European Review for Medical and Pharmacological Sciences 2019; 23 (3 Suppl): 319-326

MiR-210 inhibits apoptosis of vascular endothelial cells *via* JAK-STAT in arteriosclerosis obliterans

J.-N. YUE[1], W.-M. LI[1], W.-Z. HONG[2], J. YANG[1], T. ZHU[1], Y. FANG[1], W.-G. FU[1]

[1]Department of Vascular Surgery, Zhongshan Hospital, Fudan University, Shanghai, China
[2]Department of Cardiology, School of Medicine, South China University of Technology, Guangzhou, China

Jianing Yue and *Weimiao Li* contributed equally to this work

Abstract. – OBJECTIVE: To investigate the effect of micro ribonucleic acid (miR)-210 on the apoptosis of vascular endothelial cells in arteriosclerosis obliterans (ASO) through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway.

PATIENTS AND METHODS: In the present work, the vascular endothelial cells in ASO patients were selected as objects of study, the cell lines with miR-210 interference and overexpression were constructed with the Crisp/Case9 technique for subsequent experiments as experimental group, and the aortic endothelial cells of a healthy human were used as control group. First, the changes in the transcriptional and translational levels of such genes as JAK2 and STAT3 in the JAK-STAT signaling pathway in cell lines with miR-210 interference and overexpression were detected *via* fluorescence quantitative Polymerase Chain Reaction (qPCR) and Western blotting. The changes in the transcriptional and translational levels of nitric oxide synthase (NOS) in cells were detected in experimental group and control group to clarify the regulatory effect of miR-210 on the JAK-STAT signaling pathway. At the same time, the cell proliferation in experimental group and control group was observed *via* methyl thiazolyl tetrazolium (MTT) assay and the apoptosis rate was detected in both groups *via* flow cytometry.

RESULTS: The results of fluorescence qPCR and Western blotting revealed that the expression level of miR-210 was significantly increased in cells of ASO patients compared with that in aortic endothelial cells of healthy human with a significant difference ($p<0.05$). At the same time, the inhibition on miR-210 could significantly reduce the transcriptional and translational levels of JAK2, STAT3, and NOS, block the JAK-STAT signaling pathway, suppress the cell proliferation, and promote apoptosis. The overexpression of miR-210 could markedly increase the transcriptional and translational levels of JAK2, STAT3, and NOS, activate the JAK-STAT signaling pathway, promote the cell proliferation, and suppress the apoptosis.

CONCLUSIONS: MiR-210 can be involved in the apoptosis process of vascular endothelial cells in ASO through the JAK-STAT signaling pathway.

Key Words:
MiR-210, JAK-STAT signaling pathway, Arteriosclerosis obliterans, Vascular endothelium, Apoptosis.

Introduction

Arteriosclerosis obliterans (ASO) is a kind of common peripheral arterial occlusive disease. According to clinical statistics, the main clinical manifestation of ASO patients in China is the ischemia in the lower extremity. Statistical data show that[1] the morbidity rate of ASO is about 10% in China, slightly higher in males than in females[2]. High-risk groups are middle-aged and elderly people aged above 45 years old. The nutrition supply has been largely altered by the great changes in people's dietary structure and the excessive intake of nutrients eventually leads to the increased morbidity rates of hypertension, diabetes mellitus, hyperlipidemia, and other diseases year by year, thus increasing the incidence rate of ASO[3-5]. At present, the pathogenesis of ASO remains unclear. The treatment means mainly include the anti-platelet therapy and improvement of blood lipids and cell hypoxia. However, the above methods cannot radically treat ASO[6]. With the

Corresponding Author: Weiguo Fu, MD; e-mail: fu.weiguo@zs-hospital.sh.cn

continuous development of molecular technology in recent years, the function of micro ribonucleic acids (miRNAs) in regulating the cellular gene expression has gradually attracted people's attention[7]. Reports[8] have found that the expression level of miR-145 in vascular cells of ASO patients is significantly higher than that in healthy cells and miR-145 and other genes play important roles in cell migration, proliferation, and apoptosis. In the present study, the effect of miR-210 on the apoptosis of vascular endothelial cells in ASO through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway was explored for the first time to clarify the correlation between miR-210 and apoptosis of vascular endothelial cells in ASO, and provide a certain theoretical basis for subsequent research.

Patients and Methods

General Data

The vascular endothelial cells in ASO patients treated in our hospital in 2018 were selected as objects of study. These patients all met the criteria for ASO. Signed written informed consents were obtained from all participants before the work. This research was approved by the Ethics Committee of Zhongshan Hospital.

Main Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Roche (Basel, Switzerland), 0.25% trypsin and Ethylene Diamine Tetraacetic Acid (EDTA) reagent from Invitrogen (Carlsbad, CA, USA), the lentiviral vector system and transfection kit from TaKaRa (Dalian, China), and the Crisp/Case9 kit from ABM. The relevant recognition sites were predicted and designed through the online website (https://chopchop.rc.fas.harvard.edu/index.php). JAK2, STAT3, and nitric oxide synthase (NOS) antibodies, and methyl thiazolyl tetrazolium (MTT) assay kit were purchased from Roche (Basel, Switzerland), the animal cell total protein extraction kit from Thermo Fisher Scientific (Waltham, MA, USA), and the fluorescence quantitative Polymerase Chain Reaction (qPCR) kit from TaKaRa (Dalian, China) and the intracellular RNA extraction kit from AXYGEN (Beijing, China).

Table I. MiR-210 gene cloning primers.

Primer	Primer sequences
MiR-210-F	TGCTGATGCTGATCGTAGCTAG
MiR-210-R	GCTGATCGTAGCTAGCAGCTG

Methods

Construction of Cell Lines with MiR-210 Overexpression

The primers used in the construction of miR-200a were synthesized by Sangon (Shanghai, China), and the primer sequences are shown in Table I. Other relevant molecular operations were performed according to the *Molecular Cloning: A Laboratory Manual, 3rd Edition.*

Construction of Mutants with MiR-210 Interference

The cell lines with miR-210 interference were constructed with the Crisp/Case9 technique. First, different targets of miR-200 genes were selected through the Crisp/Case9 online design website (https://chopchop.rc.fas.harvard.edu/index.php), cleaved and knocked out. The sgRNA sequences are shown in Table II. Two groups of recognition sites were selected for each gene according to the instructions[9].

Fluorescence qPCR

RNA Extraction

The RNA was extracted according to the instructions of the AXYGEN kit (Beijing, China) as follows: (1) About 0.1 g tissue samples cryopreserved in liquid nitrogen were taken out, dissolved on ice, added with 0.45 mL RNAPlus, smashed in a pre-cooled mortar, and transferred into a 1.5 mL Eppendorf (EP) tube. Then, 0.45 mL RNAPlus was added into the mortar and transferred into a centrifuge tube after washing. (2) 200 μL chloroform was added into the centrifuge tube, shaken violently for 15 s, and placed on ice for 15 min. (3) The mixture was

Table II. MiR-200 gene recognition target sequences.

Name	Sequences
MiR-210-F1	ATCGGATCGTCAGCTACGTACGATCG
MiR-210-R1	CGTAGCCGGCATCGATCAGCTAGCTA
MiR-210-F2	CGAGCTACGAGCGCACTACGATCGC
MiR-210-R2	CGGGAGCTACGAGCATCGACTAGCTG

centrifuged at 12000 rpm and 4°C for 15 min. (4) The supernatant was transferred into an RNase-free EP tube, added with an equal amount of isopropanol, mixed evenly, and placed on ice for 10 min. (5) The mixture was centrifuged at 12000 rpm and 4°C for 10 min. (6) The supernatant was discarded, and 750 μL 75% ethanol was added and mixed evenly, followed by centrifugation at 12000 rpm and 4°C for 10 min. (7) The supernatant was discarded and the residual ethanol was removed as far as possible. (8) An appropriate amount of RNase-free water was added and the mass of RNA extracted was determined, while the remaining RNA was used for reverse transcription[10].

Fluorescence qPCR

The fluorescence qPCR kit was purchased from TaKaRa (Dalian, China) and the experiment was performed using the three-step method in accordance with the instructions. The primers used are shown in Table III.

Detection of the Protein Expression Level Via Western blotting

The total protein was extracted from the samples using the protein extraction kit (Roche, Basel, Switzerland) according to the modified instructions[11]. Next, the antibody was diluted at 1:5000 according to the instructions provided by Roche (Basel, Switzerland) and other relevant operations were performed in accordance with the Molecular Cloning Manual.

Detection of Cell Proliferation Via MTT Assay

The cells in the logarithmic growth phase were inoculated into a 12-well plate and cultured at 37°C under 5% CO_2 for transfection the next day. According to the instructions of MTT, 25 μL MTT assay solution was added into each well at 6 h before detection, and cultured on a CO_2 shaker for 3 h, followed by centrifugation at 1000 rpm. Then, the mixture was washed with phosphate-buffered saline (PBS), and the supernatant was discarded. 100 μL dimethyl sulfoxide (DMSO) was added, and the absorbance was measured at 570 nm.

Table III. Fluorescence qPCR primers.

Gene	Primer sequences
JAK2	F: 5'-CGCGCTAGCATCGATCAGCTAGC-3' R: 5'-CGGGCTAGCTACGATCGCTACG-3'
STAT3	F: 5'-CGGGCATCGATCGATAAGCTAC-3' R: 5'-CGGCGCATGCTACGATCGACTCG-3'
NOS	F: 5'-GGCGCTAGCGATCGATCGATCG-3' R: 5'-CGGCGCTAGCTACGATCGATCG-3'
GAPDH	F: 5'-TCATGGGTGTGAACCATGAGAA-3' R: 5'-GGCAGGACTGTGGTCATGAG-3'

Detection of Apoptosis Via Flow Cytometry

The cell cycle was detected via flow cytometry in experimental group at 24 h after transfection: the above cells were washed twice with PBS, collected via centrifugation at 1000 rpm, fixed with 70% ethanol for 12 h, and stained with Propidium Iodide (PI). Next, the cell cycle was determined at 488 nm. The apoptosis was detected at 72 h after transfection: above cells were washed twice with PBS, collected *via* centrifugation at 1000 rpm, and stained with 2.5 μL Annexin V and 4.5 μL PI dye in a dark place. Then, the apoptosis was determined via flow cytometry at Ex=488 nm and Em=530 nm[12].

Statistical Analysis

All experimental results were statistically processed using the Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). Data were expressed as ($\chi \pm s$), and one-way analysis of variance was adopted for the multi-sample comparison of means, *t*-test for the comparison of means between two groups, and *q*-test for the intergroup pairwise comparison. $p<0.05$ suggested that the difference was significant.

Results

Difference in the MiR-210 Expression Between Control Group and Experimental Group Detected Via Fluorescence QPCR

To explore the correlation between miR-210 and ASO, the difference in the miR-210 expression in aortic endothelial cells between ASO patients and healthy people was detected. As shown in Figure 1, the expression of miR-210 in aortic endothelial cells in ASO patients was significantly higher than that in aortic endothelial cells in healthy people, and there was a significant difference ($p<0.05$), indicating that miR-210 may have a correlation with ASO.

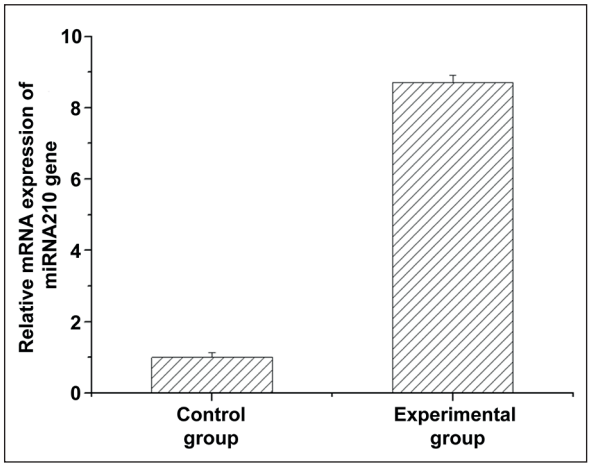

Figure 1. Difference in the miR-210 expression in aortic endothelial cells between ASO patients and healthy people. There is a significant difference in the expression of miR-210 between control group and experimental group ($p<0.05$), indicating that miR-210 may be associated with ASO.

Differences in the JAK2, STAT3, and NOS Gene Transcriptional Levels Detected Via Fluorescence QPCR

To explore the correlation between miR-210 and the JAK-STAT signaling pathway, the cell lines with miR-210 interference and overexpression were constructed with the Crisp/Case9 technique, and the JAK2, STAT3, and NOS gene transcriptional levels in different cell lines were detected. As shown in Figure 2, the JAK2, STAT3, and NOS gene transcriptional levels declined in cell lines with miR-210 interference compared with those in control group, and the differences were significant ($p<0.05$). The JAK2, STAT3, and NOS gene transcriptional levels were increased in cell lines with miR-210 overexpression compared with those in control group, and the differences were significant ($p<0.05$). Above results suggest that the down-regulation of miR-210 expression can inhibit the JAK2, STAT3, and NOS gene transcription, while the up-regulation of miR-210 expression can promote the JAK2, STAT, and NOS gene transcription.

Differences in the JAK2, STAT3, and NOS Gene Protein Levels Detected Via Western Blotting

The differences in the JAK2, STAT3, and NOS gene protein levels between control group and different experimental groups were detected

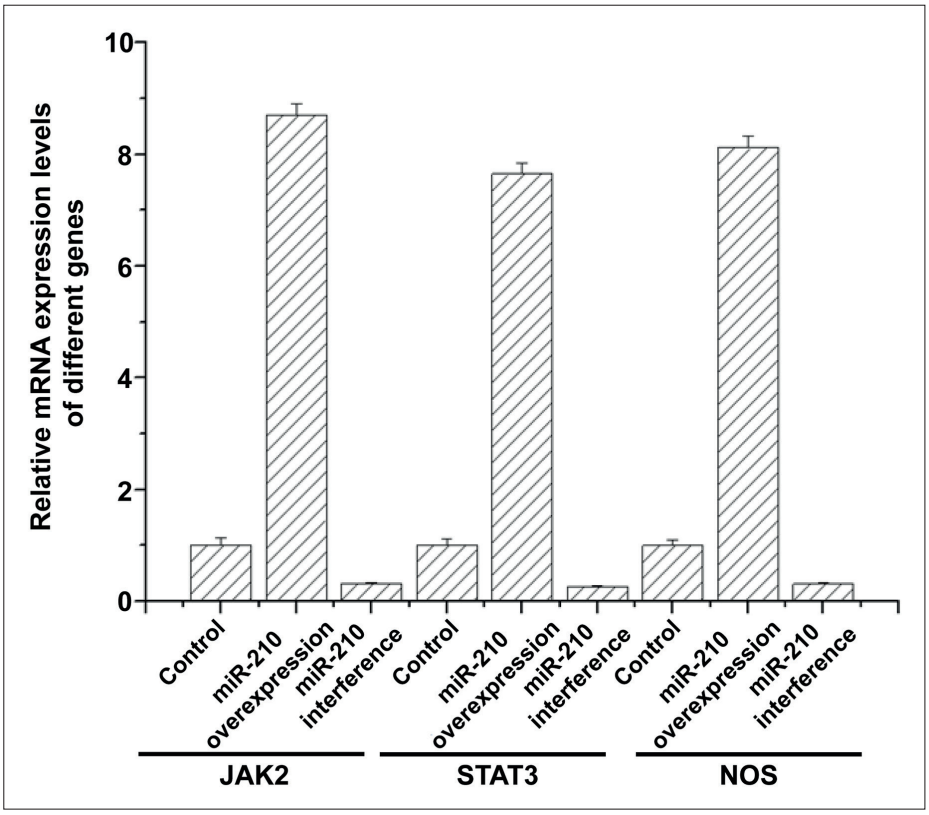

Figure 2. Differences in the JAK2, STAT3, and NOS gene transcriptional levels in experimental group detected *via* fluorescence qPCR.

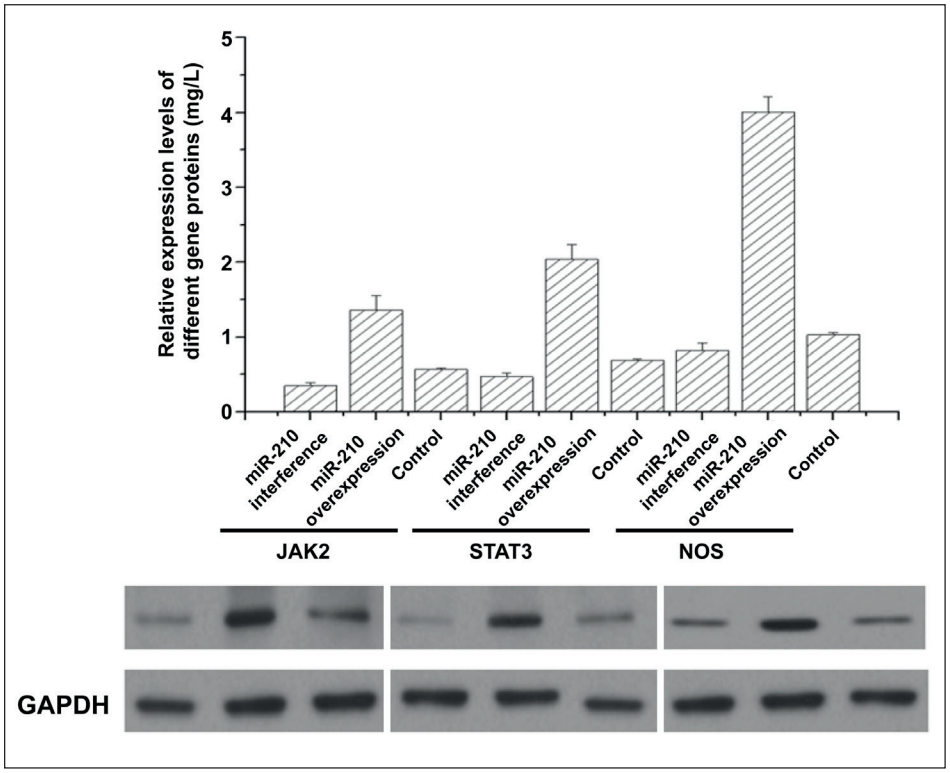

Figure 3. Differences in the JAK2, STAT3, and NOS gene protein levels.

via Western blotting. The results revealed that the JAK2, STAT3, and NOS gene protein levels significantly declined in cell lines with miR-210 interference compared with those in control group, showing significant differences ($p<0.05$). At the same time, the JAK2, STAT3, and NOS gene protein levels were significantly increased in cell lines with miR-210 overexpression compared with those in control group, with significant differences ($p<0.05$) (Figure 3).

Cell Proliferation in Experimental Group and Control Group Detected Via MTT Assay

The cell proliferation in different samples was detected *via* MTT assay and the statistical results are shown in Table IV. It could be seen that the inhibition rate of cell proliferation in cell lines with miR-210 interference markedly declined compared with that in normal cells, displaying a significant difference ($p<0.05$). At the same time, the inhibition rate of cell proliferation in cell lines with miR-210 overexpression was significantly increased compared with that in normal cells and there was a significant difference ($p<0.05$). The above results indicate that the expression level of miR-210 is associated with cell proliferation.

Apoptosis in Experimental Group and Control Group Detected Via Flow Cytometry

To explore the function of miR-210 in apoptosis, the apoptosis of control cells and cell lines with miR-210 interference and overexpression was detected *via* flow cytometry. It was found that the apoptosis rate was markedly increased in cell lines with miR-210 interference compared with that in control cells, showing a significant difference ($p<0.05$). The apoptosis rate was significantly decreased in cell lines with miR-210 overexpression compared with that in control cells, showing a significant difference ($p<0.05$) (Figure 4 and Table V). The above results suggest that the expression level of miR-210 is associated with apoptosis.

Table IV. Inhibition rate of cell proliferation in different cell lines ($\bar{x} \pm s$ %).

Cell line	Inhibition rate of cell proliferation
Control	49.43 ± 5.05
MiR-210 interference	1.245 ± 0.043[a]
MiR-210 overexpression	78.41 ± 0.26[a,b]

Note: [a]: $p<0.01$ *vs*. control group, [b]: $p<0.01$ *vs*. miR-210 interference group.

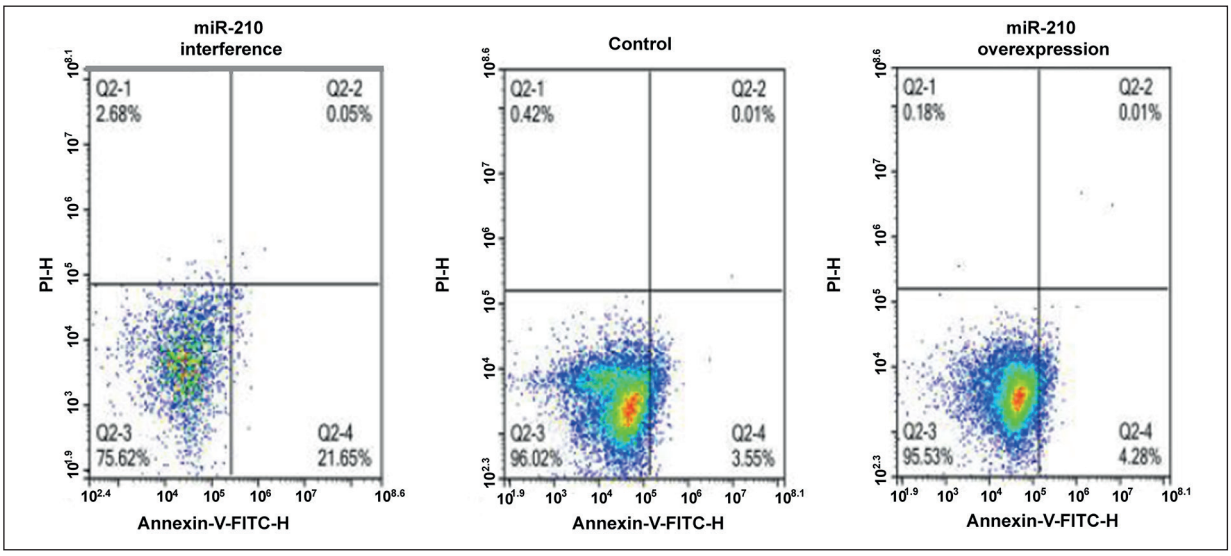

Figure 4. Apoptosis in experimental group and control group detected *via* flow cytometry.

Discussion

With the continuous improvement of people's living standards and the great changes in dietary habit, the morbidity rate of arteriosclerosis has shown an increasing trend year by year[13]. Therefore, exploring the pathogenesis of arteriosclerosis, especially ASO, has become an important direction to improve the quality of life of people, especially the middle-aged and elderly people[14]. With the rapid development of biotechnology, miRNAs, as important regulators involved in the regulation of different genes and metabolic pathways in cells in eukaryotes, especially in the human body, have gradually attracted people's attention[15-17]. For example, recent studies have found that miRNAs can participate in a variety of biochemical pathways in the human body. Moreover, researches have also found that the expression of miR-210 in cells can be significantly changed under hypoxia, and such an increase in expression has no significant tissue specificity. Therefore, it is believed that miR-210 may be involved in the response pathway of cells to hypoxia stress[18]. At the same time, further reports on intracellular miRNAs showed that they can bind to the mRNA sequence of the target gene to inhibit its translation process, thereby regulating the target gene and related metabolic pathways. In recent years, works on ASO have manifested that the intracellular energy supply rate declines in the lesion tissues, and it is presumed to be related to an insufficient intracellular oxygen supply. The lack of oxygen supply affects not only the normal physiological functions of cells, but also the cell cycle and apoptosis. In the present study, the cell lines with miR-210 interference and overexpression were constructed to explore the correlation between miR-210 and ASO. Vascular endothelial cell apoptosis, as an initial stage of ASO, plays an important role in the development of ASO. It was found in the present report that the JAK2, STAT3, and NOS gene transcriptional and translational levels declined in cell lines with miR-210 interference, suggesting that the down-regulation of miR-210 expression can inhibit the JAK-STAT signaling pathway. The previous research results[19,20] showed that the JAK-STAT signaling pathway is associated with such important signal messengers as NO. Here, it was found that the blockade of the JAK-STAT signaling pathway could reduce the intracellular NOS gene transcriptional and translational levels, thus lowering the content of intracellular NO and other important signaling molecules. At the same time, the up-regulation of miR-210 expression could promote the JAK2, STAT3, and NOS

Table V. Apoptosis rate detected in different cell lines.

Cell line	Apoptosis rate ($\bar{x} \pm s$, %)	p
Control	4.47 ± 3.42	
MiR-210 interference	3.98 ± 1.75	
MiR-210 overexpression	24.38 ± 8.68	$p < 0.01$

gene transcriptional and translational levels, thus raising the content of intracellular NO and other molecules. Moreover, the results of MTT assay and flow cytometry showed that the decline in miR-210 expression could decrease cell proliferation and promote apoptosis, while the overexpression of miR-210 could promote cell proliferation and inhibit apoptosis, indicating that miR-210 can regulate the JAK-STAT signaling pathway by regulating the transcriptional and translational of key genes in the signaling pathway, such as JAK2 and STAT3, thereby regulating the expression of such genes as NOS through the signaling pathway, and causing changes in the content of important signaling molecules related to apoptosis, such as NO, ultimately regulating cell proliferation and apoptosis. Although this study has clarified that miR-210 affects the apoptosis of vascular endothelial cells in ASO via the JAK-STAT signaling pathway, due to the limited time, it didn't show how the NO and other signaling molecules are involved in regulating apoptosis. This will be our research focus in the future.

Conclusions

We showed that miR-210 can be involved in the apoptosis process of vascular endothelial cells in ASO through the JAK-STAT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Santulli G, Wronska A, Uryu K, Diacovo TG, Gao M, Marx SO, Kitajewski J, Chilton JM, Akat KM, Tuschl T, Marks AR, Totary-Jain H. A selective microRNA-based strategy inhibits restenosis while preserving endothelial function. J Clin Invest 2014; 124: 4102-4114.
2) Chen WN, Guo SN, Wang JY, Jia LQ, Li DY, Tian Y. [Correlation between autophagy and polarization of macrophages in atherosclerosis plaque in arteriosclerosis obliterans amputees]. Yao Xue Xue Bao 2016; 51: 68-74.
3) Santulli G, Cipolletta E, Sorriento D, Del GC, Anastasio A, Monaco S, Maione AS, Condorelli G, Puca A, Trimarco B, Illario M, Iaccarino G. CaMK4 gene deletion induces hypertension. J Am Heart Assoc 2012; 1: e1081.
4) Santulli G, Trimarco B, Iaccarino G. G-protein-coupled receptor kinase 2 and hypertension: molecular insights and pathophysiological mechanisms. High Blood Press Cardiovasc Prev 2013; 20: 5-12.
5) Hao JP, Shi H, Zhang J, Zhang CM, Feng YM, Qie LY, Dong M, Ji X. Role of GSPE in improving early cerebral vascular damage by inhibition of Profilin-1 expression in a ouabain-induced hypertension model. Eur Rev Med Pharmacol Sci 2018; 22: 6999-7012.
6) Perino A, Ghigo A, Ferrero E, Morello F, Santulli G, Baillie GS, Damilano F, Dunlop AJ, Pawson C, Walser R, Levi R, Altruda F, Silengo L, Langeberg LK, Neubauer G, Heymans S, Lembo G, Wymann MP, Wetzker R, Houslay MD, Iaccarino G, Scott JD, Hirsch E. Integrating cardiac PIP3 and cAMP signaling through a PKA anchoring function of p110gamma. Mol Cell 2011; 42: 84-95.
7) Santulli G. Adrenal signaling in heart failure: something more than a distant ship's smoke on the horizon. Hypertension 2014; 63: 215-216.
8) Santulli G, Ciccarelli M, Trimarco B, Iaccarino G. Physical activity ameliorates cardiovascular health in elderly subjects: the functional role of the beta-adrenergic system. Front Physiol 2013; 4: 209.
9) Wronska A, Kurkowska-Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta Physiol (Oxf) 2015; 213: 60-83.
10) Anderson DM, Anderson KM, Chang CL, Makarewich CA, Nelson BR, McAnally JR, Kasaragod P, Shelton JM, Liou J, Bassel-Duby R, Olson EN. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell 2015; 160: 595-606.
11) Andrews SJ, Rothnagel JA. Emerging evidence for functional peptides encoded by short open reading frames. Nat Rev Genet 2014; 15: 193-204.
12) Santulli G, Ciccarelli M, Palumbo G, Campanile A, Galasso G, Ziaco B, Altobelli GG, Cimini V, Piscione F, D'Andrea LD, Pedone C, Trimarco B, Iaccarino G. In vivo properties of the proangiogenic peptide QK. J Transl Med 2009; 7: 41.
13) Ciccarelli M, Santulli G, Campanile A, Galasso G, Cervero P, Altobelli GG, Cimini V, Pastore L, Piscione F, Trimarco B, Iaccarino G. Endothelial alpha1-adrenoceptors regulate neo-angiogenesis. Br J Pharmacol 2008; 153: 936-946.
14) Santulli G, Basilicata MF, De Simone M, Del Giudice C, Anastasio A, Sorriento D, Saviano M, Del GA, Trimarco B, Pedone C, Zaccaro L, Iaccarino G. Evaluation of the anti-angiogenic properties of the new selective alphaVbeta3 integrin antagonist RGDechiHCit. J Transl Med 2011; 9: 7.
15) Hao XZ, Fan HM. Identification of miRNAs as atherosclerosis biomarkers and functional role of miR-126 in atherosclerosis progression through MAPK signalling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 2725-2733.
16) Yamamoto S, Niida S, Azuma E, Yanagibashi T, Muramatsu M, Huang TT, Sagara H, Higaki S, Ikutani M,

Nagai Y, Takatsu K, Miyazaki K, Hamashima T, Mori H, Matsuda N, Ishii Y, Sasahara M. Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes. Sci Rep 2015; 5: 8505.

17) Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D. MiR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009; 460: 705-710.

18) Iio A, Nakagawa Y, Hirata I, Naoe T, Akao Y. Identification of non-coding RNAs embracing microRNA-143/145 cluster. Mol Cancer 2010; 9: 136.

19) Batliner J, Buehrer E, Fey MF, Tschan MP. Inhibition of the miR-143/145 cluster attenuated neutrophil differentiation of APL cells. Leuk Res 2012; 36: 237-240.

20) Iio A, Takagi T, Miki K, Naoe T, Nakayama A, Akao Y. DDX6 post-transcriptionally down-regulates miR-143/145 expression through host gene NCR143/145 in cancer cells. Biochim Biophys Acta 2013; 1829: 1102-1110.