Iran J Public Health, Vol. 51, No.11, Nov 2022, pp.2494-2503

Original Article

Effect of miRNA-218-5p on Proliferation, Migration, Apoptosis and Inflammation of Vascular Smooth Muscle Cells in Abdominal Aortic Aneurysm and Extracellular Matrix Protein

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(Received 15 Apr 2022; accepted 11 Jun 2022)

Abstract

Background: To explore the effects of miRNA-218-5p on inflammation and extracellular matrix proteins of vascular smooth muscle cell line in abdominal aortic aneurysm (AAA).

Methods: miR-218-5p expression was detected with RT-qPCR. The proliferative activity of vascular smooth muscle cells (VSMCs) was detected with CCK-8, the migration was detected by Transwell, and the apoptosis was detected with flow cytometry. The expression levels of inflammatory factors (IL-1β and IL-18) were detected by ELISA. The expression levels of proteins (MMP-9 and Netrin-1) and ADAMTS5 were detected by Western blot. The targeting relationship between miR-218-5p and ADAMTS5 was verified with dual-luciferase reporter assay.

Results: Up-regulating miR-218-5p could significantly inhibit the proliferation and migration of VSMCs and induced the apoptosis (P<0.05). Down-regulating miR-218-5p could significantly promote the proliferation and migration of VSMCs and inhibit the apoptosis (P<0.05). Up-regulating miR-218-5p could inhibit the expression levels of THP-1 cytoinflammatory factors (IL-8 and IL-1β), MMP-9 and netrin-1. ADAMTS5 was the target gene of miR-218-5p. When there were both overexpression of ADAMTS5 and upregulation of miR-218-5p, the upregulation of miR-218-5p could alleviate the effects of overexpression of ADAMTS5 on the proliferation, migration and apoptosis of VSMCs.

Conclusion: miR-218-5p/ADAMTS-5 molecular axis regulates the proliferation, migration, and apoptosis of VSMCs, as well as the expression of THP-1 cell inflammatory molecules and extracellular matrix molecules.

Keywords: Abdominal aortic aneurysm; Inflammatory response; Matrix metalloproteinase

Introduction

Abdominal aortic aneurysm (AAA) refers to chronic aortic degenerative lesions caused by various factors, which occurs in middle-aged and elderly people suffering from chronic hyperten-

sion and seriously threaten their health (1). The basis for AAA lesions is the abnormal proliferation of vascular smooth muscle cells (VSMCs), abnormal proliferation, macrophage infiltration and extracellular matrix degradation and collagen degeneration due to chronic inflammation (2). The regulation mechanism of proliferation and apoptosis of VSMCs and the inflammatory related cell infiltration mechanism during the occurrence and progression of AAA has not been clarified (3,4).

microRNA (miRNA), as a short-chain RNA, can participate in the formation and development of AAA through specific expression in the tissues of AAA (5,6). Recent studies showed that microRNA-23b can regulate $FoxO_4$ by targeting, thus preventing and delaying the occurrence and progress of AAA (7). However, microRNA-23b expression will significantly decrease in the arterial lesion tissues in patients with AAA, and its circulating expression can serve as a molecular marker for the diagnosis and prognosis of AAA (8).

microRNA-218-5p (miR-218-5p) is a small RNA molecule on 5p of N13, with the length of 27nt, which participates in the occurrence and progression of emphysema (9) and osteoarthritis (10). Nonetheless, its role of miR-218-5p in the occurrence and progression of AAA is unknown. This study explored the impact of miR-218-5p on cell proliferation and chronic inflammation of aortic VSMCs, as well as potential targets in the pathogenesis of AAA We look forward to providing new molecular targets and experimental basis for the clinical treatment of AAA.

Materials and Methods

Cells and main reagents

Human aortic VSMCs (BNCC340185) and human monolithic macrophage THP-1 (BNCC100407) were purchased from the Typical Culture Conservation Center of the Chinese Academy of Sciences. RPMI-1640 and DMEM/F12 cell media were purchased from Thermo Fisher, USA. Pantrypsin, α-MEM and fetal bovine serum were purchased from Gibco. miR-218-5p inhibitor/mimics, pcDNA3.1-ADAMTS5 plasmid and negative control (scramble) were all synthesized by Shanghai Jima Company. TRIzol kit was purchased from Invitrogen Company, USA. Lipofectamine TM 3000 and reverse transcription kits were purchased from TaKaRa Company, Japan. Transwell chamber was purchased from Corning, USA. ELISA kit was purchased from Sigma Aldrich, USA. Rabbit antihuman cell ADAMTS5 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 5) monoclonal antibody, GAPDH monoclonal antibody and HRP goat anti-rat IgG were purchased from Cell Signaling Technology, USA. Penicillin, streptomycin, BCA protein quantitative kit and CCK-8 kit were purchased from Shanghai Beyotime Biotechnology Co., Ltd. Other than these, miR-218-5p inhibitor/mimic, pcDNA3.1-ADAMTS5 plasmids and negative controls were synthesized by Shanghai GenePharma Company, and dual-luciferase reporter assay kit was provided by Promega, USA.

Culture and transfection of cells

Culture of cells

Human VSMCs were cultured in DMEM/F12 medium, THP-1 cells in RPMI-1640 medium, with 10% fetal vine serum and double resistance (penicillin and streptomycin) added in advance, and then cultured in the incubator at 37 ℃, with 5% CO2. Cells growing well in logarithmic growth phase (growth fused to 80%-85%) were seeded into 6-well plates at 2×10^5/hole, which were added to the medium and continued to be cultured for 24 h, and then transfected with the cell growth density about 70%.

Transfection and grouping of cells

I. VSMCs cells were divided into miR-218-5p inhibit group, miR-218-5p mimics group, and scramble group. miR-218-5p inhibitor, miR-218-5p mimics, and scramble sequences were transfected respectively. CCK-8 cell proliferation, flow cytometry and Transwell experiments were performed in the three groups to observe the effect of miR-218-5p on the proliferation, apoptosis and invasion of VSMCs cells.

II. The THP-1 cell line was divided into scramble group and miR-218-5p mimics group. Scramble and miR-218-5p mimics sequences were transfected respectively. The concentration of IL-18 and IL-18β in scramble group and miR-218-5p mimics group were measured, and the protein expressions of MMP-9 and Netrin-1 were measured to observe the effect of miR-218-5p on inflammatory factors and extracellular matrix-related proteins of THP-1 cells.
III. VSMCs were divided into ADAMTS5 group, Scramble group and ADAMTS5+miR-218-5p mimics group. pcDNA3.1-ADAMTS5 plasmid, negative control plasmid, pcDNA3.1-ADAMTS5 plasmid+miR-218-5p mimics sequence were transfected respectively. CCK-8 cell proliferation, flow cytometry and Transwell experiments were performed to observe the rescue effect of ADAMTS5 on miR-218-5p.
IV. THP-1 cell lines were divided into Scramble group, ADAMTS5 group and ADAMTS5+miR-218-5p mimics group. Negative control plasmid, pcDNA3.1-ADAMTS5 plasmid and pcDNA3.1-ADAMTS5 plasmid+miR-218- 5p mimics sequence were transfected, the concentration of IL-18 and IL-1β and the protein expression of MMP-9 and Netrin-1 were determined to observe the rescue effect of ADAMTS5 on miR-218-5p.

RT-qPCR detection for miR-218-5p expression

Transfected cells from each group were collected. Total RNA was extracted from cells by TRIzol and RNA concentrations were measured with NanoDrop. The reverse transcription kit reverse transcripted RNA as cDNA, and relative miR-218-5p expression was detected with RT-qPCR, with U6 as the internal parameter, and the result was calculated with $2^{-\Delta\Delta}$Ct method. As for primers, upstream of miR-218-5p was 5 '-CTGUUCE TGTGCAAATCTATGC-3', downstream was 5 '-GTGCTTTUGTCCGAGGT-3', and upstream of U6 was 5'-CTGGCTTCGGCAACACATATACT-3', downstream was 5'-ACGCTTCACGATTTTGCGTGTC-3'. Each experiment was repeated 3 times to calculate the average value.

Western blot detection for the protein expression

VSMCs and THP-1 cells growing well in the logarithmic growth phase were collected, and the total protein was extracted by RAPI lysis solution, and the total protein concentration was detected. 50 μg of proteins were taken by 10% SDS-PAGE for electrophoresis, and the target proteins were transferred to PVDF membrane and sealed with 5% skim milk powder at room temperature for 1 h, and then were added with ADAMTS5 (1:200) and GAPDH antibodies (1:200) at 4℃ for incubation overnight. HRP-labeled goat antirat IgG (1:500) was added and incubated at room temperature for 2 h. The image was collected after ECL coloration, and the grayscale value was analyzed using ImageJ software. The experiment was repeated 3 times.

CCK-8 detection for the proliferation activity of VSMCs

Transfected and untransfected VSMCs were cultured to a growth density of 80%, which were transferred and seeded in 96-well plates (2×10^4/hole) with 100 μL DMEM medium, with 6 compound holes in each group. After being cultured for 0, 24, 48, 48, 72 and 96 h in the incubator at 37℃, with 5% CO2, 10 μL CCK-8 solution (0.5mg/mL) was added to each hole, which was incubated for 4 h, the optical density at 450nm (OD450) was measured.

Transwell detection for the migration capability of VSMCs

When being cultured with the growth density of 80%, transfected and untransfected VSMCs were seeded in the upper Transwell chamber (1×10^5 / hole) and the lower chamber was added 500 μL of culture medium and cultured for 24 h routinely. The cells were stained with 0.1% crystal violet for 20 min, and the number of invading and migrating cells was calculated in 5 randomly selected

fields under a light microscope. The experiment was repeated three times.

Flow cytometry detection for the apoptosis of VSMCs

Cells from different treated groups were seeded into 96-well plates (1×10^5/hole, and digested by trypsin after 12 h of culture. After washing with PBS, 300μL of Annexin V-FITC and 10μL of PI solution were added, respectively, and the cells were incubated in dark for 10min. After mixing, the apoptosis percentage was detected by flow cytometry.

Dual-luciferase reporter assay verified that the direct target gene of miR-218-5p was ADAMTS5

ADAMTS5 gene 3 'UTR was constructed, and inserted into pGL3-Promoter plasmid vector which was named pGL3ADAMTS5-3' UTR WT, and CDKN2B mutant vector, named PGL3-ADAMTS5-3 'UTR MUT, was obtained by mutation legal point mutation. VSMCs were seeded in 12-well plates (1×10^5/hole) and were transfected with miR-218-5p mimic and scramble, wild-type ADAMTS5 vectors and mutant ADAMTS5 vectors when the cell growth density reached 50%~70%. After 48 h of culture, dual-luciferase reporter assay kit was used to detect luciferase activity and repeated 3 times.

ELISA detection for the expression levels of inflammatory factors

Transfected and untransfected THP-1 cells were cultured in a serum RPMI-1640 medium containing 50% macrophage culture, and the expression levels of IL-1β and IL-18 were strictly tested in strict accordance with the instructions ELISA kit.

Statistical treatment methods

This study used SPSS 22.0 software (IBM Corp., Armonk, NY, USA) to analyze all data, where measured data were represented by mean ± standard deviation. t test is for comparing two groups, ANOVA for comparing all the groups and SNK-q test for one group of the two. P <0.05 indicates a statistical significance for the differences.

Results

Effects of elevated miR-218-5p expression on the proliferation, apoptosis and migration capacity of VSMCs

RT-qPCR results showed that miR-218-5p expression in VSMCs was significantly increased after transfecting miR-218-5p-mimic (P<0.05). miR-218-5p expression in VSMCs was significantly decreased after transfecting miR-218-5p-inhibit. The above results indicated a successful transfection (Fig. 1A). CCK-8 results show that the proliferation of VSMCs was significantly lower than that of the control group after up-regulating miR-218-5p (P <0.05), the proliferation of VSMCs was significantly higher than that of the control group and the miR-218-5p-mimics group after down-regulating miR-218-5p (P<0.05), as shown in Fig. 1B. Flow cytometry results showed that the apoptosis of VSMCs was significantly higher than that of the control group after up-regulating miR-218-5p (P<0.05), the apoptosis of VSMCs was significantly lower than that of the control group and the miR-218-5p-inhibit group after down-regulating miR-218-5p (P<0.05), as shown in Fig. 1C. Transwell results showed that the number of invaded cells in miR-218-5p mimics group was less than that in control group after miR-218-5p was up-regulated. The number of invaded cells in miR-218-5p inhibit group was more than that in control group and miR-218-5p mimics group after miR-218-5p was down-regulated (P<0.05) (Fig. 1D).

Effect of up-regulated miR-218-5p expression on THP-1 cell inflammatory cytokines and extracellular matrix-associated proteins

Up-regulating miR-218-5p expression significantly inhibited the expression levels of inflammatory factors (IL-18 and IL-1β) in THP-1 cells (P <0.05) (Fig. 2A, B). Western blot detection showed that up-regulating miR-218-5p expression significantly inhibited MMP-9 and Netrin-1

expression levels in THP-1 cells (P <0.05) (Fig. 2C, D). Up-regulating miR-218-5p significantly inhibited the expression levels of inflammatory factors and extracellular matrix-associated proteins of THP-1 cells.

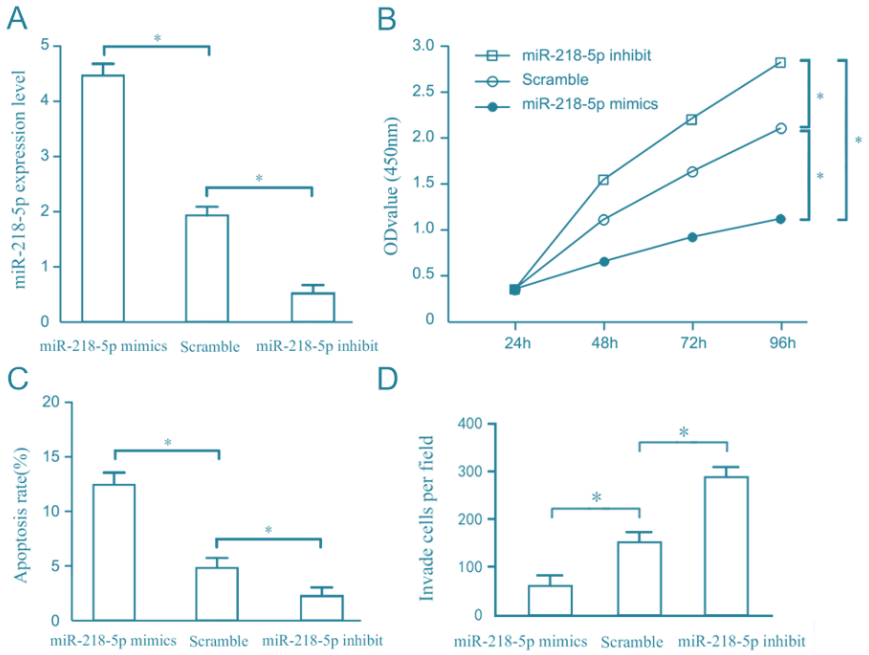

Fig. 1: The effect of miR-218-5p expression on the proliferation and migration ability of VSMCs. A: Transfection efficiency. B: Cell proliferation test. C: Comparison of apoptosis rate. D: Comparison of number of invasive cells

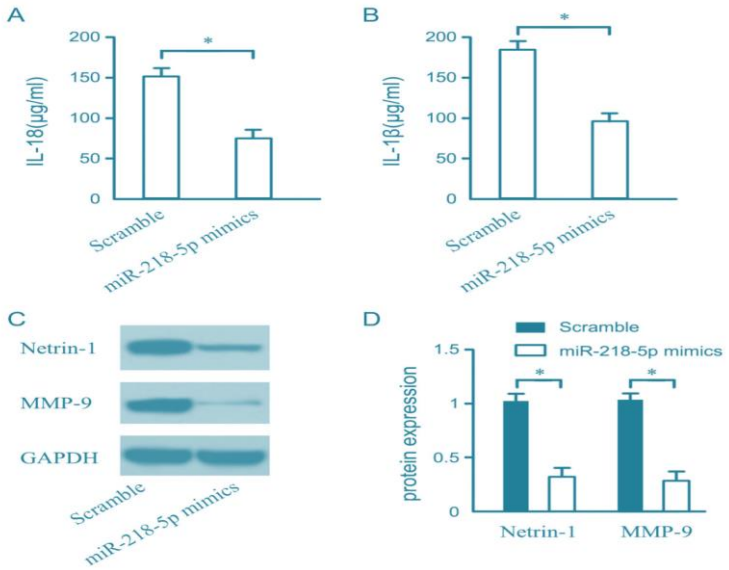

Fig. 2: The effect of up-regulation of miR-218-5p on THP-1 cell inflammatory factors and extracellular matrix-related protein expression. A: Comparison of IL-18 expression levels. B: Comparison of IL-1β expression levels. C: MMP-9 and MMP-9 Netrin-1 expressions in western blot. D: Comparison of MMP-9 and Netrin-1 expression levels

Direct target gene of miR-218-5p was ADAMTS5

The query of TargetScan database showed that ADAMTS5 was the potential direct target gene of miR-218-5p with binding sites (Fig. 3A). Dual-luciferase reporter assay showed that up-regulating miR-218-5p expression significantly reduced the luciferase activity of wild-type ADAMTS5 plasmids compared with that of the control group (scramble) (P <0.05) and had no significant effect on that of mutant ADAMTS5 plasmids (P> 0.05), as shown in Figure 3B. Western blot detection found that up-regulating miR-218-5p significantly down-regulated ADAMTS5 protein expression levels in VSMCs (Fig. 3C).

Up-regulating miR-218-5p can alleviate the regulatory effect of ADAMTS5 overexpression on the proliferation, apoptosis and migration of VSMCs

Transfection of miR-218-5p mimics can down-regulate the expression of ADAMTS5, and transfection of miR-218-5p inhibit can up-regulate the expression of ADAMTS5. On the contrary, after co-transfection of ADAMTS5 plasmid and miR-218-5p mimics, the promoting effect of miR-218-5p mimics transfection on the expression of ADAMTS5 protein can be alleviated (Fig. 3C). CCK-8 results showed that overexpressing ADAMTS5 significantly promoted the proliferative vitality of VSMCs (P <0.01). When there are both overexpression of ADAMTS5 and up-regulation of miR-218-5p, up-regulation of miR-218-5p can alleviate the promotion effect of ADAMTS5 overexpression on the proliferation of VSMCs cells, and there is no significant difference in cell proliferation compared with that in the control group (scramble) (Fig. 4A). Flow cytometry results showed that overexpressing ADAMTS5 significantly inhibited the apoptosis of VSMCs (P <0.05). After up-regulating the expression of miR-218-5p, this apoptosis inhibitory effect disappeared, and there was no significant difference from that of the control group (scramble), as shown in Fig. 4B. Transwell experiment results showed that the migration ability of VSMCs was significantly higher than that in the control group (P <0.05). After up-regulating the expression of miR-218-5p, the increase in the migration ability of VSMCs after overexpression of ADAMTS5 could be suppressed, which was not significantly different from that in the control group (scramble) (Fig. 4C). The above results showed that when there are both overexpression of ADAMTS5 and the upregulation of miR-218-5p, the regulation effect of overexpression of ADAMTS5 on the proliferation, apoptosis and migration of VSMCs can be significantly alleviated.

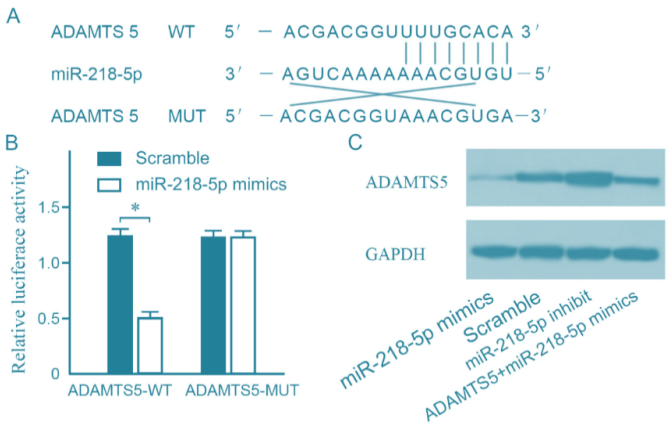

Fig. 3: ADAMTS5 is the direct target gene of miR-218-5p. A: miR-218-5p has a gene binding site with ADAMTS5. B: Dual luciferase experiment. C: The impact on the expression of ADAMTS5 after transfection of miR-218-5p and ADAMTS5 plasmid

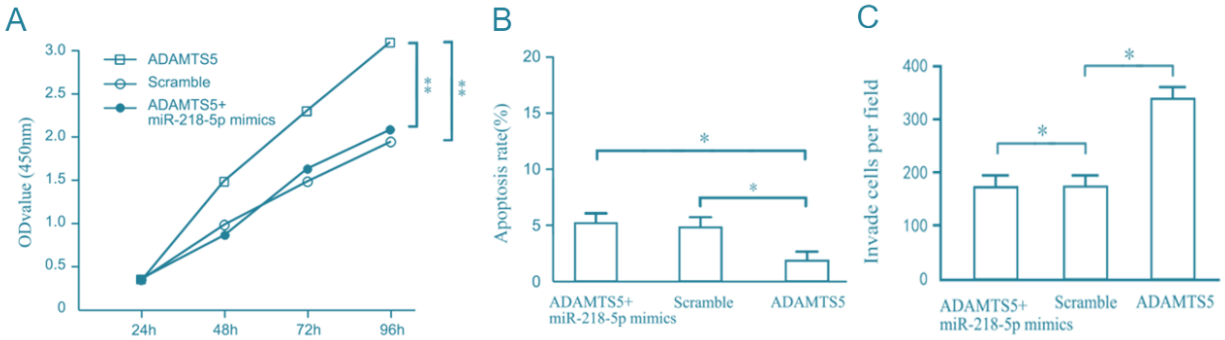

Fig. 4: miR-218-5p regulates the proliferation, apoptosis and migration of VSMCs by targeting ADAMTS5. A: CCK-8 experiment. B: Comparison of apoptosis rate. C: Comparison of number of invaded cells

Up-regulation of miR-218-5p can alleviate the promotion of inflammatory factors and extracellular matrix-related proteins caused by overexpression of ADAMTS5

ELISA results showed that, compared with the control group (scramble), overexpressing ADAMTS5 significantly up-regulated the expression levels IL-18 and IL-1β (P <0.05). Up-regulating miR-218-5p expression could relieve the promotion effect of inflammatory factors caused by overexpressing ADAMTS5 (P <0.05), as shown in Fig. 5A, B.

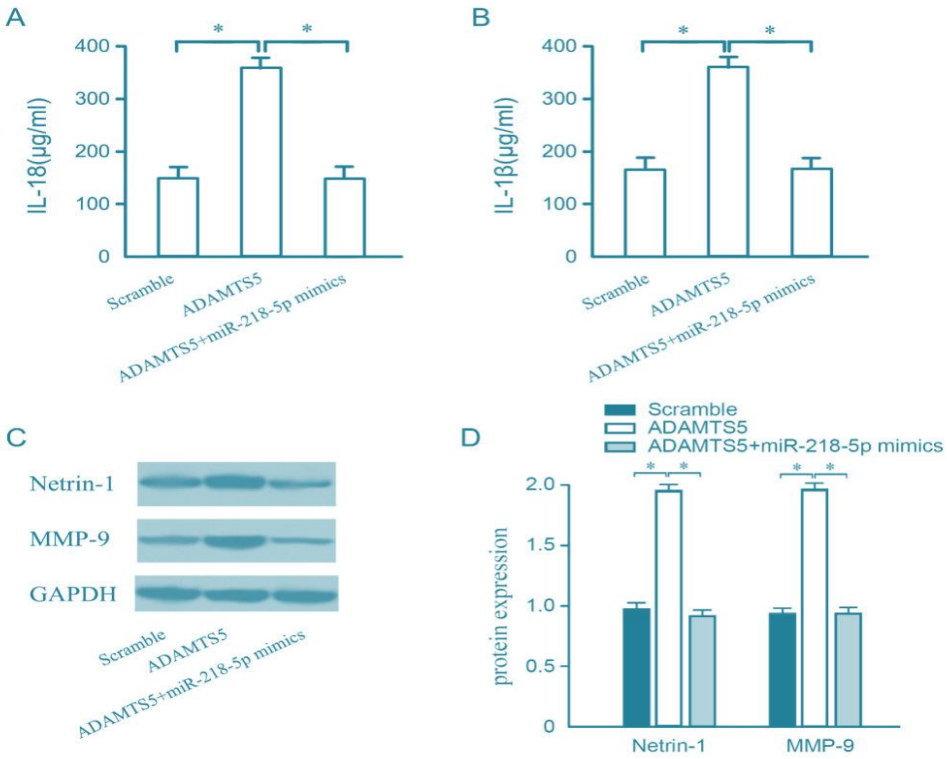

Fig. 5: Up-regulation of miR-218-5p can alleviate the promotion of inflammatory factors and extracellular matrix-related proteins caused by overexpression of ADAMTS5. A: Comparison of IL-18 concentration by ELISA. B: Comparison of IL-1β concentration by ELISA. C: MMP-9 and Netrin-1 protein expression by western blot. D: Comparison of MMP-9 and Netrin-1 protein expression levels

Western blot confirmed that, compared with the control group (scramble), overexpressing ADAMTS5 significantly suppressed the expression levels of MMP-9 and Netrin-1 proteins (P <0.05) see Figure 5C. Up-regulating miR-218-5p expression alleviated the promotion on extracellular matrix-associated proteins of THP-1 cells caused by overexpressing ADAMTS5 (P <0.01) (Fig. 5D).

Discussion

This study found that the miR-218-5p/ADAMTS-5 axis regulates the proliferation, migration and apoptosis of VSMCs, and regulates the expression of inflammatory factors and extracellular matrix proteins in THP-1 cells. AAA is a common delayed vascular disease in the elderly, its onset is closely related to long-term chronic hypertension, and atherosclerosis, thoracic aortic aneurysm, trauma, infection are all its onset factors. With the increasing incidence of hypertension, its incidence significantly increase, such as timely treatment often lead to tumor rupture and death (11). The pathogenesis of AAA is unknown, and HVSMCs are an important part of the membrane in the aortic wall, whose abnormal proliferation and local inflammatory reactions are considered to be key factors in the onset of AAA (12). In recent years, numerous studies have found that miRNA can play an important role in regulating cell proliferation and inflammatory responses, and a large number of miRNAs were identified as participating in the occurrence and progression of AAA (5,6,13). It may be an important diagnostic and prognostic molecule of AAA and potential therapeutic targets for AAA (13).

Literature studies have shown a large number of miRNA forming a miRNA network involved in the occurrence and progression of AAA (5). miR-15a expression was up-regulated in AAA tissues, knocking down miR-15a-5p expression could inhibit the proliferation of VSMCs, promote their apoptosis, regulate macrophage expression of cytokines, with targeted regulation of CDKN2B, so as to promote the occurrence and progression of AAA (14). Aneurysms-specific miRNA (including miR-221 and miR-146a) are involved in the occurrence and progression of aortic tumors in the chest and abdomen, and can be a potential target for the diagnosis and treatment of abdominal aortic tumors (15). This study found that up-regulating miR-218-5p expression significantly inhibited the proliferation and migration capacity of VSMCs and increased the apoptosis of VSMCs, suggesting that miR-218-5p may affect the proliferation and apoptotic balance of VSMCs, and promote the occurrence and progression of AAA. We found that the expression levels of inflammatory cytokines (IL-18 and IL-1β) in THP-1 cells significantly decreased, and the expression levels of extracellular strom-related proteins (MMP-9 and Netrin-1) in THP-1 cells also significantly decreased after up-regulating miR-218-5P. The above results suggested that up-regulating miR-218-5p can also inhibit the occurrence and progression of AAA by influencing the expression of inflammatory factors and extracellular matrix-related proteins in THP-1 cells. miR-218-5p plays a regulatory role in inflammation, injury and remodeling of lung tissue in patients with emphysema (9). The decreased miR-218-5p expression is associated with more severe emphysema. The results suggested that miR-218-5p up-regulation can significantly inhibit human macrophages inflammation and extracellular matrix-related protein expression and inhibit the occurrence of AAA.

Macrophages are important inflammatory immune cells in the occurrence and progression of AAA that produce local inflammatory factors (such as I L-18 and IL-1β), which bring a local inflammatory response and damage vascular walls. It could also degrade the elastic fibers and middle layers of collagen in the vascular wall by producing MMPs (such as MMP-9 and Netrin-1), causing the abdominal aortic vessels to dilate or even rupture under the impact of blood flow (16). Netrin-1 is a new marker of the newly discovered the progression of AAA, with increased

levels predicting its expansion (17). microRNA -181a can participate in the development and progression of abdominal aortic tumors via the NLRP3 pathway to affect inflammation and the expression of MMPs and low-density lipoprotein in THP-1 cells (18). This study found that up-regulating miR-218-5p inhibited the formation of AAA and may be associated with miR-218-5p regulation and affecting T HP-1 cells, and its precise mechanism remains to be further studied.

This study found that ADAMTS5 is the direct target gene of miR-218-5p, a disintegrin and metalloproteinase with thrombospondin motifs 5, which mainly plays a role in bone-arthropathy diseases in humans (19). The literature has reported increased A DAMTS-5 expression levels in abdominal aortic tumor tissues that may be involved in the progression of AAAs by regulating VSMCs and T HP-1 cells (20). We found a significant reduction in ADAMTS-5 expression levels in VSMCs after up-regulating miR-218-5p. Meanwhile, in this study, it was found that in VSMCs, miR-218-5p targeted adamts-5 to inhibit the proliferation and migration of VSMCs and promote apoptosis, and this effect partially disappeared after the addition of transfection to up-regulate the expression of miR-218-5p. Also, in THP-1 cells and episerum, miR-218-5p targeted ADAMTS-5 and reduced inflammatory cytokines and extracellular matrix molecular expression, which disappeared after up-regulating miR-218-5p expression. Upregulating miR-218-5p can inhibit the proliferation of smooth muscle cells and induce apoptosis by targeting down ADAMTS-5 expression, and alleviate the expression of inflammatory factors and MMPs in the supernatant of THP-1 cells, thus alleviating the development of AAA.

Conclusion

miR-218-5p /A DAMTS-5 molecular axis is involved in the occurrence and progression of AAA, regulating the proliferation, migration, and apoptosis of VSMCs, while regulating the expression of inflammatory and extracellular matrix molecules in THP-1 cells. Thus, up-regulating miR-218-5p expression may be a new potential and promising therapeutic target for AAA.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

No funding was received in this study.

Conflict of interest

The authors declare that there is no conflict of interest.

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