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Original Research

Effects of miR-7 on Hcy-induced rat cerebral arterial vascular smooth muscle cell proliferation, migration and inflammatory factor expression by targeting MMP-14 to regulate TLR4/NF-κB signaling pathway

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Abstract: The current research aimed to investigate the effect of miR-7 targeting matrix metalloproteinase 14 (MMP-14) on homocysteine (Hcy)-induced rat cerebral artery vascular smooth muscle cells (VSMCs) proliferation, migration and inflammatory factor expression and its possible mechanism. The expression of miR-7 and MMP-14 in Hcy-induced VSMCs were detected by real-time fluorescent quantitative PCR (RT-qPCR) and Western blot. Methyl Thiazolyl Tetrazolium (MTT) method, Transwell assays and enzyme-linked immunosorbent assay (ELISA) were performed to detect the effect of miR-7 and MMP-14 expression on the proliferation and migration, as well as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) expression of Hcy-induced VSMCs. The interaction between miR-7 and MMP-14 was detected by dual-luciferase reporter gene assay. Western blot was applied to analyse the effects of miR-7 and MMP-14 expression on the Toll-like receptor (TLR4)/nuclear transcription factor-KB (NF-κB) signaling pathway. The results showed that after induced by Hcy, the expression of miR-7 in VSMCs was significantly reduced, the expression of MMP-14 was significantly increased, and the cell viability, the number of migrating cells, IL-6 and TNF-α expression were significantly increased (P<0.05). After overexpression of miR-7, the viability, migration cell numbers, IL-6 and TNF-α expression of Hcy-induced VSMCs were significantly reduced (P<0.05). miR-7 directly binds to MMP-14 and negatively regulates the expression of MMP-14. After overexpression of miR-7, the levels of TLR4 and p-NF-κB p65 in VSMCs were significantly reduced (P<0.05); overexpression of MMP-14 could reduce the effect of miR-7 overexpression on TLR4 and p-NF-κB p65 expression in VSMCs (P<0.05). Overexpression of MMP-14 and/or activation of the TLR4/NF-κB signaling pathway could reverse the effect of miR-7 overexpression on the proliferation, migration and IL-6 and TNF-α expression of Hcy-induced VSMCs (P<0.05). It is concluded that miR-7 can inhibit Hcy-induced rat cerebral artery VSMCs proliferation, migration, and inflammatory factor expression by targeting the regulation of MMP-14 expression and inhibiting the activation of the TLR4/NF-κB signaling pathway.

Key words: miR-7; MMP-14; Homocysteine; Vascular smooth muscle cells; Proliferation; Migration; TLR4/NF-κB signaling pathway.

Introduction

Atherosclerotic (AS) is a chronic multifactorial vascular disease, which is the main cause of coronary artery disease. Vascular smooth muscle cells (VSMCs) can produce inflammatory reactions during vascular injury, and its abnormal proliferation, migration and extracellular matrix synthesis are important factors in the progress of AS (1). Therefore, the inhibition of apoptosis, proliferation, migration and inflammation of VSMCs is of great significance to delay the progression of AS. MicroRNA (miRNA) is a highly conserved non-coding short-chain RNA molecule, which plays a role in its degradation or translation inhibition by complementary binding with target mRNA. Studies have shown that miRNA participates in cell proliferation, lipoprotein homeostasis, inflammation and plaque formation and plays an important role in the pathophysiological process of AS (2-3). It has been reported that the expression of miR-7 in the brain tissue of rats decreased after transient focal ischemia, local or systemic injection of miR-7 simulator significantly reduced the lesion volume, improved motor and cognitive function, and reduced peripheral toxicity (4). Matrix metalloproteinase 14 (MMP-14) is a key enzyme in extracellular matrix metabolism and participates in the formation and fragmentation of AS plaques through the degradation of type IV collagen. Studies have shown that its high expression is related to cerebral obstruction and myocardial infarction, and inhibition of its activity is an effective treatment to reduce plaque rupture and myocardial infarction (5). Sequence analysis shows that MMP-14 is a potential target gene of miR-7. However, it is not clear whether miR-7 targets MMP-14 and plays a role in the pathogenesis of AS. Homocysteine (Hcy) can initiate the inflammatory reaction, trigger VSMCs proliferation, migration and MMPs activation. VSMCs cells induced by homocysteine are commonly used in the study of AS in vitro (6-7). In this study, we investigated the effects of miR-7 targeting MMP-14 on the proliferation, migration and expression of inflammatory factors in rat cerebral artery VSMCs induced by Hcy, and analyzed its potential molecular

mechanism, in order to provide an effective target for the prevention and treatment of AS.

Materials and Methods

Materials

Rat cerebral artery VSMCs was purchased from American ATCC; Hcy, lipopolysaccharide (LPS) was purchased from American Sigma company, reverse transcription Master Mix kit and SYBR Green PCR kit were purchased from Dalian Takara company, miR-7 mimic (mimics), mimic control (miR-con), MMP-14 overexpression vector (pcDNA-MMP-14), empty plasmid (pcDNA-con) and dual-luciferase report vector were all provided by Shanghai Shenggong Company. Rabbit anti-mouse MMP-14, cyclin D1 (CyclinD1), vascular endothelial growth factor (VEGF), P21, epithelial cadherin (E-cadherin), Toll-like receptor 4 (TLR4), nuclear transcription factor-Kappa B (NF- κB) p65, p-NF-kappa B p65 antibody and sheep anti-rabbit IgG antibody were purchased from Shanghai Saixin Biotechnology Co., Ltd. Thiazolan (MTT) kit was purchased from Shanghai Beibo Biology Co., Ltd., and Transwell cubicle was purchased from Beijing Younikang Biology Co., Ltd. Rat interleukin 6 (IL-6) and tumor necrosis factor (TNF-α) enzyme-linked immunosorbent assay ((ELISA)) kit was purchased from Nanjing Sembega Biology Co., Ltd.

Methods

Cell culture

Rat cerebral artery VSMCs were cultured in DMEM medium containing 20% fetal bovine serum and 1% penicillin double-antibody in a cell incubator with a 5% volume fraction of CO2 at 37 ℃. The cells were subcultured when 80% of the cells were fused, and the third-generation cells were taken for a follow-up experiment.

Grouping and intervention

VSMCs cells were divided into Control group (normal cultured VSMCs), Hcy group (treated with Hcy of 100mol/L for 24 h), miR-con group (transfected with miR-con to VSMCs), miR-7 group (transfected with miR-7mimics to VSMCs), Hcy+miR-con group (treated with Hcy of 100mol/L to transfect miR-con cells for 24 h), Hcy+miR-7 group (transfected miR-7 cells with Hcy of 100mol/L for 24 h), miR-7+pcDNA-con group (co-transfected miR-7mimics and pcDNA-con), MiR-7+pcDNA-MMP-14 group (co-transfection of miR-7mimics and pcDNA-MMP-14), Hcy+miR-7+pcDNA-MMP-14 group (co-transfection of miR-7mimics and pcDNA-MMP-14 cells with 100mol/L Hcy treatment for 24 hours), Hcy+miR-7+LPS group (transfection of miR-7mimics cells for 24 hours with 100mol/L Hcy treatment). And then treated with LPS of 100ng/mL for 6 hours). Lipofectamie2000 was used for transient transfection. 48 hours after transfection, real-time quantitative PCR (RT-qPCR) or/and Western blotting (Western blot) were collected to detect the transfection efficiency.

Detection of miR-7 expression by RT-qPCR

Total RNA was extracted from VSMCs by TRIzol and reverse transcribed into cDNA by Prime Script RT Master Mix kit. SYBRGreenPCR kit was used for RT-qPCR amplification. The expression of miR-7 was analyzed by the $2^{-\Delta\Delta Ct}$ method. The U6 upstream primer 5'-TGCGGGTGCTCGCTTCGGCAGC-3' ; the downstream primer 5'-GTGCAGGGT CCGAGGT-3'; the upstream primer of miR-7 was 5'-TGGAAGAC-TAGTGATTTTGTTG-3'; the downstream primer 5'-ACGCTGGAAGACTAGTGATTTTG-3'.

Detection the expression of MMP-14, CyclinD1, VEGF, p21, E-cadherin, TLR4, NF- κ B p65 and pmurNF-κ B p65 protein by Western blot

The total protein was extracted from the cells by radioimmunoassay (RIPA), and the protein concentration was determined by diquinolinecarboxylic acid (BCA). The same amount of protein samples was taken for polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. After sealing with 5% skim milk, the PVDF membrane was incubated overnight with the first antibodies of MMP-14, TLR4, NF- κB p65, p-NF-κB p65 and GAPDH diluted against 1:1000 at 4 ℃, and then incubated with the corresponding enzyme-labeled secondary antibodies. The color reaction was carried out by adding chemiluminescence reagent in the dark room, and the gray value was carried out by the gel imaging system, and the corresponding protein expression was expressed by the ratio of the target protein to the internal reference GAPDH.

Detection of cell proliferation activity by MTT method

The cells were inoculated into 96-well plates at the density of 2×10^3 cells per well. After 48 hours of culture, 20 μ L MTT solution was added to each well, and 150 μ L dimethyl sulfoxide was added to each well after incubation at 37 ℃ for 4 h. The absorbance at 490 nm was measured by an enzyme labeling instrument.

Detection of cell migration by Transwell method

The cell suspension diluted by 200 μ L serum-free medium (the number of cells was 5×10^4) was added to the supraventricular cavity of Transwell. 500 μ L medium containing 10% fetal bovine serum was added to the lower chamber. After incubation for 24 hours, the cells on the subventricular surface of Transwell were fixed with 4% paraformaldehyde and then stained with crystal violet. The number of migrating cells is expressed by the mean value of the number of cells in three visual fields randomly selected under the optical microscope.

Detection of IL-6 and TNF-a expressions by ELISA

The supernatant of cell culture medium of each group was collected, and the expression levels of IL-6 and TNF-α genes were detected according to the steps of the ELISA kit.

Double luciferase reporter gene experiment

The 24-well plate was inoculated with 3×10^4 cells per well, and luciferase reporter vector MMP-14 (WT) or MMP-14 (MUT) was co-transfected with miR-7 mimics and miR-NC with Lipofectamine 2000. Luciferase activity was detected by a double luciferase reporter gene detection system 48 hours after transfection.

Statistical analysis

SPSS 19.0 software was used for statistical analysis. The data are expressed as the average ±standard deviation ($\bar{x} \pm s$) of the three independent experiments. An independent sample t-test was used to compare the differences between the two groups, and single-factor analysis of variance and *SNK-q* test were used to compare the differences between multiple groups. The difference was statistically significant ($P < 0.05$).

Results

Expression of miR-7 and MMP-14 in rat cerebral artery VSMCs induced by Hcy

Compared with the control group, the expression of miR-7 and MMP-14 protein in VSMCs of the Hcy group decreased significantly ($P < 0.05$), as shown in Figure 1.

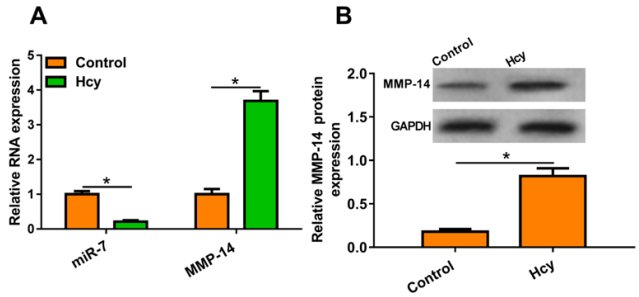

Figure 1. Expression of miR-7 and MMP-14 in vascular smooth muscle cells of rat cerebral artery induced by Hcy. A. RT-qPCR was used to detect the expression of miR-7 and MMP-14 in vascular smooth muscle cells of rat cerebral artery induced by Hcy. B. Western blot was used to detect of MMP-14 expression in vascular smooth muscle cells of rat cerebral artery induced by Hcy.

Effects of miR-7 overexpression on the proliferation, migration and inflammatory factor table of rat cerebral artery VSMCs induced by Hcy

Compared with the miR-con group, the expression of miR-7 in VSMCs of the miR-7 group was significantly higher ($P < 0.05$), see Figure 2A. Compared with the control group, the activity of VSMCs, the number of migrating cells, the expression of CyclinD1 and VEGF protein were significantly increased, the expression of P21 and E-cadherin protein were significantly decreased, and the levels of IL-6 and TNF-α mRNA were significantly increased in Hcy group ($P < 0.05$). Compared with the Hcy+miR-con group, the activity of VSMCs, the number of migrating cells, the expression of CyclinD1 and VEGF protein were significantly decreased, the expression of P21 and E-cadherin protein were significantly increased, and the levels of IL-6 and TNF-α mRNA were significantly decreased in Hcy+miR-7 group (Fig. 2B to Fig. 2G).

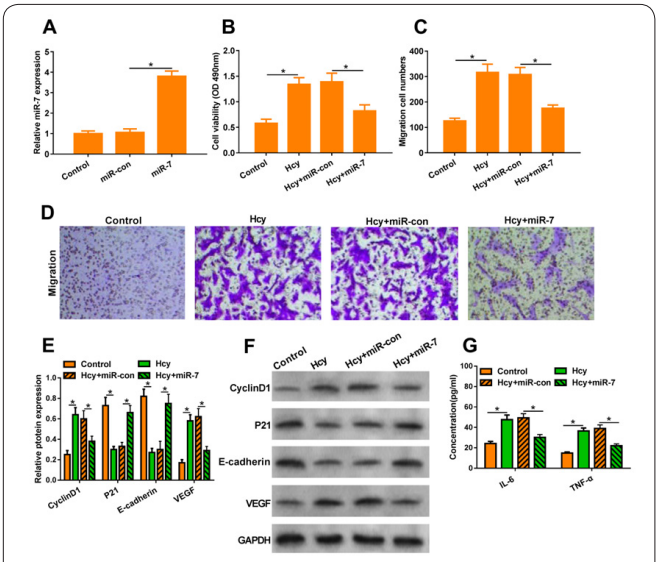

Figure 2. Effects of overexpression of miR-7 on proliferation, migration and expression of inflammatory factors in rat cerebral artery VSMCs induced by Hcy, * $P < 0.05$. A. RT-qPCR was used to detect the transfection effect of miR-7; B. The effect of miR-7 overexpression on Hcy-induced VSMCs proliferation in rat cerebral arteries was detected by MTT (48 h); C and D. The effect of miR-7 overexpression on Hcy-induced VSMCs migration in rat cerebral arteries was detected by Transwell; E and F. Western blot were used to detect the effects of miR-7 overexpression on the expression of proliferation and migration-related proteins in rat cerebral artery VSMCs induced by Hcy; G. The effect of overexpression of miR-7 on the expression of VSMCs inflammatory factors in rat cerebral arteries induced by Hcy was detected by ELISA.

Targeted regulation of MMP-14 expression by miR-7

Online analysis of the Targetscan website showed that there is a complementary nucleotide sequence between miR-7 and MMP-14, as shown in Figure 3A. Compared with miR-con and MMP-14 (WT) co-transfection, the luciferase activity of miR-7 and MMP-14 (WT) co-transfection decreased significantly ($P < 0.05$), compared with miR-con and MMP-14 (MUT) co-transfection, miR-7 and MMP-14 (MUT) co-transfection showed no significant change in luciferase activity ($P > 0.05$) , see Figure 3B. Compared with the miR-con group, the expression of MMP-14 protein in VSMCs of miR-7 group was significantly decreased ($P < 0.05$), see Figure 3C.

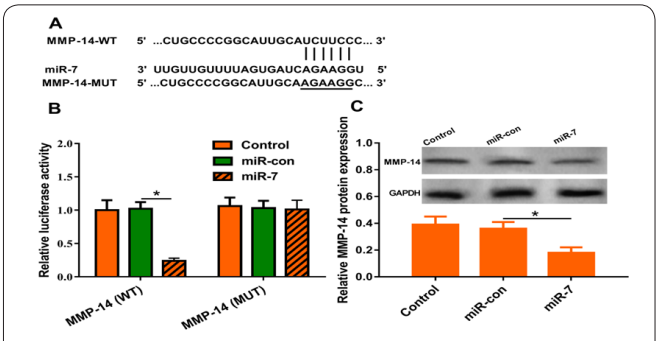

Figure 3. Targeted regulation of MMP-14 expression by miR-7, * $P < 0.05$. A. Binding site of miR-7 and MMP-14; B. Double luciferase activity assay to detect the targeting relationship between miR-7 and MMP-14; C. Effect of miR-7 overexpression on VSMCs MMP-14 expression in rat cerebral arteries detected by Western blot.

miR-7 regulated TLR4/NF- κB signal pathway through MMP-14

Compared with the pcDNA-con group, the expression of VSMCs MMP-14 in the pcDNA-MMP-14 group was significantly increased ($P < 0.05$), see Fig. 4A and 4B. Compared with the control group, the levels of VSMCs TLR4 and p-NF-κB p65 in the miR-7 group were significantly lower than those in the miR-7+pcDNA-con group, while those in the miR-7+pcDNA-MMP-14 group were significantly higher

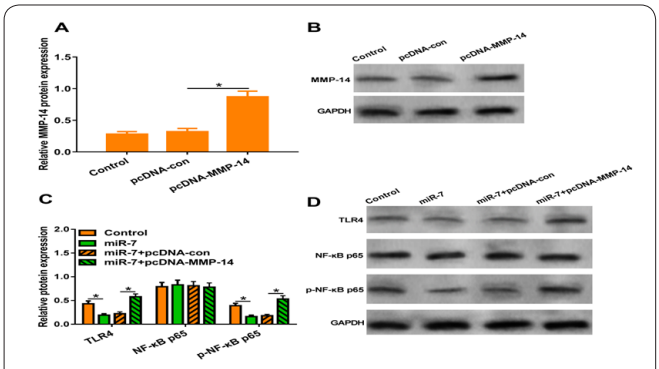

Figure 4. miR-7 regulated TLR4/NF- κB signal pathway through MMP-14, * $P < 0.05$. A and B. The transfection effect of pcDNA-MMP-14 was detected by Western blot; C and D. Western blot was used to detect the effect of overexpression of miR-7 and MMP-14 on TLR4/NF- κB signal pathway.

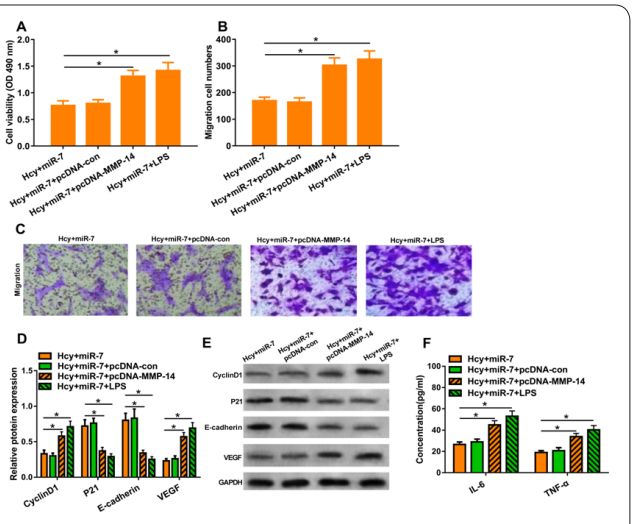

Figure 5. Overexpression of MMP-14 or activation of the TLR4/NF- κB signal pathway can reverse the effect of miR-7 on Hcy-induced VSMCs function in rat cerebral arteries, * $P < 0.05$. A. MTT method was used to detect the proliferation of VSMCs in cerebral arteries of rats in each group; B and C. Detection of VSMCs migration in cerebral arteries of rats in each group by Transwell; D and E. Western blot were used to detect the expression of VSMCs proliferation and migration-related proteins in the cerebral arteries of rats in each group; F. ELISA was used to detect the level of inflammatory factors of VSMCs in the cerebral artery of rats in each group.

than those in miR-7+pcDNA-con group ($P < 0.05$), as shown in Fig. 4C and 4D.

Overexpression of MMP-14 and/or activation of TLR4/NF- κB signal pathway can reverse the effect of miR-7 on Hcy-induced VSMCs function in rat cerebral arteries

Compared with the Hcy+miR-7 group, the activity of VSMCs, the number of migrating cells, the expression of CyclinD1 and VEGF protein were significantly increased, the expression of P21 and E-cadherin protein were significantly decreased, and the levels of IL-6 and TNF-α mRNA were significantly increased in Hcy+miR-7+pcDNA-MMP-14 group. Compared with the Hcy+miR-7 group, VSMCs activity, the number of migrating cells, CyclinD1 and VEGF protein expression were significantly increased, P21 and E-cadherin protein expression were significantly decreased, IL-6 and TNF-α protein levels were significantly increased in Hcy+miR-7+LPS group (Fig. 5).

Discussion

At present, AS is still an important medical and social problem, and it is the main cause of ischemic stroke, coronary heart disease and peripheral vascular disease. Abnormal proliferation, migration and inflammation of VSMCs are the key regulatory factors of AS. Therefore, it is very important to identify the role of VSMCs in AS to develop new AS prevention and control strategies.

MiRNA is an important participant in many biological processes such as cell proliferation, apoptosis and migration. The related research on miRNA and AS provides a new direction for the prevention and treatment of AS (8). Previous studies have shown that miR-7 has the role of tumor suppressor in cancer, and inhibits the proliferation and metastasis of tumor cells by targeting a variety of molecules and signal pathways (9). It has also been reported that the down-regulation of miR-7 expression is related to myocardial ischemia-reperfusion injury. Up-regulating its expression can improve cardiomyocyte viability, inhibit apoptosis and reduce myocardial ischemia-reperfusion injury (10). In addition, Hong et al. (11) confirmed that intracerebral injection of miR-7 can also antagonize the activation of astrocytes and protect the secondary brain injury after intracerebral hemorrhage. In this study, Hcy was used to induce VSMCs to simulate the cell state in AS to explore the biological role of miR-7 in AS. The results showed that the expression of miR-7 in VSMCs increased after Hcy induction. Further functional analysis showed that overexpression of miR-7 significantly inhibited Hcy-induced proliferation and migration of VSMCs. P21 is recognized as a proliferation inhibitor, while CyclinD1 and VEGF can promote the proliferation of VSMCs (12-13). E-cadherin is an important protein regulating cell adhesion and maintaining cell morphology. Some studies have shown that adenosine monophosphate-activated protein kinase-α 2 deficiency promotes VSMCs migration by down-regulating E-cadherin (14). In this study, overexpression of miR-7 significantly increased the expression of p21 in VSMCs induced by Hcy, while decreased the expression of CyclinD1 and VEGF, suggesting that miR-7 inhibited the proliferation and migration of VSMCs by activating p21 and E-cadherin expression and inhibiting CyclinD1 and VEGF. In addition to the abnormal proliferation and migration of VSMCs, chronic inflammation mediated by inflammatory factors such as IL-6 and TNF-α is the main pathological process of neointimal hyperplasia, in-stent restenosis and the development of AS after angioplasty (15). In this study, it was found that overexpression of miR-7 significantly decreased the expression of IL-6 and TNF-α genes, and inhibited the inflammatory response of VSMCs induced by Hcy. These results suggest that miR-7 is an important regulatory factor involved in the proliferation, migration and inflammation of VSMCs.

To further study the potential molecular mechanism of miR-7, bioinformatics analysis and double luciferase reporter genes confirmed that MMP-14 was the functional target gene of miR-7, and miR-7 negatively regulated the expression of MMP-14. Previous

studies have shown that the expression of MMP-14 in AS plaques is significantly increased, and the expression of MMP-14 in vulnerable plaques is higher than that in stable plaques (16). There was also a positive correlation between MMP-14 gene polymorphism and carotid vulnerable plaque formation (17). In addition, some studies have confirmed that miR-24 can delay the progression of AS by down-regulating the expression of MMP-14 and inhibiting the invasion of macrophages (18). This study showed that overexpression of MMP-14 could significantly reduce the effects of overexpression of miR-7 on Hcy-induced proliferation, migration and related protein expression of VSMCs, as well as the expression of pro-inflammatory cytokines IL-6 and TNF-α. It is suggested that targeted down-regulation of MMP-14 is an important way for miR-7 to inhibit the proliferation, migration and expression of IL-6 and TNF-α genes induced by Hcy in VSMCs.

TLR4 can promote pathological processes such as leukocyte infiltration and neovascularization by combining with cell surface molecular patterns. TLR4 can also activate NF- κB to further expand inflammatory response and increase the instability of AS plaques, which is very important for the occurrence and development of AS (19). Song et al. (20) found that the activation of the TLR4/NF- κB pathway is related to human VSMCs calcification induced by oxidized low-density lipoprotein. Lou et al. (21) have shown that rivaroxaban exerts its anti-atherosclerotic effect by inhibiting TLR4/NF- κ B signal pathway and activating downstream anti-inflammatory factor TGF- β. In this study, it was found that overexpression of miR-7 significantly inhibited the expression of TLR4 and pmurNF-κB p65, while overexpression of MMP-14 significantly reversed the effect of miR-7 on TLR4/NF- κB signal pathway, suggesting that miR-7 regulates TLR4/NF- κB signal pathway by targeting MMP-14. In addition, it was found that the activation of the TLR4/NF- κB signal pathway by LPS could reverse the effects of miR-7 overexpression on the proliferation, migration, IL-6 and TNF-α expression of VSMCs induced by Hcy. Using new technologies such as genome editing, gene expression can be properly controlled and the desired trait or phenotype can be accessed (22).

Conclusion

In conclusion, this study shows that miR-7 inhibits the proliferation, migration and expression of inflammatory factors of VSMCs in rat cerebral arteries induced by Hcy through targeted regulation of MMP-14 expression and inhibition of TLR4/NF- κB signal pathway, which has a potential inhibitory effect on the progression of AS. Therefore, the regulation of the miR-7/MMP-14 molecular axis and TLR4/NF- κB pathway may be a new and effective strategy for the prevention and treatment of AS.

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