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Original Research Article

MiR-126 delays the formation of aortic dissections in rats through interaction with MAPK signaling pathway

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Abstract

Purpose: *To investigate the impact of microRNA (miR)-126 on aortic dissections using rat aortic smooth muscle cells (RASMCs).*
Methods: *The cell model of AD (MA-RASMCs) was established by co-culturing RASMCs with angiotensin II (Ang II). The cells were then transfected with miR-126 control and miR-126 mimic. Transfection efficiency was assessed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The proliferative and migratory potentials of the cells were determined, as well as the expression levels of related proteins, i.e., Ras, matrix metalloproteinase-2 (MMP2), tissue inhibitor of metalloproteinase 1 (TIMP1), phosphorylated MAPK (p-MAPK), and phosphorylated ERK (p-ERK).*
Results: *Compared to RASMCs, MA-RASMCs exhibited enhanced proliferation and migration, and decreased miR-126 expression ($p < 0.05$). MA-RASMCs transfected with miR-126 mimic, reduced its proliferative potential, increased miR-126 expression, and lowered the expression levels of Ras, MMP2, p-MAPK, and p-ERK ($p < 0.05$). Furthermore, the transfected cells had higher expression levels of TIMP1 ($p < 0.05$).*
Conclusion: *MicroRNA-126 inhibits the proliferation and migration of RASMCs by modulating MAPK/ERK pathway, thereby delaying the formation of aortic dissections. Thus miR-126 is a potential therapeutic target for aortic dissections.*

Keywords: *MicroRNA-126, Aortic dissections, MAPK signaling pathway*

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INTRODUCTION

Acute aortic dissection (AAD) is a common acute disease of the aortic system, with very high morbidity and mortality rates [1]. It is currently believed that the destruction of the structure of the aorta by various factors, is the main cause of AD formation [2]. However, the specific mechanisms of AD formation have not been identified [3]. The aortic intima can rupture due to various causes, resulting in the backflow of blood into the media. This causes the separation of the vascular intima and adventitia. The blood in the aorta continuously impacts the damaged inner wall over time, causing the empty cavity of the aorta wall to continuously expand, thereby increasing the risk of a rupture and endangering the life of the patient [4,5]. The diagnostic rate of

AD has increased with the development of medical diagnostic techniques [6]. However, ruptures and aneurysms in AD remain important causes of death in patients due to the lack of early diagnosis [1,7]. Therefore, it is important to identify the molecular mechanism underlying the formation and development of AD, so as to aid and improve early diagnosis.

MicroRNAs (miRNAs) are transcribed in the nucleus in a complex form, forming the miRNA precursors in the cytoplasm. MiRNA precursors gradually become mature through splicing in the cytoplasm. Various forms of miRNAs bind to proteins, microvesicles, exosomes or multivesicular bodies to be excreted from the cells into the blood. Although miRNAs do not encode proteins, they are key participants in cell proliferation and growth. It has been reported that miR-126 plays a crucial role in vascular integrity and angiogenesis. miRNA (miR)-126 has also been studied in relation to leukocyte adhesion and the inflammatory response, whereby miR-126 targets and binds to vascular cell to exert an anti-inflammatory effect [8]. Additionally, there are reports that show that miRNAs are involved in various complex processes in diseases, such as angiogenesis and vascular repair [9-11]. Meanwhile, MAPK is a classical cell signaling pathway involved in cell proliferation and differentiation. Previous studies have demonstrated that abnormal expressions of MAPK pathway-associated proteins are associated with aortic system diseases [12,13].

This study investigated the impact of microRNA (miR)-126 on aortic dissections using rat aortic smooth muscle cells (RASMCs).

EXPERIMENTAL

Materials

Rat aortic smooth muscle cells (RASMCs; American Type Culture Collection), cell counting kit-8 (CCK-8) reagent (Applygen Technologies, Inc), BCA reagent (Beyotime Institute of Biotechnology), primers (Wuhan GeneCreate Biological Engineering Co. Ltd), TRIzol® reagent (Roche Diagnostics), Ras and matrix metalloproteinase-2 (MMP2) antibodies (Abcam; 1:1,000), a microplate reader (Thermo Fisher Scientific, Inc.), and RIPA lysis buffer (Beyotime Institute of Biotechnology) were used in the present study.

Cells and groups

The cell model of AD (MA-RASMCs) was established using angiotensin II (Ang II), with the cells divided into a control group, and Ang II group, an Ang II + mimic group and an Ang II + control group. After the MAPK/ERK pathway activator, anisomycin, was added, the cells were divided into Ang II + mimic and Ang II + mimic + anisomycin groups.

Establishment of MA-RASMCs

RASMCs were cultured in DMEM containing 10 % FBS (Gibco; Rockville, MD, USA) at 37 ^{o}C without hypoxia or oxygen enrichment. After stable growth, Ang II (1 μmol/μL) was added and the cells were cultured for 48 h. The cells were subsequently frozen prior to later use.

Transfection with miR-126 control and miR-126 mimic

The cells were cultured for 48 h. After that, they were further cultured in 6-well plates. The complete medium and miRNA-mimic/control were mixed evenly (200:1) and labelled as solution A, and DMEM and Lipofectamine were mixed evenly (50:1) at 4 ^{o}C and labelled as solution B. When the cells reached 40 % confluency after 24 h, 20 μL of AB mixture was added into the plate (1:1) and the cells were cultured for 12 h for transfections. Subsequently, the transfection mixture was discarded and replaced with DMEM.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol® and its purity determined. The RNA was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit. The RT-qPCR system consisted of 5 μL of SYBR Premix Ex Taq II (2×), 0.5 μL of PCR forward primer (10 μM), 0.5 μL of PCR reverse primer (10 μM), 1 μL of cDNA and 3 μL of dH_2O. After the reaction, the dissolution curve was analyzed. With U6 as the internal loading control, the expression levels of miR-126 were normalized with respect to the endogenous control. The data were analyzed using $2^{-\Delta\Delta Cq}$ method (Eq 1) and expressed as $2^{-\Delta\Delta Cq}$.

$$\Delta Cq = Cq_{miR\text{-}126} - Cq_{target\ miRNA} \quad \text{..... (1)}$$

The primer sequences used are shown in Table 1.

Determination of protein expression levels

Cell precipitate was collected and the protein was extracted using ultrasonication. The absorbance was measured at a wavelength of

560 nm. The total protein concentration was determined using bicinchoninic acid (BCA) assays and the concentration was adjusted to 6 μg/μL using loading buffer and radioimmunoprecipitation assay (RIPA) lysis buffer (1:1). After separation of the target band across a polyacrylamide gel, the target protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics, Basel, Switzerland) under a constant current. The proteins were sealed for 1 hour, followed by incubation with specific primary antibodies for 16 hours. After that, non-specific horse radish peroxidase-labeled secondary antibodies were applied. Finally, the image was developed and the results were calculated.

Table 1: Primer sequences

Gene		Primer sequence
miRNA-126	Forwards	GGGTCGTACCGTGAGTAAT
	Reverse	CAGTGCGTGTCGTGGAGT
U6	Forwards	CTCGCTTCGGCAGCACA
	Reverse	TGGTGTCGTGGAGTCG

Evaluation of cell proliferation and apoptosis

The MA-RASMCs were inoculated into a 96-well plate (3,000 cells/well), and absorbance was measured for 5 consecutive days. Some time was allowed for the cell to grow after which, CCK-8 reagent was added and incubated for another 2 h. The absorbance of each well was measured at a wavelength of 450 nm. Triplicate determinations were made for each well. Furthermore, for the apoptosis assay, cell precipitate was collected, and apoptosis determined.

Wound healing assays

The cells in each group were inoculated into 6-well plates and a scratch was made in a "cross" shape using the pipette. To avoid contamination, the plate was washed and the medium was replaced. The cells were cultured in the incubator for 36 h. Subsequently, crystal violet dye was added and incubated at 25 ^{o}C for 30 min. The cell migration distance was then examined under a light microscope.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for analysis of data. Univariate analyses and Student's *t*-tests were performed on the data. $P < 0.05$ indicated significant difference.

RESULTS

Expression levels of miR-126 after transfection with miR-126 control and miR-126 mimic

PCR results showed that the expression levels of miR-126 significantly reduced in the Ang II group when compared with that in the control group ($p < 0.05$), while it significantly rose in the Ang II + mimic group when compared with that in the Ang II + control group ($p < 0.01$), indicating that the expression of miR-126 decreased after the occurrence of AD (Figure 1).

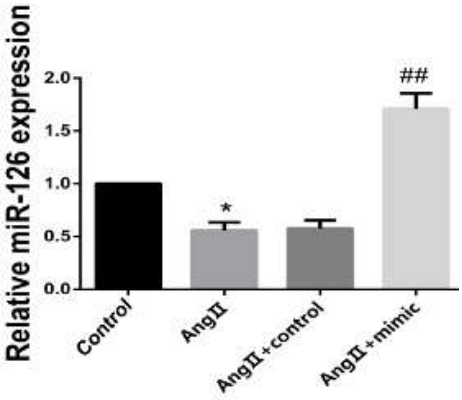

Figure 1: Expression levels of miR-126 in the control, Ang II, Ang II + control and Ang II + mimic groups. *$P < 0.05$ vs. the control group; $^{\#\#}p < 0.01$ vs Ang II + control group

Effect of miR-126 on the proliferation and apoptosis of MA-RASMCs

In the Ang II group, the proliferative rate began to increase at day 2 ($p < 0.05$), with a major rise at day 3 ($p < 0.01$), reaching its peak at day 4 ($p < 0.001$). The levels of apoptosis also decreased when compared with that of the control group. Compared with the Ang II and Ang II + control groups, the proliferative rate began to decrease at day 3 ($p < 0.05$), with an obvious decrease at day 4 ($p < 0.01$), while the rate of apoptosis was enhanced in Ang II + mimic group. These findings demonstrated that proliferation of smooth muscle cells was enhanced after the occurrence of AD, while overexpression of miR-126 inhibited and reversed the proliferation of RASMCs (Figure 2).

Effect of miR-126 on the migration of MA-RASMCs

Compared with control group, the intercellular distance was larger and the cell migratory ability was enhanced in Ang II group ($p < 0.05$). There was no significant difference in intercellular

distance between the Ang II group and the Ang II + control group. In contrast, the Ang II + mimic group exhibited reduced intercellular distance and weakened cell migratory ability compared to Ang II + control group ($p < 0.05$). These results demonstrated that overexpression of miR-126 suppressed the migratory potential of MA-RASMCs (Figure 3).

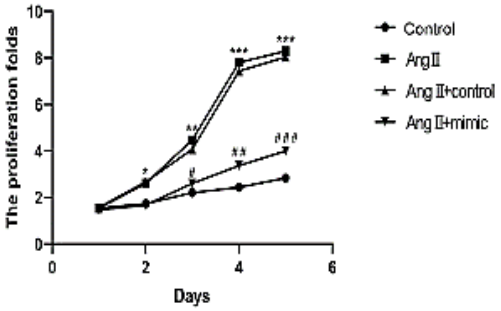

Figure 2: Effect of miR-126 on the proliferation and apoptosis of MA-RASMCs. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#}p < 0.001$ vs. the Ang II + control group

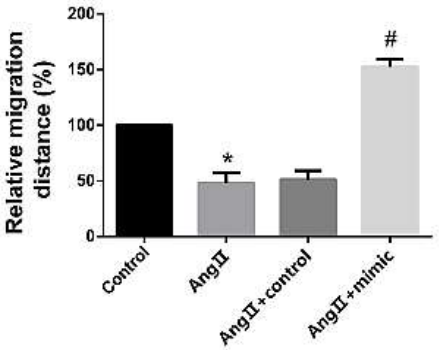

Figure 3: Effect of miR-126 on the migration of MA-RASMCs. The migratory ability of MA-RASMCs was significantly enhanced *$P < 0.05$ vs. the control group; $^{\#}p < 0.05$ vs. Ang II + control group

Expression levels of the MAPK/ERK pathway-associated proteins, Ras, MMP2 and TIMP1 in MA-RASMCs

The Ang II group exhibited significantly increased protein expression levels of Ras and MMP2, along with decreased protein expression levels of TIMP1. Additionally, they showed higher levels of p-MAPK/MAPK and p-ERK/ERK compared to the control group. There were no significant differences in the MAPK/ERK pathway associated-protein expression levels between Ang II and Ang II + control groups. The Ang II + mimic group had lower protein expression levels of both Ras and MMP2, higher protein expression levels of TIMP1 and also lower p-MAPK/MAPK and p-ERK/ERK levels than the Ang II + control group ($p < 0.05$). Thus, overexpression of miR-126 inhibited Ang II-induced AD, which is associated with MAPK/ERK (Figure 4).

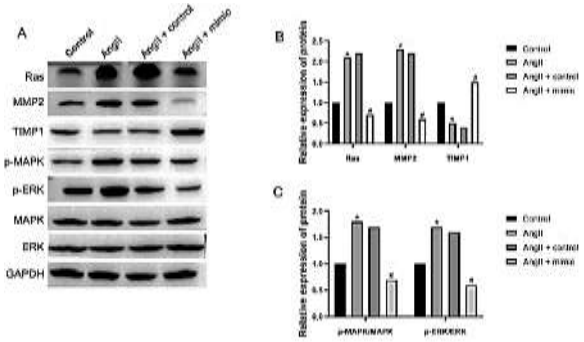

Figure 4: Changes in expression levels of Ras, MMP2, TIMP1 and MAPK/ERK pathway-associated proteins. (A & B) The Ang II group had higher protein expression levels of Ras, MMP2, p-MAPK/MAPK and p-ERK/ERK, as well as a lower protein expression levels of TIMP1 than the control group. (C) The Ang II + mimic group had lower protein expression levels of Ras and MMP2, as well as a higher protein expression level of TIMP1 and also lower p-MAPK/MAPK and p-ERK/ERK levels than Ang II + control group. *$P < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. Ang II + control group

Expression levels of Ras, MMP2 and TIMP1 after activation of the MAPK/ERK pathway

The Ang II + mimic + anisomycin group had significantly higher expression levels of Ras and MMP2, significantly lower expression levels of TIMP1, and also significantly higher p-MAPK/MAPK and p-ERK/ERK ratios than the Ang II + mimic group ($p < 0.01$), indicating that miR-126 inhibited AD formation through suppression of the MAPK/ERK signaling pathway (Figure 5).

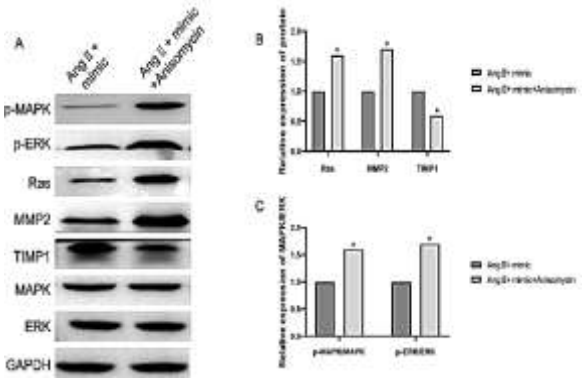

Figure 5: Effect of the MAPK/ERK pathway on protein expression levels of Ras, MMP2 and TIMP1. (A) Western blotting bands in the various treatment group. (B) Ang II + mimic + Anisomycin group had significantly higher expression levels of Ras and MMP2, and significantly lower expression levels of TIMP1 than the Ang II + mimic group ($p < 0.05$). (C) Anisomycin caused significant activation of MAPK/ERK pathway ($p < 0.05$)

DISCUSSION

Acute aortic dissection (AAD) is a life-threatening disease due to its acute onset, rapid progression and high mortality rate. The mortality rate of AAD increased from 2.49/100,000 to 2.78/100,000 people from 1990 to 2010, with men being more susceptible than women [14-16]. Despite the increase in the morbidity rate due to AAD, majority of AAD cases remain undiagnosed or misdiagnosed due to the complexity and variability of the disease, and the lack of specific clinical phenotypes [17]. Between 50 - 80 % of patients with AAD are not promptly diagnosed, and die within 3 days to 2 weeks [15]. Therefore, early and accurate diagnoses is of significant importance in preventing the development of AD, commence appropriate treatment, improve disease prognosis and reduce mortality.

The use of biomarkers such as D-dimer, cadherin, elastin, CD40L, myeloperoxidase, MMP and TIMP1 have been studied, but none have reached clinical stage [18]. In the present study, MMP2 was selected, as well as its endogenous inhibitor - TIMP1. In recent years, there has been increasing evidence that suggests that miRNAs play key roles in the physiological and pathological processes of cardiovascular development. MiRNAs are released from cells into body fluids, causing their levels to be detectable by RT-qPCR [19]. Furthermore, the expression profile of circulating miRNAs are altered in a variety of diseases, demonstrating that circulating miRNAs serve as biomarkers of the progression and recurrence of various diseases such as cancer, heart disease, abnormal pregnancy, diabetes, psychosis and various infectious diseases [11,20]. However, there are currently no reports on circulating miRNAs in AAD. Therefore, the regulatory function of miR-126 in AAD remains unknown.

In the present study, RASMCs were stimulated with Ang II for 48 h to establish the cell model of AD based on the mechanisms of action behind AD formation. To study the regulatory mechanism of miR-126 in AD, MA-RASMCs were transfected with miR-126 mimic and miR-126 control *in vitro*. The transfection efficiency was first determined using RT-qPCR. Results showed that the expression levels of miR-126 were low in Ang II group, and significantly rose in Ang II + mimic group compared to Ang II + control group. During the occurrence and development of AD, smooth muscle cells develop a strong proliferative and migratory ability. In the present study, the results of CCK-8 proliferation assays and wound healing assays revealed that in Ang II group, the proliferative rate began to increase at day 2 and reached a peak at day 4. The intercellular distance was increased in Ang II group when compared with control group. Compared with Ang II + control group, proliferative rate began to decrease in Ang II + mimic group at day 3. These results suggest that overexpression of miR-126 may reverse the proliferation and migration of MA-RASMCs, with clinical significance in the treatment of AD.

In terms of the detailed regulatory mechanisms of miR-126 in the disease, numerous reports have shown that MAPK/ERK is involved in various cardiovascular diseases. In the present study, the expression levels of Ras, MMP2, TIMP1 and MAPK/ERK pathway-associated proteins in cells were determined using western blotting. It was found that Ang II group had significantly higher protein expression levels of Ras and MMP2, significantly lower protein expression levels of TIMP1, and also significantly higher p-MAPK/MAPK and p-ERK/ERK ratios, than control group.

There were no significant differences in the aforementioned protein expression levels between Ang II and Ang II + control groups. Ang II + mimic group had lower protein expression levels of Ras and MMP2, higher protein expression levels of TIMP1, and also significantly lower p-MAPK/MAPK and p-ERK/ERK levels than Ang II + control group. Ras and MMP2 promotes the formation and development of AD, while TIMP1 inhibits it.

Results from the present study indicate that miR-126 suppressed the formation of AD, which may be associated with the MAPK/ERK signaling pathway. To further study whether MAPK/ERK is involved in the regulatory process, the MAPK/ERK pathway activator, anisomycin, was added to the cells and biomarker proteins for AD were determined. The results showed that Ang II + mimic + anisomycin group had significantly higher expression levels of Ras and MMP2, significantly lower expression levels of TIMP1, and significantly higher p-MAPK/MAPK and p-ERK/ERK levels than the Ang II + mimic group, further confirming that MAPK/ERK may affect the occurrence and development of AD.

CONCLUSION

MiR-126 inhibits the formation of AD by suppressing the activity of MAPK/ERK signaling pathway. However, the influence of miR-126 on AD was not determined *in vivo* in this study and therefore, its therapeutic effect on AD should be studied.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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