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# Effect of miR-21 on rat thoracic aortic aneurysm model by regulating the expressions of MMP-2 and MMP-9

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**Abstract. – OBJECTIVE: To explore the mechanism underlying micro ribonucleic acid (miR)-21 in the invasion of rat aortic aneurysm cells in vitro by regulating matrix metalloproteinase (MMP)-2 and MMP-9.**

**MATERIALS AND METHODS: Rats were randomly divided into three groups: control group, model group, and miR-21 group. Real Time fluorescence quantitative Polymerase Chain Reaction (qRT-PCR) was adopted to detect the levels of miR-21 in each group of cells, transwell assay was performed to measure the effect of miR-21 on the invasion of aortic aneurysm cells. Western blotting was used to examine the expression of PTEN, which is the predicted target of miR-21 in aortic aneurysm cells, as well as the expressions of invasion-related proteases, MMP-2 and MMP-9.**

**RESULTS: The expression level of miR-21 in thoracic aortic aneurysm cells in model group was significantly higher than that in normal group ($p<0.05$), and that in miR-21 group was remarkably higher than that in model group ($p<0.05$). MiR-21 group had evidently more aortic aneurysm cells and stronger cell invasion ability than normal group and model group ($p<0.05$). In addition, the expression level of PTEN in model group was significantly higher than that in normal group ($p<0.05$), while that in miR-21 group notably declined compared to model group, ($p<0.05$). Compared with normal group and model group, the expressions of MMP-2 and MMP-9 were markedly increased in miR-21 group ($p<0.05$).**

**CONCLUSIONS: In aortic aneurysm cells of rats, miR-21 could suppress the expression of PTEN and activate MMP-2 and MMP-9 signals to promote the proliferation and migration of aortic aneurysm cells.**

*Key Words:*
Aortic aneurysm, PTEN, MMP-2, MMP-9, MiR-21.

## Introduction

There are about 100,000 cases of thoracic aortic aneurysm induced by various causes in China each year. A thoracic aortic aneurysm develops slowly and can occur in multiple parts of the aorta, while the death rate of patients with aneurysm rupture is very high[1,2]. A thoracic aortic aneurysm is mainly pathologically manifested as endothelial cell injury and denudation, apoptosis of smooth muscle cells in the media, and pathological remodeling of the extracellular matrix accompanied by infiltration of inflammatory cells[3,4]. Investigations[5,6] on the aneurysm tissues manifested that the infiltration degree of inflammatory cells has a positive correlation with the aortic matrix injury. Inflammatory cell infiltration is also closely associated with collagen and elastic fiber injury and is also involved in the whole process of thoracic aortic aneurysm formation. The increased inflammatory infiltration and secretion of matrix metalloproteinases (MMPs) exert crucial effects on the formation of thoracic aortic aneurysm.

Micro-ribonucleic acids (miRNAs) are a class of non-coding single-stranded small molecule. Bartel[7] reported that miRNAs could inhibit the translation process of target genes through complete or partial base-pairing to the 3' untranslated region of target mRNA, regulating gene expression. Currently, several reports[8,9] have shown that miRNAs are closely related to the occurrence and development of a thoracic aortic aneurysm. It has been found[10,11] that the abnormally increased expression of miR-21 was detected in tumor cells of the digestive system and reproductive system. To better research the roles of miR-21 in the occurrence and progression of thoracic aortic aneurysm, this study was designed to investigate the effect of miR-21 on the invasion of thoracic aortic aneurysm cells, as well as its underlying mechanism.

## Materials and Methods

### Experimental Reagents and Materials

The main reagents employed in this study were as follows: Dulbecco's Modified Eagle's Medium



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with nutrient mixture F-12 (DMEM/F12 medium, Hyclone, South Logan, UT, USA), 0.25% trypsin (Beyotime, Shanghai, China), fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), DMEM-high glucose (Hyclone, South Logan, UT, USA), pGCMV-rno-miR-21-up lentivirus expression vector, the Hairpin-It$^{TM}$ miRNA Real Time-Polymerase Chain Reaction (RT-PCR) quantitation kit (Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-rat phosphatase and tensin homolog deleted on chromosome ten (PTEN) monoclonal antibody, rabbit anti-rat MMP polyclonal antibody and rabbit anti-rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Abcam, Cambridge, MA, USA).

### Grouping of Rats

A total of 30 female rats aged 3 months old and weighing 0.16-0.19 kg were purchased from the Animal Research Center of Shanxi Medical University. This investigation was approved by the Animal Ethics Committee of People's Hospital of Zhengzhou University Animal Center. All rats were kept under the conditions of 25°C, 45% humidity and a 12 h/12 h light/dark cycle, and had free access to food and water. After the rats were fed for adaption for 1 day, they were randomly allocated into three groups, namely, normal group (normal feeding with no treatment, n=10), model group (the rat model of thoracic aortic aneurysm, n=10), and miR-21 group (the rat model of thoracic aortic aneurysm infected with miR-21 lentiviruses, n=10).

### Establishment of the Rat Model of Thoracic Aortic Aneurysm

The rats anesthetized were intraperitoneally injected with 45 mg 2.5% pentobarbital and subjected to endotracheal intubation after tracheotomy. Then, the ventilator was fixed and connected with the respiratory quotient of 1:1.5, respiratory rate of 95 times/min, and the pressure support of 0.01 Mpa. After that, thoracotomy was performed between the 5$^{th}$ and 6$^{th}$ rib on the left side of the rat sternum to expose about 1 cm aorta. Subsequently, the aorta was soaked with 1.5 U/μL porcine pancreatic elastases, and the adventitia was covered with cotton yarn for 15-20 min. After the aorta changed and expanded by 1.5 times, the cotton yarn was removed and the thoracic cavity of the rat was rinsed with normal saline. After the ventilator was adjusted to promote the left lung recruitment, the thoracic drainage tube thread was indwelled and the thoracic cavity was sutured layer by layer. Thereafter, the thoracic drainage tube was applied to fully ventilate the air, withdraw the effusion, remove the ventilator, and thoroughly clean the respiratory secretions. Finally, Prolene slide suture was utilized to close the trachea and skin. The standard for tumor formation is that the internal diameter of the tumor must be 50% larger than that of the normal blood vessel.

### Culture of Rat Thoracic Aortic Aneurysm Cells

The rat thoracic aortic cell line collected from normal group and the rat thoracic aortic aneurysm cell line collected from model group were cultured in DMEM/F12 medium and DMEM-high glucose containing 10% FBS, respectively. The medium was replaced every two days until the cell fusion reached 85%, and finally, the cell suspension was digested with trypsin.

### Infection of Aortic Aneurysm Cells with MiR-21 Lentiviruses

Rat aortic aneurysm cells in the growth phase were collected and inoculated on 6-well plates at a density of $3\times10^4$/mL. Then, the cells were placed in an incubator with 5% $CO_2$ for 2 days of incubation at 37°C until the cell fusion reached 30-50%. Virus infection solution contained 2 μL polybrene, 40 μL miR-21 lentiviruses, 20 μL negative control lentiviruses and 4 mL enhanced infection solution. The preparation process was as follows:

1) Rat thoracic aortic aneurysm cells were first infected with miR-21 lentiviruses and negative control lentiviral vectors for 12 h.
2) The culture medium was replaced with DMEM-high glucose containing 10% serum for 3 days.
3) The infection rate of thoracic aortic aneurysm cells exceeding 85% indicated the successful expression.

### Detection of the MiR-21 Expression in Rat Thoracic Aortic Aneurysm Using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells in each group before and after infection were collected, and 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) was added to extract RNAs, which were then reversely transcribed into complementary deoxyribonucleic acids (cDNAs). Subsequently, PCR system (40 μL) was quantified using the probe method: 4 μL cDNAs, 0.8 μL miR-21 primer sets, 20 μL 2×qRT-PCR main mixture, 0.4 μL Taq DNA polymerases, 7.2 μL hydrogen per-

oxide, and 0.4 μL miR-21 probes were added in sequence. Amplification conditions were as follows: pre-denaturation at 95°C for 3 min, followed by 40 cycles of pre-denaturation at 95°C for 12 s, and pre-denaturation at 65°C for 40 s. At least 3 repeated wells were set in each group. Primer sequences used in this study were as follows: microRNA-21, F: 5'-GCCTCGTAGGCATCAACGACTG-3', R: 5'-GAGTCCTGCGTGTGGCAGCTCG-3'; MKRN3, F: 5'-AGCAGCGGCATTTGGACAA-3', R: 5'-CGTGCGAATAGCGACAGTTCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

### Detection of the Invasion Ability of Rat Aortic Aneurysm Cells Via Invasion Experiment (Transwell Assay)

The upper surface of the microporous membrane was evenly smeared with 25 μL Matrigel (Sigma-Aldrich, St. Louis, MO, USA) basement membranes, and then, the microporous membrane was incubated in an incubator at 37°C for 31 min until solidification. Subsequently, cells were collected, counted, and paved to the upper chamber**,** while the lower chamber was added with the complete medium containing 10% serum. After 24 h of culture, cells in the upper layer of the microporous membrane were wiped off, while those in the lower layer were stained. Thereafter, the microporous membrane was observed, and the cells in 5 non-overlapping fields of view were randomly counted and averaged.

### Detection of the Protein Expression in Rat Aortic Aneurysm Cells via Western Blotting

The cells in each group were lysed at a constant temperature of 5°C and centrifuged at 12,000 rpm/min. The BCA (bicinchoninic acid) method was performed to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the Tris-Buffered Saline and Tween (TBST) solution. Enhanced chemiluminescence (ECL) was used to expose the protein bands on the membrane.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS, Chicago, IL, USA) were adopted for data analysis. Differences between the two groups of test samples were detected using the two-sample *t*-test. Comparison between multiple groups was done using the One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was adopted for plotting.

## Results

### Expression Level of MiR-21 in the Three Groups of Rats

QRT-PCR was carried out to detect miR-21 expression in aortic cells in the three groups of rats. The results manifested that both the expression levels of miR-21 in rat thoracic aortic aneurysm cells in model group and miR-21 group were significantly higher than that normal group ($p<0.05$) (Figures 1 and 2).

### Effect of MiR-21 on the Invasion of Rat Thoracic Aortic Aneurysm Cells

According to the results of transwell assay, the number of invasive rat aortic aneurysm cells in normal group, model group, and miR-21 group were 14.1 ± 6.1, 49.6 ± 7.2, and 103.8 ± 7.1, respectively. Besides, the number of aortic aneurysm cells in miR-21 group was markedly larger than that in normal group and model group, which displayed statistically significant differences ($p<0.05$). Furthermore, the invasion ability of the cells in miR-21 group was also remarkably stronger than in the other two groups ($p<0.05$) (Figure 3).

### Effect of MiR-21 on the Expression of PTEN in Rat Aortic Aneurysm Cells

The expression level of PTEN in miR-21 group exhibited a significant decrease ($p<0.05$). However, the expression of PTEN in model group was evidently decreased compared to that in normal group ($p<0.05$) (Figures 4 and 5).

### Effects of MiR-21 on the Expressions of MMP-2 and MMP-9 in Rat Aortic Aneurysm Cells

The expression levels of MMP-2 and MMP-9 in each group before and after infection were detected by Western blotting. It was found that the expressions of MMP-2 and MMP-9 in miR-21 group were prominently higher than those in normal group and model group, with statistically

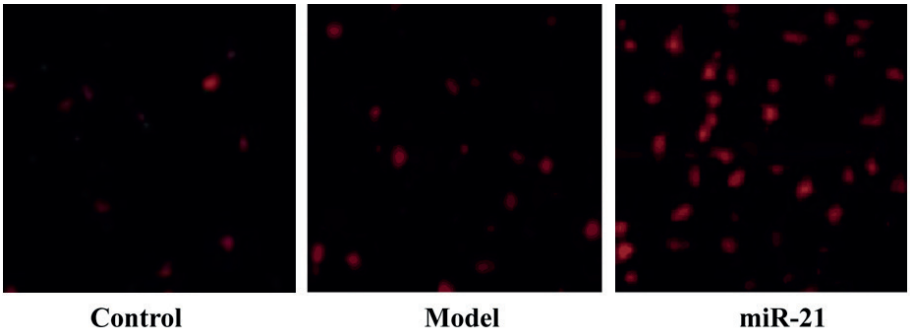


**Figure 1.** MiR-21 expression level in each group of rats detected *via* qRT-PCR (magnification × 40).

significant differences ($p<0.05$). Our data indicated that miR-21 had positive correlations with the expression levels of MMP-2 and MMP-9 in tumor cells (Figures 6 and 7).

## Discussion

The malignant degree of aortic aneurysm is very high. Therefore, even surgery combined with chemotherapy cannot achieve the desired therapeutic effect. Gene therapy has always been a hotspot in the research on the aortic aneurysm treatment. Finding effective therapeutic targets is crucial for curing aortic aneurysm. In this work, we found that the expression level of miR-21 in aortic aneurysm cells in miR-21 group was markedly higher than that in normal group and model group. Several studies have indicated that miRNAs might serve as oncogenes or cancer suppressor genes by regulating the expression of PTEN in the occurrence and development of tumors. For instance, Yang et al[12] discovered that miR-21 reduced the expression of PTEN in tumor cells to promote the proliferation of cancer cells. Increasing evidence[13,14] has demonstrated that various

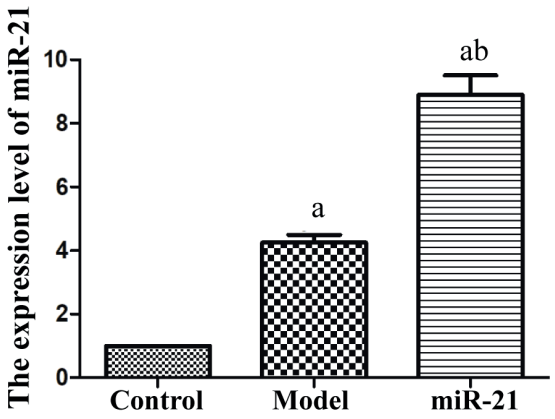


**Figure 2.** Relative expression level of miR-21 in each group of rats. [a]$p<0.05$ *vs*. model group, and [b]$p<0.05$ *vs*. model group.

biological effects of tumors are closely related to miRNAs. Zhang et al[15] reported that miR-21 could stimulate the invasion and proliferation of gastric cancer cells. Bao et al[16] also found that miR-21 inhibited the PTEN expression and activated the AKT/ERK pathway. However, few researches were reported on the role of miR-21 in the regulation of invasion and metastasis of aortic aneurysm cells, and the specific molecular mechanism un-

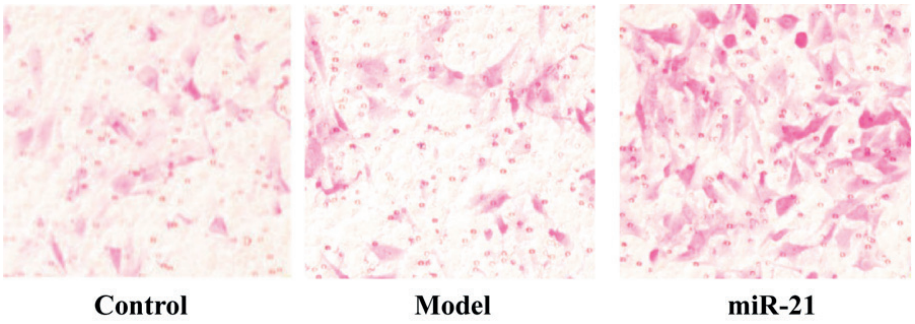


**Figure 3.** Changes in the cell invasion ability in each group detected *via* transwell assay (magnification ×200).

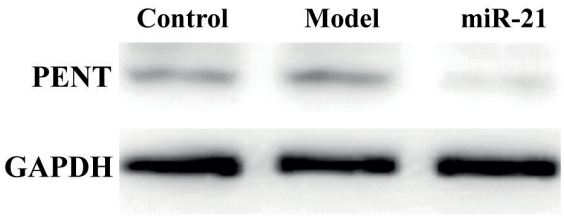


**Figure 4.** PTEN protein expression levels of the three groups of rats.

derlying is not very clear. Ziyan et al[17] showed that the expression of miR-21 was negatively related to the expression of tumor suppressor gene RECK in aortic aneurysm tissues. However, Vanas et al[18] demonstrated that miR-21 was able to suppress the expression of Sprouty2 protein in tumor cells, thus exerting a positive effect on improving the sensitivity of tumor cells to carboplatin therapy. Our results showed that the mRNA expression of miR-21 in rat thoracic aortic aneurysm cells was notably higher than that in normal thoracic aortic cells. Therefore, it could be speculated that miR-21 has close correlations with the occurrence and progression of aortic aneurysm.

In this study, the difference of miR-21expression between the rat thoracic aortic aneurysm cell line and the normal aortic cell line was examined. MiR-21 exhibited a considerable higher expression level in rat thoracic aortic aneurysm cell lines, but a lower expression level in the normal aortic cell line, which is consistent with the results of Vanas et al[18]. Moreover, the role of miR-21 in the aortic aneurysm cell line was confirmed[19,20]. According to the detection results of qRT-PCR, the expression of miR-21 was prominently increased in the rat thoracic aortic aneurysm cell line compared with that in the normal aortic cell line. To explore the effect of miR-21 on the invasion of the rat thoracic aortic aneurysm cell line, miR-21 lentiviral vectors were constructed and successfully infected into the thoracic aortic aneurysm cells. QRT-PCR detection verified that the expression of miR-21 in rat thoracic aortic aneurysm cells infected was significantly up-regulated. In the present report, the transwell assay was further performed to speculate the role of miR-21 in the invasion of rat thoracic aortic aneurysm cells and it was found that miR-21 was capable of significantly enhancing the invasion ability of rat thoracic aortic aneurysm cells.

Furthermore, this research also showed that compared with the normal thoracic cell line, the expression of PTENwas evidently decreased in the rat thoracic aortic aneurysm cell line. Besides, PTEN also showed a negative relation to the miR-21 expression, which is similar to the results of other researches. Tumor suppressor gene PTEN has long been reported to suppress the invasion of tumor cells by regulating the expression of invasion-related proteins[21]. In recent years, there has also been increasing research on the role of PTEN in aortic aneurysm[22,23]. For example, a study from Pedchenko et al[24] indicated that miR-21 negatively regulated the expression of PTEN in rat thoracic aortic aneurysm cell lines[25,26]. Hence, it can be inferred that miR-21 could inhibit the expression of PTEN to promote the invasion of the rat thoracic aortic aneurysm cell line.

According to this study, miR-21 group had significantly higher protein expressions of MMP-2 and MMP-9 than normal group and model group. Hence, the PTEN expression had negative associations with the expressions of MMP-2 and MMP-9 in rat thoracic aortic aneurysm cells. Besides, Western blotting showed that the expressions of

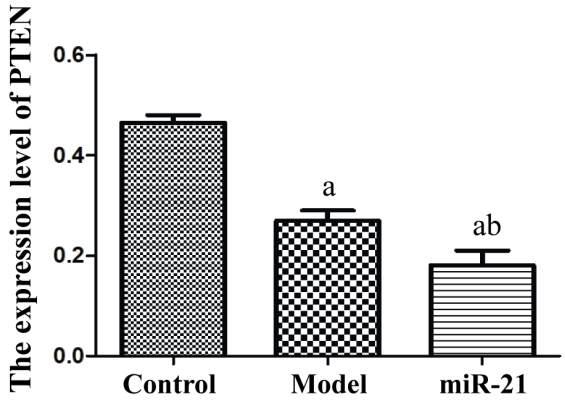


**Figure 5.** Relative expression levels of PTEN in the three groups of rats. [a]$p<0.05$ *vs*. model group, and [b]$p<0.05$ *vs*. model group.

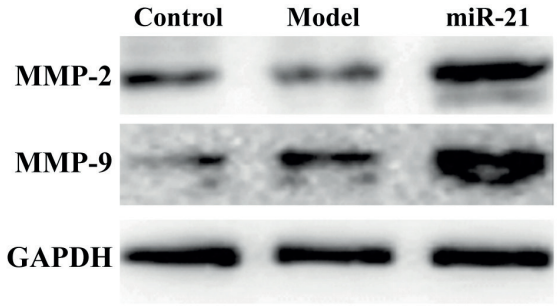


**Figure 6.** MMP-2 and MMP-9 protein expression levels of the three groups of rats.

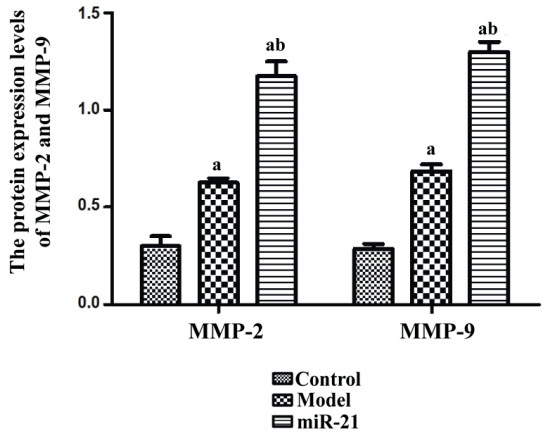


**Figure 7.** MMP-2 and MMP-9 protein expression levels in the three groups of rats. [a]$p<0.05$ *vs*. model group, and [b]$p<0.05$ *vs*. model group.

MMP-2 and MMP-9 in rat thoracic aortic aneurysm cells were positively associated with the expression of miR-21 in cells.

## Conclusions

Altogether, miR-21 could regulate the expression of PTEN in tumor cells in a targeted way and promote the secretion of invasion-related MMPs, such as MMP-2 and MMP-9, thus stimulating the invasion of thoracic aortic aneurysm cells. However, some deficiencies also existed in this work. For example, the microenvironment in organisms plays a pivotal role in the invasion and metastasis of tumors. Therefore, more investigations are warranted for further verification of the role of miR-21 in the early invasion and metastasis of the aortic aneurysm.

### Conflict of Interests

The Authors declare that they have no conflict of interests.

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