Upregulation of MicroRNA-15a Contributes to Pathogenesis of Abdominal Aortic Aneurysm (AAA) by Modulating the Expression of Cyclin-Dependent Kinase Inhibitor 2B (CDKN2B)

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Background: The objective of the present study was to identify the association between miR-15a-5p and CDKN2B, and their roles in regulating the development of abdominal aortic aneurysm (AAA).

Material/Methods: We searched the miRNA database online (www.mirdb.org) and used a luciferase reporter assay system to study the regulatory relationship between miR-15a-5p and CDKN2B. We also conducted real-time PCR and Western blot analysis to study the mRNA and protein expression level of CDKN2B among different patient groups (participants with abdominal aortic aneurysm (AAA) and normal controls) or cells treated with scramble control, miR-15a-5p mimics, CDKN2B siRNA, and miR-15a-5p inhibitors.

Results: We found that CDKN2B was a virtual target of miR-15a-5p with potential binding sites in the 3'UTR of CDKN2B (77–83 bp). We also showed that miR-15a-5p could bind to the CDKN2B 3'UTR, resulting in a significant decrease in luciferase activity compared with the scramble control. Furthermore, we found that the cells isolated from AAA participants showed an over-expression of miR-15a-5p compared to the normal controls, while the CDKN2B mRNA and protein expression level of the AAA group were much lower than the normal control group. Additionally, the expression of CDKN2B mRNA and the protein of the cells transfected with miR-15a-5p mimics and CDKN2B siRNA was downregulated, while the cells showed upregulated expression subsequent to transfection with miR-15a-5p inhibitors compared to the scramble control.

Conclusions: The data revealed a negative regulatory role of miR-15a-5p in the apoptosis of smooth muscle cells via targeting CDKN2B, and showed that miR-15a-5p could be a novel therapeutic target of AAA.

MeSH Keywords: Aortic Aneurysm, Abdominal • Apoptosis • MicroRNAs • Myocytes, Smooth Muscle

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Background

An abdominal aortic aneurysm (AAA) elevates the risk of fatal rupture, as it is a dilated infrarenal aorta that seems to be caused by chronic weakness of the arterial wall. Additionally, AAA is correlated with an elevated risk of other cardiovascular conditions in patients with aneurysm [1]. The prevalence of AAA is estimated to be increasing in elderly people worldwide. AAAs are usually silent and there is no available routine screening, so it is often recognized during an imaging test for evaluation of other health problems [2]. The progression of vessel dilation is usually present, and because there are no available prognostic indicators or therapeutic drugs, monitoring patients by follow-up imaging to inspect AAA expansion is necessary [3]. Monitoring persists until the diameter of the aorta reaches 50 to 55 mm, when intervention with surgery is often provided, because it is believed that the risk of rupture outweighs perioperative risks for majority of patients [4,5].

AAA ranks tenth in the list of causes of death. During a process called phenotypic switching, smooth muscle cells (SMCs) are significantly plastic and switch to a proliferative migratory state from a quiescent contractile state [6]. This process is facilitated by downregulated indicators of differentiated SMCs such as SM α-actin, smooth muscle myosin heavy chain (SM-MHC), SM22α, and genes necessary for contraction of SMC [6]. Following injury, SMC phenotypic plasticity is thought to have evolved for optimized vascular recovery, though it is generally recognized that the switching of SMC phenotype serves a crucial role in the regulation of plaque stability, and the development and progression of atherosclerotic sites [7]. We previously conducted a laboratory study to reveal that the switch of SMC phenotypes was an early event in the formation of aorta aneurysm [8]. Nevertheless, few data have described the mechanism of switching of SMC phenotypes in the formation of aneurysm [8].

The pathologic features of AAA include oxidative stress, extracellular matrix degradation, vascular SMC apoptosis, and inflammation [9]. In AAA patients, pro-inflammatory cytokines are secreted by T cells and enhanced cytotoxicity is shown in natural killer cells [10,11]. In the development and progression of AAA, autoimmunity may serve a role [12].

There exist endogenous methods to regulate genes at the post-transcriptional level, and these mechanisms are important in the determination of cell behavior and cell fate, as shown in studies over the past 10 years [13]. MicroRNAs (miRNAs), as single-stranded non-coding RNAs consisting of ~22 nucleotides, exert their effect by triggering translational depression or degradation of certain target mRNAs [14]. It is currently understood that over 30% of the genome is modulated in such a pattern, although less than 1,000 human miRNAs have been identified [15,16]. It has been previously shown that miR-15a-5p is differentially expressed in the SMCs collected from AAA [17]. Furthermore, cyclin-dependent kinase inhibitor 2B (CDKN2B) encodes a cyclin-dependent kinase inhibitor, lying close to the tumor suppressor gene CDKN2A, and is frequently dysregulated in human malignancies. CDKN2B prevents activation of CDK4 or CDK6 by forming a complex with them. Dysregulation of CDKN2B has also been reported to be involved in the molecular mechanism of the apoptosis of SMCs [18,19]. By searching the online miRNA database, we found that CDKN2B is a virtual target of miR-15a-5p. In this study, we validated CDKN2B as a target of miR-15a-5p and verified the involvement of miR-15a-5p and CDKN2B in the development of AAA.

Material and Methods

Subjects

A group of 32 patients with AAA were included in our study; samples of abdominal aorta were collected between December 2013 and December 2014 when the patients underwent surgery to treat AAAs. Meanwhile, a group of 29 aorta samples from autopsies were collected during the same period of time as normal controls, and these specimens were further confirmed to make sure no patients with AAAs and collagen disease were included. All procedures and the use of tissue samples were approved by the Ethics Committee of No. 1 People’s Hospital of Jining, and written consents were obtained from all of the donors or their first-degree relatives.

SMC isolation and culture

Abdominal aorta segments from the patients were further sectioned to small pieces and incubated with 1.5 mg/mL collagenase containing Hanks’ buffer for 20 minutes. A fine forceps was used to remove the thin layer of adventitia, and endothelium was further removed by gently scratching the intimal surface with a surgical blade. Further digestion of the remaining smooth muscle with 0.5 mg/mL elastase and 2.0 mg/mL collagenase was performed at 37°C for 35–45 minutes. Cells were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin at 37°C. After initial incubation for about 48 hours, cells were passaged by trypsin and seeded at the appropriate density for further experiments.

RNA isolation and real-time PCR

RNAlater (Qiagen, Hilden, Germany) was used to submerge the blood vessel samples at 4°C according to the manufacturer’s protocol. TissueLyser II homogenizer (Qiagen, Hilden, Germany) was used to homogenize the blood vessel samples. The mirVana miRNA and mirVana RNA isolation kits (Ambion, Austin, TX) were used to isolate the RNA. The RNA was then treated with DNase I and reverse transcribed into cDNA using random primers. The primers were designed using Primer3, and the primers were validated using the NCBI database. The cDNA was then amplified using a real-time PCR mixture (Applied Biosystems, Foster City, CA) and the CFX Connect software (Bio-Rad, Hercules, CA). The relative expression levels were normalized to 18S and calculated using the comparative C(q) method.
reporter constructs including the targeting sequence from the region of the miR-15a-5p binding sites was mutated to generate DNA to obtain the melt curves. GAPDH or U6 was used as housekeeping genes for comparison to standardize the levels of CDKN2B mRNA and miR-15a-5p. Each test was repeated three times.

**Cell culture and transfection**

Dulbecco’s modified Eagle’s medium/F12 (Invitrogen, CA, USA) containing 10% (v/v) FBS (fetal bovine serum), 100 μg/mL streptomycin, and 100 units/mL penicillin (Invitrogen, CA, USA) were used to maintain the SMCs in an atmosphere of 5% CO2/95% air at 37°C, as described previously. DharmaFECT transfection reagent (Dharmacon, MA, USA) was used to transfect the SMCs with miR-15a-5p mimics, antimiR-15a-5p siRNA (On-TargetPlus SMARTpool; Dharmacon, MA, USA), negative-control RNA (Dharmacon, MA, USA) and non-targeting miRNA mimics (Dharmacon, MA, USA) according to the manufacturer’s recommendation. Quantitative real-time PCR (RT-PCR) was used to confirm the resultant changes in miR-15a-5p levels.

**Cell proliferation assay**

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay was used to measure the cell proliferation of SMCs. Then 48 hours after transfection, 100 μL of fresh serum-free medium with 0.5 g/L MTT was added to change the transfection medium in each well, and the cells were incubated for an additional four hours. Then aspiration was used to remove the MTT medium, and 50 μL of DMSO was added into each well. A plate reader was used to measure the A540 of each sample after incubating for 10 minutes at 37°C. Each test was repeated three times.

**Luciferase assay**

A fragment from the 3′-untranslated region (3′-UTR) of the CDKN2B gene was amplified by PCR. The PCR products were inserted into the multiple restrictive site of the pGL3 firefly luciferase reporter vector (Promega, Madison, WI, USA). The seed region of the mir-15a-5p binding sites was mutated to generate the corresponding mutant constructs. Lipofectamine 2000 (Invitrogen, CA, USA) was used to transfect the SMCs with the reporter constructs including the targeting sequence from the CDKN2B 3′-UTR and its mutant according to the protocol from the supplier. The pRL-TK vector (Promega, Madison, WI, USA) was used to co-transfect the internal control in order to normalize the efficiency of transfection. A Lumat LB 9507 luminometer (Berthold Technologies, Germany) was used to determine the luciferase activities, as described previously. Each experiment was performed in triplicate.

**Western blot analysis**

β-Actin (1:10,000, Cell Signaling Technology, Boston, MA, USA) was used as an internal control and specific antibodies against CDKN2B (1:1,000, Cell Signaling Technology, Boston, MA, USA) were used to perform the Western blotting. The SMCs and tissue samples were harvested and washed with ice-cold PBS (Invitrogen, CA, USA) three times, and RIPA buffer (Sigma, Japan) containing 10 mM Tris/HCl, pH 7.4, 0.5% Triton X-100, 150 mM NaCl, and protease inhibitors (Sigma, Japan) was used to lyse the cells according to the manufacturer’s recommendation, and homogenized on ice. Then the cellular lysates were centrifuged for 15 minutes at 13,500 g at 4°C. The Bradford assay (Bio-Rad Laboratories, Berkeley, CA, USA) was used to determine the concentration of protein. Then 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Bio-Rad Laboratories, Berkeley, CA, USA) was used to isolate the protein extracted, followed by semi-dry transferring to a nitrocellulose membrane (Whatman, UK). Then 5% (w/v) non-fat dried skimmed milk powder was used to treat the membranes to avoid nonspecific binding, followed by washing the membranes with Tris-buffered saline with 0.05% Tween 20. The primary antibodies (1:1,000) were used to incubate the membranes at 4°C for 12 hours or at 25°C for one hour. PBST (PBS containing 0.05% Tween 20) was used to wash the membranes three times, and then appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (1: 10,000, Cell Signaling Technology, Boston, USA) was used to maintain the membranes for another one hour, followed by washing the membranes three times with PBST. The Immun-Star HRPChemiilluminescence Kit peroxide buffer and luminol/enhancer (Bio-Rad Laboratories, Berkeley, California, USA) were used to treat the membranes in order to detect the protein bands.

**Apoptosis analysis**

The apoptosis of the SMCs was performed using the AnnexinV-PI dual staining assay kits (BD Biosciences, CA, USA) according to the kit protocol. A FACSCalibur (BD Bioscience, CA, USA) was used to measure the fluorescence signals of AnnexinV and PI by flow cytometry. Cell Quest Pro software (BD Bioscience, CA, USA) was used to analyze the data.
All the statistical results in the experiments were presented as mean ± SEM; difference analysis was performed by two-sided Fisher’s test. Tests for normality were performed for all experimental data using non-parametric or parametric unpaired t-test, and one-way or two-way ANOVA using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). A p-value less than 0.05 was considered significant statistically for all the analysis.

**Results**

**The miR-15a-5p level in different groups**

RT-PCR was used to evaluate the expression of miR-15a-5p in the abdominal aortic aneurysm (AAA) group and the normal group; as shown in Figure 1, the cells isolated from participants diagnosed with AAA showed an over-expression of miR-15a-5p mRNA compared to the normal group, suggesting that the upregulation of miR-15a-5p could promote the development of AAA.

**CDKN2B is a target gene of miR-15a-5p in SMC cells**

Furthermore, based on computational screening (www.mirdb.org) and previous reports, CDKN2B was identified as a virtual target gene of miR-15a-5p with two potential binding sites at 3’UTR of CDKN2B (Figure 2). A Quick Change XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to generate the two mutations at 3’UTR of CDKN2B: 77–83 bp and 80–86 bp, respectively. Furthermore, we conducted a luciferase assay to study the regulatory relationship between miR-15a-5p and CDKN2B; as shown in Figure 3, the luciferase activity of cells transfected with wild-type CDKN2B was much higher than the scramble control, while the luciferase activity of cells transfected with mutant 1 CDKN2B was comparable to the scramble control; furthermore, the luciferase activity of cells transfected with mutant 2 CDKN2B was similar with that of cells transfected with wild-type CDKN2B, indicating that the CDKN2B is a virtual target of miR-15a-5p with potential binding site in the 3’UTR of CDKN2B (77–83 bp).

**The negative regulatory relationship between CDKN2B and miR-15a-5p**

To further confirm the miRNA-mRNA regulatory relationship, RT-PCR was used to examine the expression level of CDKN2B mRNA and the expression of miR-15a-5p. The results confirmed...
the negative regulatory relationship between miR-15a-5p and CDKN2B, and the negative correlation coefficient was \(-0.5965\) (\(r=–0.5965\)), as shown in Figure 4.

**Determination of expression of CDKN2B mRNA and protein in different groups**

RT-PCR and Western blotting were used to estimate the expression of CDKN2B mRNA and protein in participants with AAA and normal controls. As shown in Figure 5, the expression of CDKN2B mRNA (Figure 5A) and protein (Figure 5B) of cells isolated from people with AAA were much lower compared to those without AAA, indicating that the downregulation of CDKN2B could promote the development of AAA.

**Effect of miR-15a-5p on the expression of CDKN2B**

WE investigated the role of miR-15a-5p in the control of AAA, using scramble control, different concentrations of miR-15a-5p mimics and its inhibitors were transfected into SMC cells, as shown in Figure 6. We found that the CDKN2B protein (upper panel) and mRNA (lower panel) expression level of SMCs treated with 50 nM miR-15a-5p mimics were apparently lower than the scramble control, and those of the cells treated with 100 nM miR-15a-5p mimics were even lower than the 50 nM treatment group, indicating a concentration-dependent effect of miR-15a-5p on the expression of CDKN2B. Meanwhile, the 50 nM miR-15a-5p inhibitor treatment group showed obviously higher expression levels of CDKN2B protein (upper panel) and mRNA (lower panel) when compared to the scramble control; the miR-15a-5p mimic treatment groups, and those of the cells treated with 100 nM miR-15a-5p inhibitors, were even higher than the 50 nM miR-15a-5p inhibitor treatment group, validating the negative regulatory relationship between miR-15a-5p and CDKN2B.

**Effect of miR-15a-5p on cell viability and apoptosis**

To understand the biological function of miR-15a-5p in SMCs miR-15a-5p was upregulated and downregulated by transfecting miR-15a-5p mimics or inhibitors into SMC cell lines. In the first of these experiments, we transfected SMCs with miR-15a-5p mimics or inhibitors and performed MTT assays to evaluate the effects of miR-15a-5p expression on cell apoptosis and viability. Over-expression of miR-15a-5p promoted the viability of SMCs (Figure 7A), while downregulation of miR-15a-5p significantly inhibited the viability of SMCs (Figure 7B).

**Figure 4.** The expression level of CDKN2B mRNA and the expression of miR-15a-5p were measured using real-time PCR to validate a miRNA-mRNA regulatory relationship. We confirmed the negative regulatory relationship between miR-15a-5p and CDKN2B, and the negative correlation coefficient was \(-0.5965\) (\(r=–0.5965\))

**Figure 5.** (A) CDKN2B mRNA expression level in PASMCs from patients with abdominal aortic aneurysm was significantly lower compared with normal controls; (B) CDKN2B protein expression level in SMCs with abdominal aortic aneurysm was significantly lower compared with normal controls.
Discussion

As a member representing the pro-apoptotic miRNA cluster miR-15a/16, hsa-miR-15a-3p serves a pro-apoptotic role in mammalian cells. Previous studies have demonstrated that some members of this cluster, including miR-16 and miR-15a-5p, could suppress growth and trigger apoptosis in certain tumors with high expression of Bcl-2. The current observations...
extend the known possible effects of the miR-15a/16 cluster to include triggering activation of apoptosis cascade in tumors with overexpressed Bcl-xL by miR-15a-3p [20]. These results broaden the possibility of this miRNA cluster as a tumor inhibitor in miRNA therapies for cancers [20]. The cluster may be more appropriate for therapies that regulate apoptosis rather than the artificially developed antisense suppressors of these genes, as the members of miR-15a/16 cluster are the endogenous suppressors of Bcl-2, Bcl-xL and possibly other genes that are anti-apoptotic [21]. Moreover, the sensitivity of selected cancers to radiotherapy and chemotherapy can be promoted by the ectopic expression of pro-apoptotic miRNAs, including the entire miR-15a/16 cluster or miR-15a-3p [22,23]. Possibly, the application of miRNAs described above will synergize with chemotherapeutic agents in the treatment of cancers, or play a role independently; therefore, they should be investigated as promising agents against cancers. In 2002, Calin et al. reported that the role of microRNAs in apoptotic regulation was first revealed in peripheral blood cells of people with confirmed chronic lymphocytic leukemia (CLL), where a missing miR-15a/16 cluster was observed in most patients. Subsequent studies demonstrated that the members of this cluster, miR-16 and miR-15a-5p, enhance apoptosis in malignant B cells by post-transcriptionally affecting the expression of Bcl-2 expression [20]. In addition, miR-15a-3p, which is also one member of miR-15a/16 cluster, was shown to trigger apoptosis by affecting Bcl-xL, thus mediating the activation of caspase-3/7 and decreasing viability in an array of cancers [24]. A microRNA known as miR-21 was found to be increased in an array of human cancers and featured as an oncogene, the silencing of which in glioblastoma cells resulted in apoptosis by active caspases 3 and 7 [25]. Cheng et al. discovered an array of microRNAs associated with apoptotic regulation by application of large-scale antisense microRNA suppression in HeLa cells. The apoptosis was elevated by suppression of miR-1d, 7, 148, 204, 210, 216, and 296 via active caspase 3, while the opposite effect could be obtained by suppression of miR-214 [26]. In this study, we performed RT-PCR to estimate the expression in two groups, an abdominal aortic aneurysm (AAA) group and a normal control group, and found that the miR-15a-5p mRNA level was much higher in people with AAA compared to normal controls. We searched the bioinformatics algorithms TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) to study CDKN2B as a target gene of miR-15a-5p, and further confirmed its role using luciferase assay. We conducted the luciferase assay by inserting the wild-type 3′UTR of CDKN2B and mutant 3′UTR of CDKN2B into the luciferase reporter, and found that the cells showing lower luciferase activity were significantly downregulated compared to the scramble control following transfection with wild-type 3′UTR of CDKN2B, which evidently abolished the effects subsequent to transfection with mutant1 3′UTR of CDKN2B. The correlation of 9p21 with either non-atherosclerotic intracranial aneurysms or AAA disease may be explained by the finding that CDKN2B is closely correlated with apoptosis of SMCs. A main feature of these disorders, especially for berry aneurysms, is a reduction of integrity of the vascular wall associated with vascular wall medial SMC number [27]. There is also a correlation between neuronal cell death and SMC apoptosis, as well as upregulated p53, in human aneurysms [28]. Pathologically downregulated CDKN2B would promote p53-dependent apoptosis, and finally enhance medial thinning in carriers of the C9CAR risk haplotype. It is likely that even slightly imbalanced proliferation and apoptosis might have a significant influence on the progression of AAA, especially over a long term period of several decades, since vascular SMC turnover develops at an extremely low rate [29–31].

It seems that signaling downstream of CDKN2B associated with apoptosis is more complicated than previously estimated. In health, both CDKN2A and CDKN2B are well-identified suppressors of tumor development that suppress the transition of the G1 to S by retinoblastoma pathway signaling. The hypermethylation or deletion of the two genes are coordinate-ly observed in human cancer, contributing to tumor growth [32]. In the vasculature, CDKN2B has a similar regulatory role in cell-fate decisions, but seems to play a more important regulatory role in the apoptosis responsive to stress. A loss of CDKN2B leads to active p53 axis and an array of downstream effector molecules, instead of achieving this via the RB pathway. Previously, p53 has been noted to be involved in vascular remodeling [33]. However, its role in progression of atherosclerotic plaque remains controversial [34]. The signaling between CDKN2B and p53 may be mediated by multiple potential mechanisms. The upregulation of expression of vascular ARF responding to lower CDKN2B expression may be one potential mechanism discovered in these studies that link ARF to p53. As demonstrated by these data, both indirect regulation of ARF through intermediate modulation of CDKN2B or direct regulation of ARF by causal alteration at C9CAR could render a common disease pathway. An argument against a pure role for ARF in downregulation of CDKN2B is supported by data derived from ARF knockout mice in the ApoE model [35]. In our study, we further performed RT-PCR and Western blot analysis to evaluate the CDKN2B mRNA and protein expression levels in AAA and normal control groups, and found that the cells isolated from patients with AAA showed lower expression of CDKN2B mRNA and protein compared to normal controls. We also found that the CDKN2B protein showed a clear band with the miR-15a-5p inhibitor treatment group, and a barely visual band with the miR-15a-5p mimic treatment group, compared to the scramble control. Furthermore, the qRT-PCR analysis results revealed that the CDKN2B mRNA expression level was significantly enhanced subsequent to transfection with miR-15a-5p inhibitors, while notably lowered following...
transfection with miR-15a-5p mimics, compared to the scramble control. We further performed MTT assay and flow cytometry analysis to evaluate cell viability and apoptosis, and found that the viability of miR-15a-5p mimics group was considerably promoted, while the miR-15a-5p inhibitor group showed markedly lower viability. Meanwhile, we found that apoptosis of the miR-15a-5p mimic group was considerably lowered, while the miR-15a-5p inhibitor group showed markedly higher apoptosis compared to the scramble control.

**Conclusions**

The findings of this study revealed a negative regulatory role of miR-15a-5p in the apoptosis of SMCs via targeting CDKN2B, and found that miR-15a-5p could be a novel therapeutic target for AAA.

**Conflicts of interest**

None.

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