Original Article

Involvement of MicroRNA-133a in the Development of Arteriosclerosis Obliterans of the Lower Extremities via RhoA Targeting

Yongxin Li^{1,2}, Mao Ouyang³, Zhen Shan⁴, Jieyi Ma¹, Jie Li⁴, Chen Yao⁴, Zhengrong Zhu⁴, Longjuan Zhang¹, Lianzhou Chen¹, Guangqi Chang⁴, Shenming Wang⁴ and Wenjian Wang¹

Yongxin Li, Mao Ouyang and Zhen Shan contributed equally to this work.

¹Laboratory of General Surgery, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, People's Republic of China

²Department of Vascular Surgery, The Affiliated Hospital of Qingdao University, Qingdao, People's Republic of China

³Department of Clinical Laboratory, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, People's Republic of China

⁴Department of Vascular Surgery, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, People's Republic of China

Aim: RhoA is a critical factor in regulating the proliferation and migration of arterial smooth muscle cells (ASMCs) in patients with arteriosclerosis obliterans (ASO). RhoA is modulated by microRNA-133a (miR-133a) in cardiac myocytes and bronchial smooth muscle cells. However, the relationship between miR-133a and RhoA with respect to the onset of ASO in the lower extremities is uncertain.

Methods: We employed in situ hybridization (ISH) and immunohistochemistry (IHC) to detect the location of miR-133a and RhoA in ASO clinical samples, respectively. 5-ethynyl-2'-deoxyuridine (EdU), cell counting kit-8 (CCK-8), Transwell and wound closure assays were utilized to determine the features of human ASMC (HASMC) proliferation and migration. The expression of miR-133a in the HASMCs was assessed using quantitative real-time PCR (qRT-PCR), while that of RhoA was examined via qRT-PCR and Western blotting.

Results: We found miR-133a and RhoA to be primarily located in the ASMCs of ASO. miR-133a was significantly downregulated in the ASO tissues and proliferating HASMCs. In contrast, RhoA was upregulated in the ASO samples. The proliferation and migration of HASMCs was markedly promoted by the downregulation of miR-133a and inhibited by the upregulation of miR-133a. The Luciferase assay confirmed that RhoA was a direct target of miR-133a. The upregulation of miR-133a in the HASMCs decreased the RhoA expression at the protein level. Inversely, the downregulation of miR-133a increased the RhoA protein expression. Of note, the overexpression of RhoA in the HASMCs attenuated the anti-proliferative and anti-migratory effects of miR-133a.

Conclusions: Our data indicate that miR-133a regulates the functions of HASMCs by targeting RhoA and may be involved in the pathogenesis of ASO. These findings may lead to the development of potential therapeutic targets for ASO of the lower extremities.

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Address for correspondence: Wenjian Wang, Laboratory of General Surgery, The First Affiliated Hospital, Sun Yat-Sen University, 58# Zhongshan II Road, Guangzhou 510080, People's Republic of China

E-mail: w166w2000@aliyun.com

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Introduction

Approximately 20% of elderly subjects (≥ 65 years) are found to have large-vessel peripheral arterial disease upon noninvasive testing¹⁾; most of these cases involve ASO. The proliferation and migration of ASMCs, the main cells comprising the media of the

arteries, play vital roles in ASO development². However, the regulatory mechanisms underlying the proliferation and migration of ASMCs in the setting of ASO have not been fully elucidated.

microRNAs (miRNAs) are small (~22-nucleotide), diverse non-coding RNAs that regulate the target gene expression by either degrading mRNA or inhibiting protein translation³⁻⁵. Established studies have confirmed that some miRNAs, such as miR-21, miR-143 and miR-221, play a role in the development of atherosclerosis by regulating the function of ASMCs⁶⁻⁸. Our previous study⁷ showed that miR-133a is downregulated in tissues involving ASO of the lower extremities, indicating that miR-133a is potentially involved in ASO formation. In addition, the downregulation of miR-133a promotes cardiac hypertrophy by targeting RhoA⁹, and the expression level of RhoA has been found to be associated with the level of miR-133a in bronchial smooth muscle cells¹⁰. It is well known that miRNAs from different arteries and species have different functions in ASMCs¹¹. However, whether miR-133a modulates the HASMC function by targeting RhoA in the setting of ASO of the lower extremities remains uncertain.

Methods

Cell Culture

Primary HASMCs were obtained from the normal femoral arteries of amputees who suffered serious trauma with the consent of the donors and approval of the ethics committee of the First Affiliated Hospital of Sun Yat-sen University. Smooth muscle cells were cultured and characterized as previously described⁷. Cells from passages III to V were used.

In Situ Hybridization and Immunohistochemical Staining

The localization of miR-133a and RhoA was determined using *in situ* hybridization (ISH) and immunohistochemistry (IHC), respectively. Six normal femoral arteries (obtained from amputees who had suffered serious trauma) and 16 ASO artery samples (10 men, six women) were histopathologically diagnosed at the Department of Pathology between 2012 and 2013. All patients provided their written informed consent prior to recruitment.

In situ detection of miR-133a was performed using 4- μ m sections of the arteries with a digoxigenin-labeled oligonucleotides miR-133a detection probe (Exiqon, USA), as previously described⁷. The temperatures of prehybridization and hybridization were both set at 55°C, and the tissues were treated with

anti-digoxigenin-AP (Roche, Switzerland) at 4°C overnight. BCIP/NBT staining was performed in the dark for six hours, and the reaction was stopped by washing the tissues in TE buffer (pH 8.0). The nuclei were stained with nuclear fast red solution, and images were acquired under a microscope (Olympus CX41, Japan).

IHC was performed with a two-step IHC kit (ZSGB, China), as previously described⁷.

Transfection

We used an miR-133a mimic to imitate miR-133a and an miR-133a inhibitor to deplete endogenous miR-133a. Both items were designed by and purchased from Ribobio (Ribobio, China), as well as their negative controls. RhoA cDNA mutant (RhoA^{mut}) was constructed by mutating the miR-133a seed region in the 3' untranslated region (UTR) of RhoA (GeneChem, China). Briefly, 24 hours after being seeded into the wells, the cells were transfected with Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol.

Cell Proliferation

The cells (3×10^3 cells per well) were plated in triplicate in 96-well culture plates and incubated for 12 hours before transfection. Platelet-derived growth factor-BB (PDGF-BB) (20 μ g/L) was added to the cells 48 hours after transfection, and the mixture was incubated for 24 hours.

For the CCK-8 (Dojindo, Japan) assay, CCK-8 solution (10 μ l) was added to each well, and the mixture was incubated for one hour. Then, the absorbance was measured at 450 nm using a spectrophotometer.

For the EdU (Ribobio, China) assay, the cells were exposed to EdU (50 μ M) for two hours and then fixed with 4% paraformaldehyde for 30 minutes. The cells were subsequently treated with 100 μ L of Apollo reaction cocktail for 30 minutes, followed by 100 μ L of 1% Hoechst 33342 for 30 minutes for nuclear staining. Finally, an inverted fluorescence microscope was used for visualization. The number of EdU-positive nuclei per the total number of nuclei was calculated in 10 random microscope fields in each well using the Image Pro-Plus 5.1 analysis system.

Cell Migration

A Transwell assay and wound closure assay were performed to assess cell migration, as previously described⁷.

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

Total RNA was prepared from ASO and normal

Fig. 1. Features of the miR-133a expression in normal arteries, ASO arteries and HASMCs

(A) The miR-133a expression in the ASO arteries ($n=16$) was higher than that observed in the normal arteries ($n=6$), as determined on qRT-PCR. (B) In situ hybridization (ISH) of miR-133a (violet) in the normal and ASO arteries. Original magnification: a, b, e, f: $50\times$, c, d, g, h: $100\times$. (C) After 48 hours of starvation, HASMCs were stimulated with PDGF-BB ($20\text{ }\mu\text{g/L}$). Then, miR-133a was tested immediately (T0h) or at one, four, eight and 24 hours (T1h, T4h, T8h and T24h, respectively) later. miR-133a was significantly downregulated in the proliferating HASMCs, especially at eight hours after PDGF-BB stimulation. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

femoral arteries as well as HASMCs using TRIzol reagent (Invitrogen). qRT-PCR was performed as previously described⁷⁾.

Western Blotting Analysis

HASMCs were lysed in RIPA lysis buffer (CST, USA) supplemented with protease inhibitor (Roche, Switzerland), and the protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific, USA). A Western blotting analysis was performed as previously described⁷⁾.

3' Untranslated Region (UTR) Luciferase Assay

The RhoA 3'UTR binding sites or mutated binding sites for miR-133a were amplified via PCR and cloned into the XbaI site of the GV306 vector (GeneChem, China). 293T cells, cultured to 80% confluency in a 96-well plate, were co-transfected with

a luciferase reporter vector and either 100 nmol/L of the miR-133a mimic or negative control using Lipofectamine 2000 (Invitrogen, USA). After 48 hours, the cells were lysed, and the luciferase activity was measured using the Dual-GloTM Luciferase Assay kit (Promega, USA).

Statistical Analysis

All data were expressed as the mean $\pm$ SD from at least three independent experiments and were analyzed using Student's *t*-test. A probability value of 0.05 was accepted as being statistically significant. All data were processed using the GraphPad Prism 5.0 software program (GraphPad, USA).

Fig. 2. Effects of miR-133a on the proliferation of HASMCs

HASMC cells were transfected with the miR-133a mimic (50 nmol/L) and inhibitor (100 nmol/L) for eight hours, respectively. EdU (A, B) and CCK-8 assays (C) were performed to determine the rate of cell proliferation. Original magnification: 100 $\times$. ** P <0.01, *** P <0.001, compared with the controls.

Results

Expression of miR-133a in the ASO Arteries

Our qRT-PCR results confirmed that the miR-133a expression was significantly decreased in the ASO arteries compared to the normal arteries (P <0.01) (Fig. 1A). ISH showed that miR-133a was primarily localized in the HASMCs and that miR-133a staining was much weaker in the ASO arteries (Fig. 1B).

Expression of miR-133a in the Quiescent and Proliferating HASMCs

After 48 hours of serum starvation, the HASMCs were coaxed back into quiescence. Then, the quiescent cells were stimulated into a proliferating state by PDGF-BB¹². As a result, the miR-133a expression was significantly downregulated in the proliferating HASMCs, especially at eight hours after PDGF-BB stimulation, compared to that observed in the quiescent cells (P <0.05) (Fig. 1C).

miR-133a Inhibits HASMC Proliferation and Migration

In order to explore the role of miR-133a in the proliferation and migration of HASMCs, we conducted CCK-8 and EdU assays to evaluate the HASMC proliferation capacity, and Transwell and wound closure assays to assess HASMC migration. Consequently, the miR-133a mimic significantly suppressed cell proliferation and migration, whereas the miR-133a inhibitor significantly promoted cell proliferation and migration (P <0.01) (Fig. 2 and Fig. 3), indicating that miR-133a negatively regulates HASMC proliferation and migration.

RhoA is a Direct Target of miR-133a

The Starbase 2.0 system predicted that RhoA is a potential target of miR-133a, as it exhibits seed sequence complementary to miR-133a (Fig. 4A). In order to investigate whether RhoA is a direct target of miR-133a, we performed a luciferase reporter assay.

Fig. 3. Effects of miR-133a on the migration of HASMCs

HASMC cells were transfected with the miR-133a mimic (50 nmol/L) and inhibitor (100 nmol/L) for eight hours, respectively. Transwell (A, B) and wound closure (C, D) assays were performed to determine the rate of cell migration. Original magnification: 100 $\times$. * P <0.05, ** P <0.01, *** P <0.001, compared with the controls.

The miR-133a mimic significantly reduced the luciferase activity in the presence of the RhoA 3'UTR (wild-type 3'UTR) compared to the negative control. Furthermore, we mutated the 3'UTR of RhoA mRNA, which lacks the binding site for miR-133a. Consequently, the inhibitory effect of miR-133a on the relative luciferase activity was abrogated (Fig. 4B), suggesting that RhoA is a direct target of miR-133a.

miR-133a has been confirmed to affect the RhoA expression at the protein level, but not mRNA level, in cardiac myocytes⁹. However, these interactions have not been studied in HASMCs. In order to investigate the regulatory effect of miR-133a on RhoA, we treated HASMCs with the miR-133a mimic or inhibitor. As shown in Fig. 4C, the miR-133a inhibitor induced the upregulation of the RhoA protein (P <0.001), whereas transfection with the miR-133a mimic resulted in the downregulation of RhoA protein in the HASMCs (P <0.001). In contrast, alteration of the miR-133a expression in the HASMCs did

not affect the levels of RhoA mRNA (Fig. 4D). These results indicate that miR-133a regulates the RhoA expression by inhibiting RhoA protein translation in HASMCs.

RhoA is Involved in miR-133a-Induced Anti-Proliferative and Anti-Migratory Effects on HASMCs

In order to determine whether miR-133a controls the HASMC function via RhoA, we constructed an RhoA^{mut} plasmid in which the 3'UTR binding site for miR-133a was mutated. Thereafter, HASMCs were transfected with the RhoA^{mut} plasmid or empty vehicle vector together with the miR-133a mimic or negative control. As a result, the overexpression of RhoA^{mut} enhanced HASMC proliferation and migration (Fig. 4F-H), while the upregulation of miR-133a significantly suppressed HASMC proliferation and migration (P <0.05) (Fig. 2, Fig. 3 and Fig. 4F-H). In contrast, the overexpression of RhoA^{mut} in the presence of the miR-133a mimic significantly attenuated

Fig. 4. RhoA is a direct target of miR-133a in HASMCs

(A) Target sequences of miR-133a in the RhoA 3'UTR and mutant site in RhoA 3'UTR. (B) Relative luciferase activity of the RhoA 3'UTR and mutant RhoA 3'UTR in the miR-133a mimic-transfected 293T cells. (C) Effects of the miR-133a inhibitor (100 nmol/L) and miR-133a mimic (50 nmol/L) on the RhoA protein expression in the cultured HASMCs. (D) Neither the miR-133a mimic (50 nmol/L) nor miR-133a inhibitor (100 nmol/L) altered the RhoA mRNA levels, as determined on qRT-PCR. (E) Expression of RhoA in the HASMC cells transfected with wild-type RhoA or the RhoA^{mut} construct, respectively, as determined using Western blotting. (F) The overexpression of RhoA attenuated the miR-133a-induced anti-proliferative effects on the HASMCs in the presence or absence of the miR-133a mimic, as determined in a CCK-8 analysis. (G, H) The overexpression of RhoA attenuated the miR-133a-induced anti-migratory effects on the HASMCs in the presence or absence of the miR-133a mimic, determined using a Transwell assay. Original magnification: 100 $\times$. # P <0.05, * P <0.05, ** P <0.01, *** P <0.001, compared with the controls.

the anti-proliferative and anti-migratory effects of miR-133a (P <0.05) (Fig. 4F-H). These results suggest that RhoA is involved in the miR-133a-mediated

negative regulation of HASMC proliferation and migration.

Fig. 5. Expression of RhoA in the ASO samples

(A) HE and IHC staining of the ASO arteries showed RhoA as brownish-yellow granules located in the cytoplasm of the HASMCs in the media of the arteries. (B) Comparison of the IOD values of RhoA between the normal ($n=6$) and ASO ($n=16$) arteries. Original magnification: $200\times$. * $P<0.05$, compared with the normal arteries.

The RhoA Expression is Increased in the ASO Arteries Compared with the Normal Arteries

As shown in **Fig. 5A**, RhoA was primarily located in the cytoplasm of the HASMCs in the media of the arteries. In addition, the RhoA expression was significantly higher in the ASO arteries than in the normal arteries ($P<0.05$) (**Fig. 5B**). These findings indicate that RhoA plays a potential role in the pathogenesis of ASO.

Discussion

The progression of ASO is associated with the activation of multiple genes that induce HASMC proliferation and migration¹³. Therefore, exploring the molecular mechanisms underlying the activation of these genes may yield potential targets for preventing and treating ASO.

In this study, we first observed that miR-133a was primarily located in artery smooth muscle cells (ASMCs) of the human artery wall and its expression was significantly downregulated in the ASO arteries compared to the normal arteries. These findings indicate that miR-133a may take part in the onset of ASO by regulating the function of HASMCs. miR-133a has been demonstrated to inhibit proliferation^{14, 15}, migration^{16, 17}, invasion¹⁷⁻¹⁹ and differentiation^{20, 21} and promote apoptosis^{17, 22} in various types of cells. In addition, miR-133a has been shown to suppress proliferation and promote apoptosis in osteosarcoma cells by targeting Bcl-xL and Mcl-1¹⁴. The overexpression of miR-133a suppresses the proliferation of mice

cardiomyocytes by targeting SRF and cyclin D2²³. Moreover, the downregulation of miR-133a enhances the proliferation and migration of ASMCs in injured rat carotid arteries by targeting Sp-1²⁴. Hence, we propose that an aberrant expression of miR-133a contributes to dysfunction of the proliferation and migration of HASMCs.

Enhanced proliferation and migration of HASMCs is a common phenomenon in cases of ASO²⁵, and the proliferation and migration of HASMCs play vital roles in the pathogenesis of ASO. During the course of ASO, HASMCs migrate from the media to the intima, where they proliferate²⁵. In order to confirm that the miR-133a expression is associated with a state of HASMC proliferation, we evaluated the miR-133a expression in both proliferating and quiescent HASMCs and subsequently found that the expression levels of miR-133a were significantly downregulated in the proliferating HASMCs versus the quiescent HASMCs, consistent with the findings of a previous report²⁴. This result suggests that the miR-133a expression is associated with HASMC proliferation. Furthermore, we found that the upregulation of miR-133a significantly suppresses the proliferation and migration of HASMCs, whereas the downregulation of miR-133a significantly promotes the proliferation and migration of HASMCs, indicating that miR-133a acts as an anti-miR for HASMC proliferation and migration. However, which factors downregulate the miR-133a expression in the setting of ASO despite the proliferation of HASMCs remain unclear, and whether miR-133a is involved in the dedifferentiation

of HASMC types must be further investigated.

We utilized the Starbase 2.0 program to predict targets of miR-133a. The results suggested that RhoA is a potential target of miR-133a. The target sequence of miR-133a in the RhoA 3'UTR is poly conserved in mammals. Although miR-1 and miR-133b have the same seed sequence as miR-133a, miR-133b and miR-1 are almost absent in arterial smooth muscle cells²⁴. We suppose that miR-133a plays a dominant role in arterial smooth muscle cells. Thereafter, we performed a 3'UTR luciferase assay and confirmed that RhoA is a direct target of miR-133a, consistent with the results of a previous study⁹. miRNAs has an effect on the target gene by either degrading its mRNA or inhibiting its protein translation⁴. Recent studies have shown that miR-133a negatively regulates the expression of RhoA in cardiomyocytes⁹ and bronchial smooth muscle cells¹⁰ at the protein level. However, the modality of this interaction in HASMCs remains uncertain, as miRNAs have distinct functions in different species¹¹. In order to investigate the regulatory effects of miR-133a on RhoA in HASMCs, we downregulated the miR-133a expression and found that the expression levels of RhoA proteins significantly increased, while the upregulation of miR-133a significantly decreased the expression levels of RhoA proteins. In contrast, alteration of the miR-133a expression in HASMCs did not change the levels of RhoA mRNA. The results suggest that miR-133a regulates RhoA by inhibiting RhoA protein translation instead of degrading RhoA mRNA in HASMCs, which is also consistent with the findings of previous studies^{9, 10}.

RhoA plays a crucial role in the pathogenesis of ASO by regulating ASMC proliferation and migration^{26, 27}. In order to further investigate whether miR-133a has an effect on HASMC proliferation and migration by targeting RhoA, we overexpressed a mutated form of RhoA in the presence of miR-133a and found that the anti-proliferative and anti-migratory capacity of miR-133a was significantly attenuated. Furthermore, our IHC results showed that the expression of RhoA was significantly higher in the tunica media of the ASO artery samples than in the normal arteries, which may be associated with the downregulation of miR-133a described previously in this context. Our findings also indicate that miR-133a may inhibit HASMC proliferation and migration in the setting of ASO by targeting RhoA. As previously reported⁶⁻⁸, miR-21, miR-143 and miR-221, etc. each play a role in the development of atherosclerosis by regulating the function of ASMCs. Whether miR-133a has a synergistic effect with these miRNAs is

worth studying in the future. Moreover, whether a predominant miRNA-target regulatory pathway exists in diseased HASMCs is uncertain.

In conclusion, we herein demonstrated that the miR-133a expression is downregulated in ASO arteries, whereas that of RhoA is upregulated. Alterations in the miR-133a expression may affect the capacity of HASMCs for proliferation and migration via RhoA, which is involved in the onset of ASO. Therefore, our findings may provide new insight into the mechanisms underlying the pathogenesis of and potential therapeutic targets for ASO.

Conflicts of Interest

None to declare.

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