195 promoted the levels of IL-1β and IL-6, induced the protein expression of MMP-2 and MMP-9, upregulated the protein expression of TNF-α and NF-κB, and suppressed the protein expression levels of VEGF, PI3K and p-Akt in angiotensin II-vascular smooth muscle cells. Combined, these results suggested that miR-195 suppressed AAA inflammation through the TNF-α/NF-κB and VEGF/PI3K/Akt pathways.

Introduction

Abdominal aortic aneurysm (AAA) is a disease with a high mortality rate, which manifests as permanent abdominal aortic dilation with potential rupture and substantial hemorrhage, which eventually leads to patients succumbing to mortality (1,2). AAA ranks as the highest among all types of aneurysm in terms of morbidity rates. The disease is characterized by local and permanent dilation of the abdominal aortic wall, with the tumor body diameter being higher than normal; this leads to a weakened abdominal aortic wall, which is life-threatening once the tumor body ruptures (1,2).

AAA is currently considered to be associated with multiple factors, including heredity factors, biochemistry, immunity, inflammation and hemodynamics (3). The AAA continues to dilate until rupture when it occurs, and it is associated with a poor prognosis (3). Nuclear factor (NF)-κB p65 is a type of endonuclear transcription factor. The NF-κB p65 signaling pathway is important in regulating inflammatory, immune and cell apoptotic responses, the abnormal activation of which in the aorta promotes the expression of inflammatory factors and matrix metalloproteinase (MMP)-hydrolyzed proteins, and becomes one of the factors inducing aneurysm genesis (4). Tumor necrosis factor (TNF)-α is an important pro-inflammatory cytokine, which is most closely associated with the genesis and development of AAA inflammation (5). It has been suggested that the level of TNF-α in the synovial fluid of patients with rheumatoid arthritis is positively correlated with the severity of AAA. Inhibiting the overexpression of TNF-α can prevent AAA, whereas antibody therapy targeting TNF-α can effectively alleviate AAA symptoms in patients and delay the progression of inflammation (6). TNF-α has been identified in in vitro experiments to induce the cell production of multiple inflammatory mediators, including interleukin (IL)-6, IL-8, prostaglandin E2, collagenase and metalloproteinase, promoting AAA inflammation and leading to articular damage (6).

Key words: miRNA-195, abdominal aortic aneurysm, tumor necrosis factor-α/nuclear factor-κB, vascular endothelial growth factor/phosphoinositide 3-kinase/Akt
MicroRNAs (miRNAs) are a type of highly conserved, small, non-coding RNA, which is distributed extensively in eukaryotes (7). They are also novel gene expression regulatory factors, which exert their function mainly through inhibiting target gene protein translation or degrading target mRNA (7). The majority of miRNAs in animals are considered to exert their functions through inhibiting post-transcriptional translation, whereas they function mainly through degrading target mRNAs in plants (8). miRNAs are involved in cell differentiation, proliferation and apoptotic processes; therefore, they are closely associated with multiple diseases, including tumors, cardiovascular diseases, fibrotic diseases and viral infections (9). Multiple miRNAs regulating extracellular matrix degradation and smooth muscle cell apoptosis have been identified in studies investigating the genesis and development of AAA in previous years. They are reported to potentially be involved in regulation (10). Zampetaki et al. showed that miR-195 may contribute to the pathogenesis of AAA (11).

Vascular endothelial growth factor (VEGF) can promote endothelial formation and angiogenesis; however, it cannot enhance the proliferation of other cell types, which is an important characteristic of VEGF. In addition, VEGF can promote plasmin activity, prevent thrombosis, and increase capillary permeability and vasodilation. Therefore, VEGF is of significance in the repair of vascular injury and prevention of restenosis (12). It is known that the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is one of the important downstream signaling pathways of VEGF, which is involved in numerous processes, including tumor proliferation and metastasis (13). The PI3K/AKT signaling pathway is extensively distributed in cells, and is a signal transduction pathway involved in cell growth, proliferation, differentiation, cell survival, adhesion, migration and anti-apoptosis (14). Signaling pathways can regulate expression at the transcription level, promoting tumor angiogenesis. The aim of the present study was to identify the function of miR-195 on AAA and its possible mechanism.

Materials and methods

Ethics statement and patients. All experiments were approved by the Ethics Committee of the General Hospital of People's Liberation Army (Beijing, China). Whole blood samples from patients with AAA (n=6, 61.5±8.5 years old, male) and normal volunteers (n=6, 55.5±9.5 year age, male) were collected from General Hospital of People's Liberation Army (March and May 2015) and centrifuged at 1,000 x g for 10 min at 4°C. Serum was stored at -70°C for subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was isolated from serum samples using TRIzol according to the manufacturer's protocol. The following primers were used for the PCR: miR-195 forward, 5'-GGGGAGCCAAAGGGTCTCATCCT-3' and reverse, 5'-GAGGGGCATCCACGTTCTCT-3'; U6 forward, CTCGCTTCGCCAGCACA, and reverse, 5'-AACGCTTCA CGATTTCTGT-3'. The following thermocycling conditions were used for PCR: Initial at 95°C for 5 min; 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The data were analyzed using the 2-ΔΔCq method (15).

Culture cell and transfection. Primary human umbilical vein endothelial cells (HUVECs; PromoCell GmbH, Heidelberg, Germany) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with penicillin/streptomycin (100 U/ml and 100 µg/ml), L-glutamine (2 mM) and FBS (10%, Thermo Fisher Scientific, Inc.) at 37°C in humidified air containing 5% CO2. The miR-195 mimics and negative mimics were obtained from iGene Biotechnology, Inc. (Shanghai, China). The cells were transfected with miR-195 mimics and negative mimics using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 24h. Subsequently, the cells (1x105 cell/well) were seeded in 6-well-plates and treated with 120 nM angiotensin II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 20 h at 37°C, prior to treatment with 100 µl of lipopolysaccharide (LPS, Beyotime Institute of Biotechnology, Nanjing, China) for 4 h at 37°C.

ELISA. The supernatants of the cells were collected and the concentrations of IL-1β ELISA kit (cat. no. PI305) and IL-6 ELISA kit (cat. no. PI330) were assayed (both Beyotime Institute of Biotechnology). Absorbance detection was performed using a microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm.

Western blot analysis. Total cellular lysates were prepared with radioimmunoprecipitation assay buffer, according to the manufacturer's protocol (Beyotime Institute of Biotechnology). The protein concentrations were determined using the Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.). Subsequently, 40 µg of the protein samples were separated by 8-12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk powder-TBST and western blot analysis was performed with the following primary antibodies: MMP-2 (cat. no. 40994, 1:2,000), MMP-9 (cat. no. 13667, 1:2,000), NF-κB (cat. no. 11948, 1:2,000), VEGF (cat. no. 9698, 1:2,000), PI3K (cat. no. PI3K, 1:2,000), p-AKT (cat. no. 4060, 1:1,000) and GAPDH (cat. no. 5174, 1:5,000), Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (cat. no. 7076, 1:5,000; Cell Signaling Technology, Inc.) was used as a secondary antibody at 37°C for 1 h and visualized using enhanced chemiluminescence detection (EMD Millipore, Billerica, MA, USA).

Statistical analysis. The results are expressed as the mean ± standard deviation and analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Statistical analysis was performed using one-way analysis of variance followed by Bonferroni’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference.
**Results**

**Expression levels of miR-195 in patients with AAA.** With the aim of examining the miRNAs involved in the bone regeneration process of AAA, the present study analyzed the expression levels of miR-195 in patients with AAA and normal volunteers. As shown in Fig. 1A, the expression levels of miR-195 in patients with AAA were higher, compared with those in the normal volunteers. In the angiotensin II-induced cell model, the expression level of miR-195 was also increased, compared with that in the control group (Fig. 1B).

**miR-195 promotes the levels of IL-1β and IL-6 in angiotensin II-vascular smooth muscle cells.** In order to determine whether miR-195 affects inflammation in angiotensin II-vascular smooth muscle cells, the levels of IL-1β and IL-6 were examined for the array. As shown in Fig. 2A and B, miR-195 effectively increased the levels of IL-1β and IL-6 in the angiotensin II-vascular smooth muscle cells.

**miR-195 promotes the protein expression of MMP-2 and MMP-9 in angiotensin II-vascular smooth muscle cells.** To confirm the protein expression of MMP-2 and MMP-9 following induction of the overexpression of miR-195, the protein expression levels of MMP-2 and MMP-9 in the angiotensin II-vascular smooth muscle cells were measured. As shown in Fig. 3A-C, miR-195 effectively promoted the protein expression levels of MMP-2 and MMP-9 in the angiotensin II-vascular smooth muscle cells.
miR-195 upregulates the protein expression of TNF-α and NF-κB in angiotensin II-vascular smooth muscle cells. To confirm the expression of inflammatory proteins in the AAA model, the protein expression levels of TNF-α and NF-κB were determined following the overexpression of miR-195. As shown in Fig. 4A-C, miR-195 significantly upregulated the protein expression of TNF-α and NF-κB in the angiotensin II-vascular smooth muscle cells.

miR-195 suppresses the protein expression of VEGF, PI3K and p-Akt in angiotensin II-vascular smooth muscle cells. To further elucidate the effect of miR-195 during AAA, the protein expression levels of VEGF, PI3K and p-Akt were determined in angiotensin II-vascular smooth muscle cells following the overexpression of miR-195. The protein expression levels of VEGF, PI3K and p-Akt in the angiotensin II-vascular smooth muscle cells were significantly downregulated by miR-195 (Fig. 5A-D).

TNF-α promotes the protein expression of TNF-α and NF-κB by miR-195. To characterize the effect of TNF-α on the function of miR-195 in AAA, the present study investigated the protein expression of TNF-α and NF-κB following treatment with miR-195 and TNF-α protein. As shown in Fig. 6A-C, the TNF-α recombinant protein significantly promoted the protein expression of TNF-α and NF-κB in the angiotensin II-vascular muscle cells.
smooth muscle cells by miR-195. In addition, immunofluorescence was used to observe the protein expression of NF-κB in angiotensin II-vascular smooth muscle cells overexpressing miR-195. As shown in Fig. 7, the protein expression of NF-κB in the miR-195-overexpressing group was higher, compared with that in the control group, and the combination of miR-195 and TNF-α recombinant protein significantly increased the protein expression of NF-κB in the angiotensin II-vascular smooth muscle cells, compared with that in the miR-195 group.

**TNF-α promotes the generation of IL-1β and IL-6 by miR-195.** To confirm whether the levels of IL-1β and IL-6 induced by miR-195 were affected by TNF-α in AAA, the levels of IL-1β and IL-6 were quantified using ELISA kits. The increased levels of IL-1β and IL-6 in the angiotensin II-vascular smooth muscle cells induced by miR-195 were significantly promoted by the TNF-α recombinant protein (Fig. 8A and B).

**TNF-α promotes the protein expression of MMP-2 and MMP-9 by miR-195.** It was also found that TNF-α significantly promoted the protein expression of MMP-2 and MMP-9 by miR-195 in the angiotensin II-vascular smooth muscle cells (Fig. 9A-C). These data showed that miR-195/ TNF-α affected inflammation in the development of AAA.

**PI3K affects the protein expression levels of VEGF, PI3K and p-Akt by miR-195.** The present study subsequently investigated whether PI3K was a potential target for miR-195 in the development of AAA. The PI3K inhibitor, LY294002, suppressed the PI3K signaling pathway in angiotensin II-vascular smooth muscle cells; the protein expression levels of VEGF, PI3K and p-Akt in the angiotensin II-vascular smooth muscle cells decreased by miR-195 were suppressed significantly by the PI3K inhibitor (Fig. 10A-D). It was found that miR-195 significantly suppressed the protein expression of VEGF in the angiotensin II-vascular smooth muscle cells, compared with that in the control group. The PI3K inhibitor led to significant suppression of the protein expression of VEGF in the angiotensin II-vascular smooth muscle cells with miR-195, compared with that in the miR-195 group without the inhibitor (Fig. 11).

**PI3K affects the protein expression of MMP-2 and MMP-9 by miR-195.** To determine whether miR-195 directly binds PI3K in the development of AAA, the protein expression levels of MMP-2 and MMP-9 were examined in angiotensin II-vascular smooth muscle cells. The PI3K inhibitor significantly induced the protein expression of MMP-2 and MMP-9 in the angiotensin II-vascular smooth muscle cells with miR-195, compared with that in the miR-195 group without the inhibitor (Fig. 12A-C).
Figure 8. TNF-α promotes the generation of IL-1β and IL-6 by miR-195. TNF-α promoted the generation of (A) IL-1β and (B) IL-6 by miR-195. **P<0.01, vs. control group; ***P<0.01, vs. miR-195 group. Control, negative control group; miR, microRNA; TNF-α, tumor necrosis factor-α; IL, interleukin; miR-195, miR-195 mimics group; TNF-α, miR-195 mimics+TNF-α group.

Figure 9. TNF-α promotes the generation of MMP-2 and MMP-9 protein by miR-195. TNF-α promoted the protein expression of MMP-2 and MMP-9 by miR-195, determined using (A) western blot analysis with statistical analysis of (B) MMP-2 and (C) MMP-9. **P<0.01, vs. control group; ***P<0.01, vs. miR-195 group. Control, negative control group; miR, microRNA; miR-195, miR-195 mimics group; TNF-α, tumor necrosis factor-α; miR-195 mimics+TNF-α group.

Figure 10. PI3K affects the generation of VEGF, PI3K and p-Akt proteins by miR-195. PI3K affected the protein levels of VEGF, PI3K and p-Akt by miR-195, determined using (A) western blot analysis with statistical analysis of (B) VEGF, (C) PI3K and (D) p-Akt. **P<0.01, vs. control group; ***P<0.01, vs. miR-195 group. Control, negative control group; miR, microRNA; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated Akt; miR-195, miR-195 mimics group; Anti-PI3K, miR-195 mimics+PI3K inhibitor group.
AAA is a serious, life-threatening vascular disease, which predominantly affects older men (15). Its morbidity rate has increased gradually, and it has become one of the top 10 causes of mortality in the elderly worldwide (15). Its risk factors include old age, being male, smoking, family history, central obesity, low high-density lipoprotein cholesterolemia and hypertension (15). AAA includes the following major pathogeneses: First is extracellular matrix degradation; extracellular matrix of the aorta is composed of collagen, elastin, fibronectin and laminin, which is the major component for maintaining the structural integrity and elasticity of vascular wall (15). Extracellular matrix degradation is mainly mediated by MMPs, among which, MMP-2 and MMP-9 are the most important and have been investigated extensively (2). The enhanced expression of MMP or reduced expression of its specific inhibitor induces increased MMP activity, which results in extracellular matrix degradation,

Discussion

AAA is a serious, life-threatening vascular disease, which predominantly affects older men (15). Its morbidity rate has increased gradually, and it has become one of the top 10 causes of mortality in the elderly worldwide (15). Its risk factors include old age, being male, smoking, family history, central obesity, low high-density lipoprotein cholesterolemia and hypertension (15). AAA includes the following major pathogeneses: First is extracellular matrix degradation; extracellular matrix of the aorta is composed of collagen, elastin, fibronectin and laminin, which is the major component for maintaining the structural integrity and elasticity of vascular wall (15). Extracellular matrix degradation is mainly mediated by MMPs, among which, MMP-2 and MMP-9 are the most important and have been investigated extensively (2). The enhanced expression of MMP or reduced expression of its specific inhibitor induces increased MMP activity, which results in extracellular matrix degradation,
loss of integrity and reduced elasticity of the vascular wall, eventually leading to aortectasia or aortic aneurysm formation (16). Second is smooth muscle cell apoptosis; it has been shown in human AAA tissue specimens that loss of smooth muscle cells in the tunica media is associated with smooth muscle cell apoptosis, and extracellular matrix degradation can lead to anokis of smooth muscle cells (17). Third is inflammation. Angiogenesis is closely associated with the inflammatory response, and it the former is commonly considered an important factor promoting aortic rupture, whereas inflammation can regulate the genesis and development of AAA through stimulating angiogenesis (18). These important pathogeneses are closely associated, among which extracellular matrix degradation is the most important. In the present study, the expression levels of miR-195 were we analyzed in patients with AAA and were found to be higher, compared with those in normal volunteers. miR-195 effectively promoted the protein expression of MMP-2 and MMP-9 in angiotensin II-vascular smooth muscle cells. Cai et al also reported that miR-195 inhibited the tumor progression of prostate cancer via MMP-9 and VEGF (19).

miRNAs are a novel type of gene expression regulatory factor, which inhibits the translation process of mRNAs encoding proteins through binding with the target mRNA 3'untranslated region and inducing regulatory effects; it is also important in cell differentiation, proliferation, apoptosis and metabolism (20). Previously, it was found that multiple miRNAs are associated with the genesis and development of AAA, having regulatory effects on extracellular matrix degradation, vascular smooth muscle cell apoptosis, inflammation and angiogenesis (21). In the present study, it was found that miR-195 significantly increased the levels of IL-1β and IL-6 in angiotensin II-vascular smooth muscle cells. Chen et al reported that miR-195 suppressed ulcerative colitis-induced inflammation through targeting small mothers against decapentaplegic 7 (22).

NF-κB p65 predominantly exists in the cytoplasm in the form of an inactive precursor at rest. It can induce the expression of target genes once it is activated, including inflammatory mediators IL-1 and IL-6, and MMPs (23). Of these, the transcriptional activation of target MMP-2 and MMP-9 can lead to the degradation and destruction of abdominal aortic extracellular matrix (24). NF-κB inhibitor has been shown in animal experiments to significantly inhibit the formation of AAA (24). In addition, the present study demonstrated that miR-195 significantly upregulated the protein expression of TNF-α and NF-κB in angiotensin II-vascular smooth muscle cells. TNF-α promoted the pre-inflammatory effect of miR-195 on the protein expression of TNF-α and NF-κB, the levels of IL-1β and IL-6, and the protein expression of MMP-2 and MMP-9 in angiotensin II-vascular smooth muscle cells. Ding et al showed that miR-195 suppresses cancer cell proliferation and migration in hepatocellular carcinoma through the TNF-α/NF-κB pathway (25).

Angiogenesis refers to the process of growing new blood vessels from endothelial cells in original blood vessels through budding, migration and proliferation (12). VEGF can increase number of vesicles in endothelial cells as the most specific key precipitating factor of angiogenesis, which increases vascular permeability (12). VEGF is an agent with the highest selectivity in promoting endothelial cell mitosis; it can promote the proliferation of smooth muscle cells, epithelial cells and fibroblasts, and induce angiogenesis. It also stimulates endothelial cells to produce nitric oxide, thus exerting a function of vascular maintenance (26). VEGF binds with its receptor, releases multiple growth factors and cytokines, induces the proliferation and migration of endothelial cells, and eventually promotes angiogenesis (13). The present study demonstrated that miR-195 significantly downregulated the protein expression of VEGF in angiotensin II-vascular smooth muscle cells. Almeida et al suggested that miR-195 regulates important mechanisms for bone regeneration through the expression of VEGF (27).

PI3K and its downstream AKT constitute an important signaling pathway, which is termed the PI3K/AKT signaling pathway and is vital for the survival, differentiation, proliferation and apoptosis of cells (14). The association between PI3K and tumors is supported in numerous studies, and an imbalance of PI3K/AKT is involved in multiple human tumor diseases, including lung cancer, nasopharyngeal carcinoma, liver cancer, gastrointestinal cancer, breast cancer, ovarian cancer, renal carcinoma, prostate cancer, lymphoma, malignant glioma and medulloblastoma (28). The correlation between PI3K/AKT and non-tumor diseases, including hepatic fibrosis, Alzheimer’s disease, diabetes and cardiovascular diseases, has gradually attracted attention (29). The present study found that miR-195 significantly downregulated the protein expression of PI3K and p-AKT in angiotensin II-vascular smooth muscle cells. The suppression of PI3K promoted the pre-inflammatory effect of miR-195 on the protein expression of PI3K, p-Akt and VEGF, levels of IL-1β and IL-6, and protein expression of MMP-2 and MMP-9 in angiotensin II-vascular smooth muscle cells. Sun et al indicated that miR-195 has a tumor suppressive effect in ACHN cells through the PI3K/Akt signaling pathways (30).

In conclusion, the present study demonstrated that miR-195 suppressed AAA through the TNF-α/NF-κB and VEGF/PI3K/Akt pathways (Fig. 13). Taken together, these observations revealed that miR-195 functioned as an anti-inflammatory gene in AAA through the TNF-α/NF-κB and VEGF/PI3K/Akt pathways.

Competing interests

The authors declare that they have no competing interests.

References