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Original Research Article

MiR-22 inhibits angiotensin II-induced aortic dissection and protects aortic vessel wall in mice by targeting MAPK-14

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Abstract

Purpose: *To study the effect of miR-22 on angiotensin II-induced aortic dissection in mice, and its protective effect on aortic vessel wall, as well the involvement of MAPK-14 in these processes.*
Methods: *A mouse aortic dissection model was established via subcutaneous implantation of angiotensin II (1 µg/kg/min) micropump in the dorsal region. The mice (n = 30) were assigned in equal numbers to 5 groups (n = 6). All injections were given via the tail vein. The miR-22 expressions in aortas of mice in each group were determined with quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Western blot assay was used to determine the expressions of MAPK-14 protein, while H&E staining was used to measure the ratio of aortic thickness-to-diameter, and contents of collagen and elastic fibers.*
Results: *The expression of miR-22 in aorta of mice in miR-22 overexpression group was significantly higher than that in overexpression control group, but significantly lower in miR-22 inhibition mouse than in inhibition control mouse ($p < 0.05$). There was significantly lower protein expression of MAPK-14 in mice aorta in miR-22 overexpression mice than in overexpression control mice, but significantly up-regulated in miR-22 inhibition mice, relative to that in inhibition control mice ($p < 0.05$). In the miR-22 overexpression mice, the ratio of membrane thickness-to-diameter was higher than the corresponding value in miR-22 inhibition mice. There were significantly higher contents of aortic elastic and collagen fibers in miR-22 overexpression mice than in overexpression control and miR-22 inhibition groups ($p < 0.05$).*
Conclusion: *Overexpression of miR-22 inhibits the up-regulation of expression of its target gene mapk14, increases thickness of aortic media and aortic elasticity in mice, and increase the content of collagen fibers, thereby exerting protective effect on aortic wall structure.*

Keywords: *miR-22, MAPK-14, Angiotensin II, Aortic dissection, Vessel wall*

INTRODUCTION

Aortic dissection is caused by the tearing of the aortic intima due to various factors. The blood enters the aortic media, resulting in its isolation from intima, and formation of true and false lumens of the aortic wall. The false lumen extends along the longitudinal axis of the aorta, eventually leading to aortic rupture [1]. The clinical manifestations in patients are sudden chest and back pain, and a large difference in blood pressure between the upper and lower limbs. The disease is life-threatening, and it is characterized by rapid progression and high mortality rate (as high as 50 – 68 %). Epidemiological studies have shown that there are about 7 - 9 cases of acute aortic dissection per 100,000 people every year, and in recent years, with increased prevalence of hypertension, its prevalence increased by 5 % every year [2]. In clinical practice, the early diagnosis rate of aortic dissection is very low due to the rapid onset of the disease. Thus, some patients die of dissection rupture before diagnosis or treatment. Therefore, an understanding of the pathogenesis of aortic dissection and identification of biomarkers and intervention indicators for evaluating the development of aortic dissection, are crucial for timely diagnosis-cum-prevention of the disease. The pathological processes associated with aortic dissection are relatively complex. More and more studies have shown that abnormal gene expression in patients with aortic dissection is the genetic basis of the disease.

The miRNAs belong to a class of small RNAs that do not code for proteins, but which participate widely in regulating various cellular processes through regulation of target gene expression levels after mRNA synthesis [3]. It has been shown that miRNAs are involved in regulation of vascular reconditioning by regulating the proliferative and migratory potential of vascular smooth muscle cells [4]. The present research was focused on identifying the influence of miR-22 on occurrence of angiotensin II-induced aortic dissection in mice and protection of aortic vascular wall, and the underlying mechanism.

EXPERIMENTAL

Reagents and instruments

Angiotensin II was purchased from Sigma, USA; miR-22 overexpression and inhibition vectors were purchased from Shanghai Jima Pharmaceutical Technology Co. Ltd, while PBS buffer, antigen retrieval solution and antibody diluent were bought from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd. TRIzol extraction reagent was purchased from Invitrogen, USA. Reverse transcription kit was obtained from Takara, Japan. The MAPK-14 antibody was purchased from Beijing Boasen Biological Co. Ltd.

Sterile ultra clean workbench was bought from Suzhou Purification Equipment Company. Inverted microscope was product of Leica, Germany, and PCR instrument was purchased from Takara Company in Japan. Micropipettes were purchased from Eppendorf Research, USA. Vertical electrophoresis apparatus was purchased from Bio-Rad, USA. Gel imaging analysis system was bought from Syngene g:box, USA. Automatic paraffin embedding machine was purchased from Leica, Germany, while low-temperature high-speed centrifuge was obtained from Hitachi-cf16rx, Japan.

Establishment of experimental animal models and grouping

Thirty male mice aged 3 weeks and weighing 19 – 22 g, were selected and housed in the animal laboratory of Zibo Central Hospital, China at temperature range of 22 - 24 ℃ and 50 – 60 % relative humidity. The mice were fed with normal diet. β-Aminopropionitrile was added to the drinking water given the animals at a dose of 1 g/kg/day. After feeding for 7 weeks, the mice were locally anesthetized, and angiotensin II micropump was implanted subcutaneously in the back of each mouse for 24 h. The micropump delivered angiotensin II at a rate of 1 µg/kg/min. The experiment lasted for 29 days. At the end of the experiment, the surviving mice were injected with high-dose phenobarbital anesthesia prior to sacrifice. The aortic vessels at the bifurcation of abdominal aorta and iliac artery were isolated, and their morphology was examined closely. The lesion site was subjected to fixation in formalin (10 %) and paraffin-embedding. When the mice were fed to age of 1½ months, they were assigned to miR-22 overexpression group and miR-22 inhibition group. The miR-22 overexpression (miR-22 agomir) and inhibition vectors (miR-22 antagomir) were slowly injected into the tail vein with 1-mL syringe, while mice in the model group received equivalent volume of normal saline injection via the same route. Over-expression control group (agomir NC) and inhibition control groups (antagomir NC) were set up, with 6 mice in each group.

Determination of parameters

The expressions of miR-22 in the aortas of mice in each group were determined using RT-qPCR. Total RNA was extracted from 100 g of aortic tissue of mice in each group, using lqiazol lysing reagent, and its purity and concentration were measured using spectrophotometry. The RNA extract was used as the template to reverse-transcribe and synthesize cDNA. The reverse transcription reaction was carried out according to the instructions on the reverse transcription kit. β-Actin was used as reference, and PCR was done viz: pre-denaturation at 94 °C for 15 s, 55 °C for 30 sec, and 70 °C for 30 sec. Relative miR-22 mRNA level was calculated with $2^{-\Delta\Delta Ct}$ formula.

Immunoblot assay was used to determine protein levels of MAPK-14 in the aortas of mice. The extraction of total aortic tissue total protein was done using 300 µL of RIPA buffer, and the protein content was measured using BCA protein assay. Then, addition of 10 µL of loading buffer to each extracted protein sample was done, followed by denaturation at 100 °C for 5min, and storage at -80 °C prior to use. Equal amounts of protein samples were subjected to 12 % SDS-PAGE, followed by transfer to PVDF membrane. After blocking the membranes for 1 h at room temperature, the membranes were incubated at 4 °C overnight with of MAPK-14 polyclonal antibody (1:500 dilution). Thereafter, the membranes were rinsed three times with TBST for 10 min, followed by incubation with the corresponding secondary antibody for 1 h at room temperature on a shaker, after which the membrane was again washed three times with TBST for 10 min. ImageJ software was used for band analysis.

The ratios of aortic media thickness-to-diameter of mice in each group were measured with H&E staining. The aortic tissue of mouse in each group was sectioned, fixed with 10 % formalin and refrigerated overnight at 4 °C. The aortic tissue was rinsed with running water for 24 h, dehydrated with different concentrations of ethanol, and cleared twice in xylene, each for 10 min. Then, the tissue was paraffin-embedded and sectioned, followed by H&E staining in line with standard procedures, and microscopic examination. Then, the sections were sealed in neutral resin glue. Image-Pro Plus6.0 software was used to measure the aortic diameter and media thickness of mouse in each group, and the ratio of the two was calculated.

The collagen fiber contents of the aortas of mice in each group were determined using Masson staining. Paraffin aortic sections were dried, immersed in Weigert iron hematoxylin solution, differentiated with 1 % hydrochloric acid alcohol solution, stained with acid Fuchsin Ponceau Orange G solution, moistened with 0.2 % acetic acid, differentiated with phosphomolybdic acid solution, moistened with 0.2 % acetic acid, soaked in aniline blue, moistened with 0.2 % acetic acid, moistened with 95 % ethanol, dehydrated, cleared, and resin-sealed. The collagen fibers were examined under the microscope where they appeared blue in color.

The aortic elastic fibers of mice in each group were stained. The paraffin sections were dried, dewaxed with xylene and ethanol, impregnated with Weigert elastic fiber staining solution, differentiated with hydrochloric acid ethanol, impregnated with van Gieson staining solution, differentiated with 95 % ethanol, dehydrated with 100 %, sealed with transparent and resin glue, and on examination under the microscope, the elastic fiber tail was black or dark blue in color.

Statistical analysis

Data analysis was done with SPSS 20.0 software package, and all data are presented as mean ± standard deviation (SD). Two-group comparison of mean values was done with independent sample *t*-test, while ANOVA was used to compare multiple groups. Values of $p < 0.05$ indicated significance.

RESULTS

Impact of miR-22 transfection on miR-22 expression in mouse aorta

The results of real-time quantitative PCR demonstrated significantly higher miR-22 expression in aorta of mouse in miR-22 overexpression mice than in overexpression control group, but it was significantly lower in miR-22 inhibition group than in inhibition control group ($p < 0.05$; Figure 1).

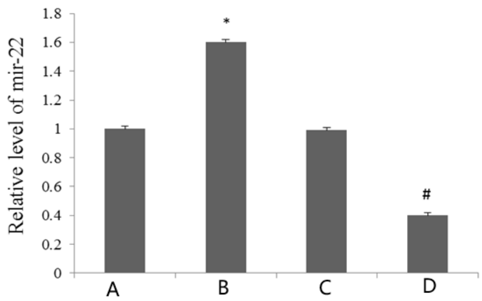

Figure 1: Effect of miR-22 transfection on miR-22 in mouse aorta. *$P < 0.05$; vs. overexpression control; #$p < 0.05$, vs. inhibition control. A: Overexpression control, B: miR-22 overexpression, C: Inhibition control, and D: miR-22 inhibition

Influence of miR-22 transfection on MAPK14 protein level in mouse aorta

Western blot assay showed that the protein expression of MAPK-14 in mouse aorta in miR-22 overexpression group was significantly lower than that in overexpression control group, but it was significantly higher in miR-22 inhibition group than in inhibition control group ($p < 0.05$), as shown in Table 1 and Figure 2.

Table 1: Effect of miR-22 transfection on MAPK-14 protein expression in mouse aorta (n = 6)

Group	MAPK-14 protein
Overexpression control	0.75±0.06
miR-22 overexpression	0.41±0.04*
Inhibition control	0.76±0.07
miR-22 inhibition	$1.05 \pm 0.08^{\#}$

*$P < 0.05$, vs. overexpression control; $^{\#}p < 0.05$, vs. inhibition control

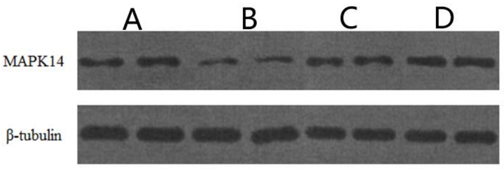

Figure 2: Effect of miR-22 transfection on Mapk14 protein expression in mouse aorta. A: Overexpression control, B: miR-22 overexpression, C: Inhibition control, and D: miR-22 inhibition

Effect of miR-22 transfection on the ratio of aortic media thickness to diameter

The ratio of aortic media thickness-to-diameter in miR-22 overexpression group was significantly higher than that in overexpression control group, but it was significantly lower in miR-22 inhibition group than in inhibition control group ($p < 0.05$). However, the ratio of aortic media thickness-to-diameter was higher in miR-22 overexpression group than in miR-22 inhibition group ($p < 0.05$; Table 2).

Table 2: Effect of miR-22 transfection on the ratio of aortic media thickness to diameter in mice (n = 6)

Group	Media thickness	Aortic lumen diameter	Ratio
Model	73.65 ± 4.48	2.53 ± 0.19	29.11±2.36
Overexpression control	81.12 ± 3.04	3.13 ± 0.20	25.92±1.52
miR-22 overexpression	118.83 ± 5.65	1.56 ± 0.12	76.17±2.71*
Inhibition control	94.65 ± 4.11	1.55 ± 0.11	61.06±3.74
miR-22 inhibition	65.60 ± 3.63	3.75 ± 0.21	$17.49 \pm 1.73^{\times\Delta}$

*$P < 0.05$, vs. overexpression control; $^{\times}p < 0.05$, vs. inhibition control; $^{\Delta}p < 0.05$, vs. miR-22 overexpression

Effect of transfection of miR-22 on elastic fibers and collagen fibers

The contents of aortic elastic fibers and collagen fibers were significantly raised in miR-22 overexpression group, relative to overexpression control and miR-22 inhibition groups, but they were significantly lower in miR-22 inhibition mice than in inhibition control mice ($p < 0.05$; Table 3).

Table 3: Effect of transfection of miR-22 on elastic fibers and collagen fibers of mouse aorta

Group	Elastic fiber content	Collagen fiber content
Model	0.98 ± 0.05	0.95 ± 0.07
Overexpression control	1.00 ± 0.08	1.01 ± 0.04
miR-22 overexpression	1.85 ± 0.12*	2.18 ± 0.13*
Inhibition control	1.01 ± 0.09	0.94 ± 0.11
miR-22 inhibition	$0.47 \pm 0.05^{\times\Delta}$	$0.50 \pm 0.07^{\times\Delta}$

*$P < 0.05$, vs. overexpression control; $^{\times}p < 0.05$, vs. inhibition control; $^{\Delta}p < 0.05$, vs. mir-22 overexpression

DISCUSSION

In the cytoplasm, the precursor miRNA is acted upon by dicer enzyme and extended into 20 - 25 nucleotide double-stranded RNA which then differentiates into mature, single-stranded miRNA bodies. The mature body forms an RNA-related silencing complex with RNA and protein, and then functions through its 5' terminal seed sequence, thereby inhibiting the gene translation process. In particular, miR-22 has been confirmed to play an important role in the regulation of vascular smooth muscle cell phenotype [5]. Research on animal model of aortic aneurism has shown that miR-22 is significantly up-regulated in the abdominal aorta [6]. In addition, a study has shown the likely involvement of miR-22 down-regulation in the pathogenesis of aortic dissection through suppression of vascular smooth muscle proliferation [7]. In this study, results from real-time quantitative PCR showed that miR-22 was significantly up-regulated in mouse aorta in miR-22 overexpression group, relative to that in overexpression control mice, and aortic miR-22 level was significantly lower in miR-22 inhibition mice than in inhibition control mice. These results confirm high miR-22 expression level in aortic dissection.

Previous research found that *mapk14* is a potential gene targeted by miR-22 in the regulation of apoptosis of human aortic smooth muscle cells [8]. Immunoblot assay data showed that mouse aortic MAPK-14 protein in miR-22 overexpression group was significantly down-regulated, relative to that in overexpression control mice, but it was significantly higher in miR-22 inhibition mice than in inhibition control mice. Thus, down-regulation of miR-22 may significantly enhance the expression of MAPK-14 protein in human aortic smooth muscle cells, thereby participating in aortic vascular remodeling. Cystic degeneration of the aortic media is an important part of aortic vascular remodeling [9-11]. The greater the ratio of thickness of the media to diameter of the tube, the greater the extent to which vascular remodeling is dominated by increase in thickness of the media. Histopathologically, the degeneration of aortic media was characterized by reduction of population of vascular smooth muscle cells, apoptosis and necrosis, and absence of elastic fibers [12]. Elastic fiber is the crucial material that enhances the elasticity and compliance of aortic wall, and elastic fiber dysfunction is one of the early manifestations of aortic dissection. There was a reduction in collagen fibers in the adventitia of aortic dissection tissue. The results of this study revealed that overexpression of miR-22 increased the thickness of aortic media.

CONCLUSION

Overexpression of miR-22 inhibits the up-regulation of expression of its target gene *mapk14*, increases the thickness of aortic media and aortic elasticity in mice, and raise the content of collagen fibers, thereby exerting a protective effect on aortic wall structure.

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

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Liu Bing designed the study, supervised the data collection, and analyzed the data. Xinming Yu interpreted the data and prepared the manuscript for publication. Zonggang Zhao, Lili Tao and Xiao Shun supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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