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MiR-223 alleviates thrombus and inflammation in thromboangiitis obliterans rats by regulating NLRP3

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Abstract. – OBJECTIVE: The aim of this study was to observe the regulatory effects of micro ribonucleic acid (miR)-223 on thromboangiitis obliterans (TAO) rats, and to explore the potential regulatory mechanism.

MATERIALS AND METHODS: Online database TargetScan was used to predict the downstream regulatory targets of miR-223. A total of 45 Sprague Dawley (SD) rats were randomly divided into three groups, including sham operation group (Sham group), Model group, and miR-223 agonist group (miR-223 mimic group). TAO model was successfully established in rats through the injection of lauric acid via the femoral artery. The content of serum thromboxane B2 (TXB2) and endothelin (ET) was measured via enzyme-linked immunosorbent assay (ELISA). The pathological changes in the left hind limb were detected via hematoxylin-eosin (HE) staining. Moreover, the expressions of interleukin-6 (IL-6) and IL-1β in the tissues of the rat left hind limb were determined via immunohistochemistry. In addition, the protein expression of Nod-like receptor protein 3 (NLRP3) in tissues was determined using Western blotting.

RESULTS: TargetScan database predicted that NLRP3 was the downstream target gene of miR-223. Compared with the Sham group, Model group exerted significantly higher content of serum TXB2 and ET, severe lesions in the rat left hind limb, as well as significantly increased expressions of IL-6 and IL-1β and protein expression of NLRP3 in tissues of the rat left hind limb ($p<0.05$). Besides, compared with the Model group, miR-223 mimic group showed remarkably lower content of serum TXB2 and ET, improved lesions in the rat left hind limb, as well as decreased expressions of IL-6 and IL-1β and protein expression of NLRP3 in the tissues of the rat left hind limb ($p<0.05$).

CONCLUSIONS: MiR-223 agonist can alleviate thrombus and inflammatory response in TAO rats. The possible underlying mechanism may be related to targeted regulation on NLRP3 inflammasome expression.

Key Words:
MiR-223, Thromboangiitis obliterans, NLRP3 inflammasome, Inflammatory response.

Introduction

Thromboangiitis obliterans (TAO), also known as Buerger's disease, is a common peripheral vascular disease mainly occurs in the limbs[1]. With the development of the disease, gangrene, and ulceration will occur in severe cases[2,3]. Currently, TAO has been effectively controlled with the development of drugs in clinical practice. However, it fails to be cured. Therefore, searching for novel biomarkers and the pathogenesis of TAO is of great significance for the treatment of the disease.

Inflammasome is an important component of innate immunity, which also plays an important role in the occurrence of immune response and disease development. Inflammasome also has a critical effect in the pathogenesis of TAO[4,5]. Inflammasome is a complex composed of multiple proteins with a molecular weight of about 72 kDa. It can mainly be classified into 5 categories, including Nod-like receptor protein 1 (NLRP1), NLRP3, NLRC4, IPAF, and AIM2 inflammasomes[6]. As the most widely studied inflammasome, NLRP3 can identify a variety of pathogens and related risk factors. Meanwhile, its regulatory role in TAO has attracted increasingly attention from researchers[7]. NLRP3 could play a crucial role in the pathogenesis of TAO.

Micro RNAs (miRNAs) are a kind of non-coding ribonucleic acids (RNAs) with about 22 nucleotides in length. With the in-depth research of molecular biology, miRNAs have been discov-

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ered[8,9] to possess multiple important regulatory functions in cells. MiR-223 is a miRNA closely related to inflammation[10]. It can also inhibit inflammatory response in the intestine and reduce the release of inflammatory factors *via* regulating NLRP3 during the onset of enteritis[11]. Moreover, miR-223 plays an important regulatory role in acute lung injury, which is able to regulate inflammatory response by regulating the activation of macrophages. In the case of miR-223 overexpression, the pulmonary inflammatory response of lipopolysaccharide-induced acute lung injury can be inhibited to a certain extent[12]. However, the regulatory role of miR-223 in TAO has been rarely reported. In this experiment, therefore, the rat model of TAO was established through injection of lauric acid *via* the femoral artery. Our study aimed to explore the regulatory effect of miR-223 on TAO rats and its regulatory mechanism.

Materials and Methods

Reagents

Lauric acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), hematoxylin-eosin (HE) staining kit from Beijing Solarbio Technology Co., Ltd. (Beijing, China), immunohistochemistry kit, TRIzol and first-strand reverse transcription kit from Invitrogen (Carlsbad, CA, USA), miR-223 agonist from Shanghai GenePharma Co., Ltd. (Shanghai, China), thromboxane B_2 (TXB_2) and endothelin (ET) enzyme-linked immunosorbent assay (ELISA) kit from R&D (Minneapolis, MN, USA), NLRP3 and β-actin primary antibodies from Cell Signaling Technology (Danvers, MA, USA), and horse radish peroxidase (HRP)-labeled secondary antibodies from Abcam (Cambridge, MA, USA).

Instruments

Full-automatic biochemical analyzer was purchased from Abbott (Abbott Park, IL, USA), gel imager from Shanghai Clinx Science Instruments Co., Ltd. (Shanghai, China), NanoDrop instrument from Thermo Fisher Scientific (Waltham, MA, USA), microplate reader and electrophoresis instrument from Bio-Rad (Hercules, CA, USA), centrifuge from Hunan Pingfan Science and Technology Co., Ltd. (Changsha, China), and microscope from Nikon (Tokyo, Japan).

Experimental Rats

Healthy adult male Sprague Dawley (SD) rats weighing (240±10) g were provided by Shandong First Medical University. All rats were routinely fed in the laboratory, with free access to water and food. This study was approved by the Animal Ethics Committee of Shandong First Medical University Animal Center.

Prediction of Relation Between MiR-223 and NLRP3 Using the TargetScan Database

TargetScan is a website specialized in the prediction of miRNA target genes, including those of human, mouse, rat, fruit fly, nematode, and zebrafish[13]. The query process was as follows: first, open the TargetScan website (http://www.targetscan.org), and select "Rat" in "Select a species". Then, enter "NLRP3" in "Enter a mouse gene symbol" and click "Submit". Finally, wait for the database query results.

Establishment of TAO Model in Rats

After preoperative fasting for 12 h, healthy male SD rats were anesthetized with isoflurane using an anesthesia machine. The groin was cut open at the midpoint, and subcutaneous tissues and muscle tissues were bluntly stripped off. After stripping off the femoral artery, the proximal end was clamped using the artery clamp to block the blood flow. In the Model group, 0.2 mL of lauric acid solution (5 g/L) was injected into the distal end below the artery clamp. Next, absorbent cotton was used to stop bleeding. After 15 min, the skin would be sutured if there was no bleeding. After operation, penicillin was injected intraperitoneally to prevent infection. In Sham group, 0.2 mL of normal saline was injected as a control, and the rats were fed separately after operation. In the miR-223 mimic group, miR-223 agonist was injected *via* the caudal vein every 3 d. After treatment for 15 d, the content of TXB_2 and ET in the serum was determined.

Detection of Pathological Changes in the Left Hind Limb Via HE Staining

According to the instructions of HE staining, the sections were first deparaffinized with xylene, and dehydrated with ethanol at a concentration gradient from low to high. Subsequently, the sections were stained with hematoxylin dye for 15 s. After washing with double distilled water, the sections were stained with eosin dye for 15 s, followed by washing again. Next, the

sections were sealed with neutral resins. The staining was finally observed under a fluorescence microscope.

Determination of Interleukin-6 (IL-6) and IL-1Ð Protein Expressions in Tissues of the Left Hind Limb Using Immunohistochemistry

Tissues of the rat left hind limb were first cut into sections and soaked in xylene for 20 min, followed by dehydration. Endogenous peroxidase activity was blocked with H_2O_2. Next, 5% bovine serum albumin (BSA) blocking buffer was added for 30 min of blocking for antigen exposure. The sections were then incubated with IL-6 and IL-1β antibodies (1:500, diluted with 1% BSA) for 1 h, followed by washing with phosphate-buffered saline (PBS) for 3 times. After that, the sections were incubated again with secondary antibodies. Color development was conducted with diaminobenzidine (DAB; Solarbio, Beijing, China). After counterstaining, the sections were sealed with neutral resins. The staining was finally observed under a microscope.

Determination of NLRP3 Protein Expression in Tissues of the Left Hind Limb Using Western Blotting

The tissues of the rat left hind limb were first lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and total protein was extracted. Protein concentration was determined using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Subsequently, the protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel at 90 V, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were sealed with 5% skimmed milk powder solution and washed with cleaning solution for 3 times, with 5 min for each. According to the size of marker protein, the membrane was incubated with NLRP3 primary antibody (1:1000) at 4°C overnight. On the next day, the membrane was incubated with corresponding secondary antibody (1:2000). Color development was finally conducted with DAB solution.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. GraphPad Prism 6.0 software (La Jolla, CA, USA) was used for plotting. Experimental data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between two groups. $p<0.05$ suggested statistically significant difference.

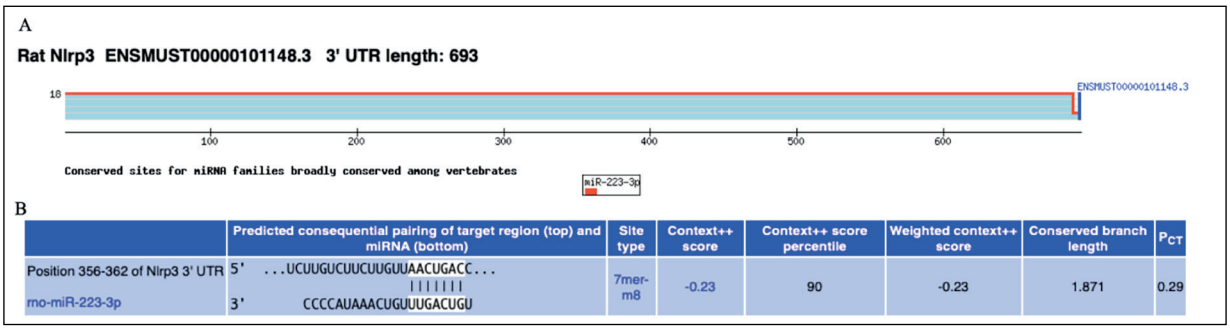
Results

NLRP3 Was the Target Gene of MiR-223 According to TargetScan Database

MiRNAs that were able to regulate target "NLRP3" were inquired in the species "Rat" using TargetScan database. It was found that miR-223 could regulate NLRP3 in a targeted way. Meanwhile, it was highly homologous in rat, mouse, and human (Figure 1A). The relationship between miR-223 and NLRP3 was shown in Figure 1B. There were potential binding sites at 356-362 in the NLRP3 3'UTR for miR-223, indicating that NLRP3 was a target gene of miR-223.

MiR-223 Agonist Could Inhibit Content of Serum TXB2 and ET in TAO Rats

The content of serum TXB_2 and ET in TAO rats was detected using ELISA. As shown in Fig-



	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 356-362 of Nlrp3 3' UTR	5' ...UCUUGUCUUCUUGUUAACUGACC...	7mer-m8	-0.23	90	-0.23	1.871	0.29
mo-miR-223-3p	3' CCCCAUAAACUGUUUGACUGU						

Figure 1. Prediction results using TargetScan database. **A,** MiRNAs was able to bind to NLRP3 predicted using TargetScan database. **B,** Binding sites between miR-223 and NLRP3.

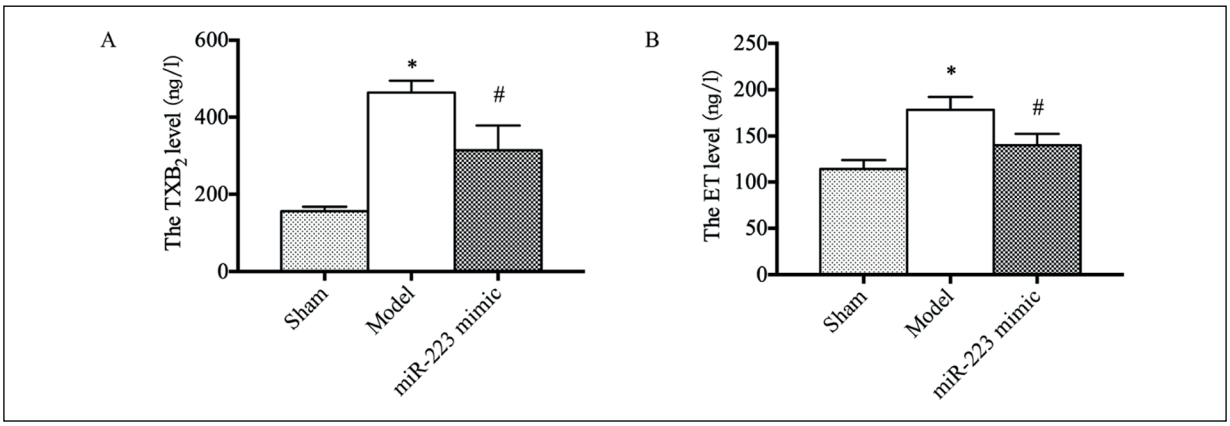


Figure 2. Content of serum TXB_2 and ET in TAO rats. **A,** TXB_2 content. **B,** ET content.

ure 2A-2B, the content of serum TXB_2 and ET rose significantly in the Model group when compared with the Sham group (*p<0.05, *p<0.05). However, it declined significantly in the miR-223 mimic group when compared with the Model group ($^{\#}p$<0.05, $^{\#}p$<0.05). These findings suggested that miR-223 agonist could inhibit thrombus formation in TAO rats.

MiR-223 Agonist Could Improve Pathological Damage in the Left Hind Limb of TAO Rats

The skin color and swelling degree of left hind limb were observed roughly in rats. In the Model group, the left hind limb was pale and had lower skin temperature. After treatment with miR-223 agonist, muscles of the rat left hind limb became flexible, and the skin temperature rose. Meanwhile, limb ulcers were relieved, and perivascular edema was significantly improved (Figure 3). The above findings indicated that miR-223 agonist could improve the pathological damage in the limbs of TAO rats.

MiR-223 Agonist Suppressed Inflammatory Response in the Rat Left Hind Limb of TAO Rats

The effect of miR-223 on inflammatory response in the left hind limb of TAO rats was determined using immunohistochemical staining. The results manifested that the expressions of IL-6 and IL-1β in tissues of the rat left hind limb significantly increased in the Model group compared with Sham group (*p<0.05). However, they evidently declined in the miR-223 mimic group compared with the Model group (*p<0.05, Figure 4). The results demonstrated that miR-223 agonist could suppress inflammatory response in the affected limb of TAO rats.

MiR-223 Agonist Inhibited NLRP3 Protein Expression in Tissues of the Left Hind Limb of TAO Rats

NLRP3 protein expression in the tissues of the rat left hind limb was determined *via* Western blotting. The results revealed that the Model group exhibited remarkably higher protein ex-

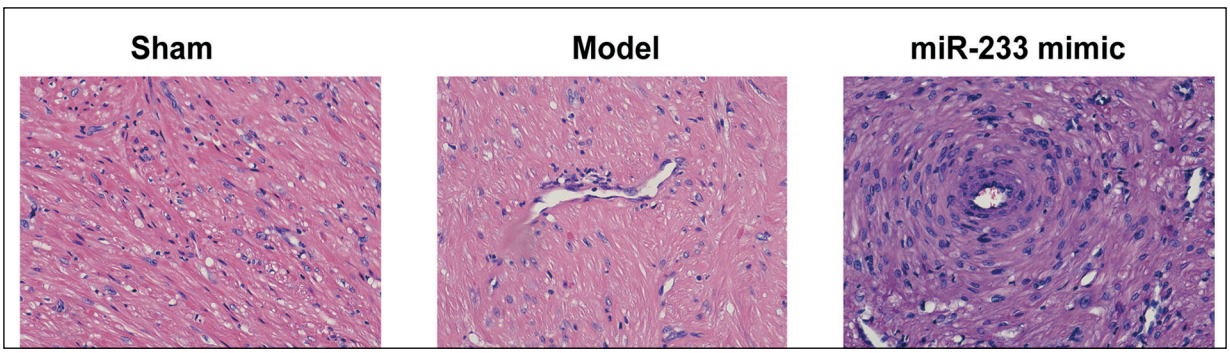


Figure 3. Pathological changes in the tissues of the affected rat limb detected *via* HE staining (magnification: 20×).

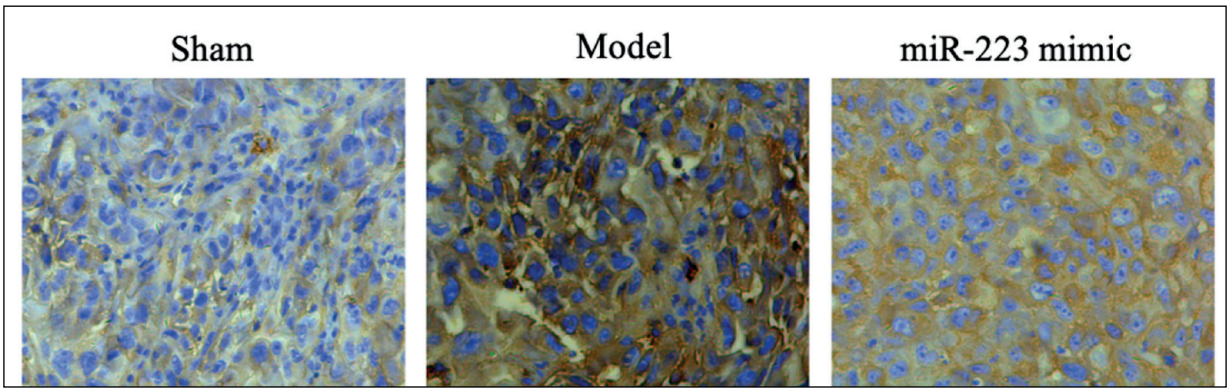


Figure 4. Levels of IL-6 and IL-1β in the rat left hind limb detected using immunohistochemical staining (magnification: 20×).

pression of NLRP3 in the tissues of the rat left hind limb than the Sham group (*p<0.05). However, miR-223 mimic group had significantly lower NLRP3 protein expression in the tissues of the rat left hind limb than the Model group ($^{\#}p$<0.05) (Figure 5). It could be concluded that miR-223 agonist inhibited the protein expression of NLRP3 in tissues of the affected limb of TAO rats.

Discussion

TAO is a vascular inflammatory disease characterized by recurrent, progressive, and phased occurrence. Epidemiological data have shown that TAO is closely related to smoking, infection, immune inflammation, and genetics[14]. Pathological manifestations of TAO in patients include coldness and numbness of limbs, and intermittent claudication. Currently, the main clinical treatment methods for TAO are conservative therapy and surgery. Despite some progresses[15] have been made in its treatment with the constant advance in medicine, TAO fails to be cured.

MiRNAs are a class of non-coding RNAs. Each miRNA can regulate multiple target genes, while several miRNAs can regulate the same gene[16]. MiRNAs regulate about one-third of genes. Meanwhile, miRNAs can promote degradation or inhibit translation of target genes into proteins by binding to the 3'UTR of target genes[17]. For example, miR-223 is closely related to the regulation on biological processes and is highly conserved among species, such as rat, mouse, and zebrafish. Its promoter is highly conserved as well. In recent years, it has been disclosed that miR-223 plays a key role in lipid metabolism and the occurrence and development of malignant tumors[18]. In par-

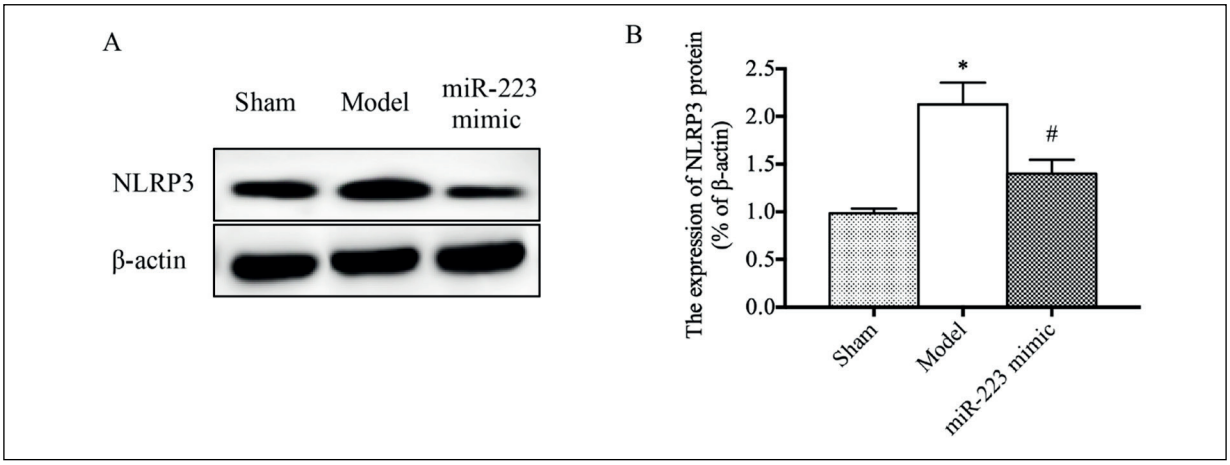


Figure 5. NLRP3 protein expression in the affected limb. **A,** Western blotting bands, with β-actin as an internal reference. **B,** Statistical chart of NLRP3 protein expression.

ticular, miR-223 has an important effect on the occurrence of inflammation. Ding et al[19] have found that miR-223 can suppress the occurrence of glioblastoma and inflammatory response. The possible mechanism is related to the regulation on NLRP3. It can be concluded that miR-223 may be an inhibitor and potential target in the treatment of glioblastoma[19]. As an inflammasome, NLRP3 is highly expressed in TAO. Therefore, inhibiting the expression of NLRP3 will open up a new way for exploring the pathogenesis of TAO[20].

In this study, the relationship between miR-223 and NLRP3 was first detected using the online database. The results showed that miR-223 could regulate NLRP3 in a targeted way. There were also binding sites in the NLRP3 3'UTR for miR-223. The rat model of TAO was successfully established through injection of lauric acid *via* the femoral artery. TXB2 and ET were measured to evaluate the degree of thrombus aggregation and vascular injury in rat hind limb, respectively. Our results showed that TAO Model group exerted significantly higher content of serum TXB2 and ET, more severe lesions in the rat left hind limb, compared with the Sham group. Meanwhile, the rats were treated with miR-223 agonist. Subsequent results indicated that miR-223 agonist could inhibit the content of serum TXB_2 and ET and the formation of thrombus in TAO rats. Next, the pathological changes in affected rat limb were detected *via* HE staining. The results manifested that miR-223 agonist could significantly improve the pathological damage and ameliorate the function of affected limb. Moreover, the expressions of IL-6 and IL-1β in the affected rat limb were determined *via* immunohistochemical staining. Yellow brown color revealed IL-6- and IL-1β-positive cells. Our findings found that yellow brown color significantly became light in the miR-223 mimic group compared with the Model group. This suggested that miR-223 agonist could remarkably inhibit the expressions of IL-6 and IL-1β and inflammatory response in the affected limb of TAO rats. To further explore the regulatory mechanism of miR-223 and verify the prediction results of TargetScan database, Western blotting was conducted. The results revealed that miR-223 mimic group exhibited remarkably lower protein expression level of NLRP3 in the tissues of the affected rat limb than the Model group. All these findings indicated that miR-223 agonist could exert a therapeutic effect on TAO rats *via* downregulating NLRP3 expression.

Conclusions

Taken together, our results showed that miR-223 could inhibit inflammatory response and thrombus formation in TAO rats by regulating NLRP3 protein expression. In addition, miR-223 exerted a protective effect against TAO, which might be a new therapeutic target for TAO.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Modaghegh MS, Hafezi S. Endovascular treatment of thromboangiitis obliterans (Buerger's disease). Vasc Endovascular Surg 2018; 52: 124-130.
2) Sapkota P, Budhathoky P, Mathew S. Smoking and thromboangitis obliterans- are they related? JNMA J Nepal Med Assoc 2014; 52: 802-805.
3) Olin JW, Young JR, Graor RA, Ruschhaupt WF, Bartholomew JR. The changing clinical spectrum of thromboangiitis obliterans (Buerger's disease). Circulation 1990; 82: V3-V8.
4) Rathinam VA, Fitzgerald KA. Inflammasome complexes: emerging mechanisms and effector functions. Cell 2016; 165: 792-800.
5) Song N, Li T. Regulation of NLRP3 inflammasome by phosphorylation. Front Immunol 2018; 9: 2305.
6) Elliott EI, Sutterwala FS. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. Immunol Rev 2015; 265: 35-52.
7) Guo H, Sun L, Ling S, Xu JW. Levistilide A ameliorates NLRP3 expression involving the Syk-p38/JNK pathway and peripheral obliterans in rats. Mediators Inflamm 2018; 2018: 7304096.
8) Tafrihi M, Hasheminasab E. MiRNAs: biology, biogenesis, their web-based tools, and databases. Microrna 2019; 8: 4-27.
9) Lu TX, Rothenberg ME. MicroRNA. J Allergy Clin Immunol 2018; 141: 1202-1207.
10) Guo C, Xie S, Chi Z, Zhang J, Liu Y, Zhang L, Zheng M, Zhang X, Xia D, Ke Y, Lu L, Wang D. Bile acids control inflammation and metabolic disorder through inhibition of NLRP3 inflammasome. Immunity 2016; 45: 944.
11) Neudecker V, Haneklaus M, Jensen O, Khailova L, Masterson JC, Tye H, Biette K, Jedlicka P, Brodsky KS, Gerich ME, Mack M, Robertson A, Cooper MA, Furuta GT, Dinarello CA, O'Neill LA, Eltzschig HK, Masters SL, McNamee EN. Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. J Exp Med 2017; 214: 1737-1752.

12) Zhang D, Lee H, Wang X, Groot M, Sharma L, Dela CC, Jin Y. A potential role of microvesicle-containing miR-223/142 in lung inflammation. Thorax 2019; 74: 865-874.

13) Tian T, Wang J, Zhou X. A review: microRNA detection methods. Org Biomol Chem 2015; 13: 2226-2238.

14) Fazeli B, Keramat S, Assadi L, Taheri H. Angiogenesis induction in Buerger's disease: a disease management double-edged sword? Orphanet J Rare Dis 2019; 14: 189.

15) Shapouri-Moghaddam A, Saeed MM, Rahimi HR, Ehteshamfar SM, Tavakol AJ. Molecular mechanisms regulating immune responses in thromboangiitis obliterans: a comprehensive review. Iran J Basic Med Sci 2019; 22: 215-224.

16) Zealy RW, Wrenn SP, Davila S, Min KW, Yoon JH. MicroRNA-binding proteins: specificity and function. Wiley Interdiscip Rev RNA 2017; 8. doi: 10.1002/wrna.1414.

17) Mellis D, Caporali A. MicroRNA-based therapeutics in cardiovascular disease: screening and delivery to the target. Biochem Soc Trans 2018; 46: 11-21.

18) Yuan X, Berg N, Lee JW, Le TT, Neudecker V, Jing N, Eltzschig H. MicroRNA miR-223 as regulator of innate immunity. J Leukoc Biol 2018; 104: 515-524.

19) Ding Q, Shen L, Nie X, Lu B, Pan X, Su Z, Yan A, Yan R, Zhou Y, Li L, Xu J. MiR-223-3p overexpression inhibits cell proliferation and migration by regulating inflammation-associated cytokines in glioblastomas. Pathol Res Pract 2018; 214: 1330-1339.

20) Taniguchi T, Higuchi T, Tazaki J, Saito N, Kimura T. Successful percutaneous transcatheter angioplasty of radial artery in thromboangiitis obliterans (Buerger's disease). JACC Cardiovasc Interv 2017; 10: e205-e206.