MiR-513a-5p Aggravates Atherosclerosis Phenotype by Downregulating TFPI2 in Vitro

Running title: MiR-513a-5p aggravates atherosclerosis phenotype

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Abstract

Background: Atherosclerosis (AS) induced cardiology disease is largely associated with morbidity and mortality. The dysfunction of vascular smooth muscle cells (VSMCs) is considered to contribute to the etiology of AS. However, the mechanism underlying VSMCs dysfunction remains largely unclear. Our study aimed to explore novel molecules mediating VSMCs function.

Methods: Bioinformatical analysis was applied to identify the key miRNAs that was aberrantly expressed in AS mouse and potentially targeted TFPI2. The AS-like cell model was generated by treating VSMCs with ox-LDL. The expression level of miR-513a-5p and TFPI2 in VSMCs and the serum of AS patients was evaluated by RT-qPCR, and the expression level of TFPI2 and PCNA was measured by western blot. The cell viability and migration capacity of VSMCs were determined by CCK-8 and wound healing assay, respectively. The target relationship between miR-513a-5p and TFPI2 was validated by dual-luciferase assay.

Results: MiR-513a-5p was highly expressed while TFPI2 presented a low expression in AS patient serum. Treatment with 100 μg/mL ox-LDL overtly facilitated the cell viability and migration of VSMCs, also promoted miR-513a-5p expression while limit the expression of TFPI2. Moreover, silencing miR-513a-5p inhibited the cell viability, migration and the expression of proliferative marker in ox-LDL treated VSMCs, while inhibition of TFPI2 enhanced that. It was further found that miR-513a-5p could target TFPI2 and silencing miR-513a-5p compromised the aggresive effect of TFPI2

inhibition on the viability and migration ox-LDL treated VSMCs.

Conclusion: miR-513a-5p could contribute to the dysfunction of VSMCs in AS through targeting and inhibiting TFPI2.

Keywords: atherosclerosis; vascular smooth muscle cells; miR-513a-5p; TFPI2

Introduction

In the past half of century, atherosclerosis (AS) derived cardiovascular disease including myocardial infarction, angina, stroke and heart failure sustains to be the critical cause of death throughout the world, imposing a considerable social and economic burden (World Health Organization. The top 10 causes of death. WHO https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death). The occurrance of AS is a compex process of plaque formation and rupture in the intima of arteries, which involves lipid retention, inflammatory cell infiltration, vascular smooth muscle cells (VSMCs) modulation and migration, as well as fibrous tissue deposition (1). Although evidences points out the pathogenesis of AS attributes to maladaptive immune response and dysregulated lipid metabolism in the arterial wall (2-4), accumulating reports unravel the crucial role of VSMCs who possess remarkable plasticity in the initiation and progression of AS. VSMCs are the dominant cell type at almost each stage of AS progression and undergo multiple phenotypic conversion during the plaque formation. They will switch from a quiescent contractile phenotype into a proliferative synthetic phenotype in the context of arterial injury and lipid retention (1, 5-7). In addition to potentiated ablility of proliferation and migration, the synthetic VSMCs also induced large amount synthesis of extracellular matrix (ECM), which together with synthetic VSMCs constitutes the major structure of atherosclerotic plaque (8, 9). At the stage of plague rupture, VSMCs released matrix metalloproteinases (MMPs) initiate the ECM degradation and the spontaneous apoptosis of VSMCs further

hasten the plaque instability (10). Given the significance of VSMCs in AS development, it is much valuable to explore the molecules regulating VSMCs function, thus provide novel molecular targets for developing AS theraputic strategies.

Tissue factor pathway inhibitor-2 (TFPI-2), which was first identified from the placental tissue and named as placental protein 5 (PP5), retains a homology Kunitz-inhibitory domain and is classified as a member of Kunitz-type serine proteinase inhibitor family (11, 12). However, distinct from the typcial Tissue factor pathway inhibitors such as TFPI-1 which presents strong inhibtion to coagulation proteins including factor Xa and factor VIIa/TF complex, TFPI-2 can only weakly regulate the expression of these proteins and the process of blood coagulation (13). Due to its high expression in endothelial cells (ECs) and VSMCs (14), the function of TFPI2 in AS arouses wide concern in the following study. Herman et al. demonstrated that TFPI2 could diminish ECM degradation wthin human atheroma through inactivating several interstitial collagenases such as MMP-1, MMP-2, MMP-9 and MMP-13, thus impeding the atherosclerotic plaque rupture (15). Hong et al. revealed conditional knockout of TFPI2 in vascular endothelial cells aggravate atherosclerotic progression in mice (16). Moreover, in oxidized low density lipoprotein (ox-LDL) stressed VSMCs cells, TFPI2 was discovered to be downregualted, and overexpression of TFPI2 could restrain the migration and proliferation of VSMCs in this AS cell model (17). Despite these advances, the molecules regulating TFPI2 in AS remains to be defined.

MicroRNAs, also termed miRNAs, are a cluster of small noncoding RNAs with the

capacity to target and suppress protein-coding genes expression via binding to the 3'-UTRs of these genes (18). Since the miRNA was identified in the early 1990s, emerging reports elaborate the pivotal role of miRNAs in varieties of pathophysiological processes such as organogenesis, immune response and tumorigenesis (19-22). Additionally, miRNAs are also reported to widely participate in the development of AS (23, 24). For instance, miR-663 was found as an inhibitor of human VSMCs switching from contractile phenotype to synthetic phenotype (25). MiR-let-7g was proved to inhibit VSMCs proliferation and apoptosis, thus ameliorate the AS phenotype both in oxLDL-induced AS-like cell model and high-fat fed Apoe-deficient mice model (26, 27). MiR-223 was another miRNA that was demonstrated with increased expression within atherosclerotic plaques of AS patients and exerted an atherogenic role in vitro and in vivo, mainly through controling VSMCs functional behaviour (28). To this respect, we speculated that some miRNAs might act as the regulator of TFPI2 to mediate AS progression, which need to be explored.

Here, we employed bioinformatical analysis in a GEO dataset with dysregulated miRNAs in AS cell and mice model and a miRNA dataset that targets TFPI2 to identify miRNAs potentially regulating TFPI in the ox-LDL induced AS cell model. As the most promising candidate, the role miR-513a-5p and its interaction with TFPI were further studied, which will provide a further insight into the pathogenesis of AS.

Materials and Methods

Clinical tissue samples

Forty AS patients examined with more than 70% coronary artery stenosis were recrtuited as patients donors. Meanwhile, Forty healthy individuals were recruited as controls. After the informed consents were obtained, the vein blood was collected from the healthy donors or AS patient donors. The fresh blood was then centrifuged at 4°C for 15 minutes and the upper serum was collected and stored at -80°C for further assay. The present study was approved by the Ethics Committee of the Sixth Hospital of Wuhan (Wuhan, China). The clinical tissue samples were collected and processed by following the ethical standards of the Declaration of Helsinki. Informed consent has been obtained from all individuals included in this study.

Cell culture

Human primary aortic smooth muscle cells (HASMC, here we called VSMCs) were bought from the Global Bioresource Center: ATCC (USA). Dulbecco's modified Eagle's medium (DMEM) containing10% fetal bovine serum (FBS), 10 ng/mL of fibroblast growth factor, 100 μg/mL streptomycin and 100 U/mL penicillin served as the complete culture media of VSMCs. The cells were incubated in a humidified container with 5% CO_2 at 37°C. The subculturing was performed by trypsinization with digestion buffer (0.25% trypsin and 0.02% EDTA in PBS), and VSMCs from Passage 3~7 were applied for the experiments.

Cell treatment and transfection

Ox-LDL was used to treat VSMCs for 24 h to establish an AS-like cell model according

to the previous report (17). For cell transfection, the well-growth VSMCs were dissociated and seeded into 6-well plates with 2×10^4/well. After overnight culture, VSMCs were transiently transfected with miR-513a-5p inhibitor, TFPI2 siRNA, or their corresponding negative control by lipofectamine 3000 (Invitrogen, USA). After 24-hour incubation, The medium was updated with the complete culture medium supplemented with 100 μg/mL ox-LDL treatment for anther 24-h culture. Then after, the cells were collected to apply for transfection efficiency assay and other functional assays. The miR-513a-5p inhibitor, TFPI2 siRNA, and their corresponding negative control (inhibitor-NC and si-NC) were bought from RiboBio (Guangzhou, China), and their sequences were listed in Table 1.

RNA isolation and RT-qPCR

Total RNA in clinical serum samples or vascular smooth muscle cells were isolated by using the RNAprep Pure Cell/Bacteria Kit (Tiangen Biochemical, China) according to the manufacture's guideline. The RNA concentration was quantified with NanoDrop2000 (Thermofisher Scientific, USA). Afterwards, 2 μg RNA was first subjected to reverse transcription by using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA), and then the quantitative real-time PCR (RT-qPCR) was conducted to determine the expression of TFPI2 through the use of SYBR Green PCR Master Mix (Applied Biosystems, USA). On the other hand, the miR-513a-5p expression level was deternimed by usomg All-in-One™ miRNA qRT-PCR detection kits (Genecopoeia, USA) was employed to detect. GAPDH and U6 served as the reference control for

TFPI2 and miR-513a-5p, respectively, and the primer sequences of targets and references were listed in Table 2.

Cell viability assay

The VSMCs viability was evaluated with the Cell Counting Kit-8 (CCK-8) (YEASEN, China). Firstly, the VSMCs underwent transfection and ox-LDL treatment were harvested and seeded into a 96-well plates with 2×10^3/well. Then the cells were constantly incubated at 37°C in a humidified container containing 5% CO_2. At culture time of 0 h, 24 h, 48 h and 72 h, the VSMCs were harvested, and 2 hours before harvest, 10 μL CCK8 solution were added into each cell well. At last, the microplate reader (Bio-Rad, USA) was applied to measure the optical density at 450 nm within 30 minutes.

Wound healing assay

The wound-healing assay was applied for the evaluation of the migration capability of VSMCs as previously reported (29). In brief, 2×10^5 VSMCs underwent transfection and ox-LDL treatment were first seeded into each well of a 6-well plate and cultured upto the cell confluence reached to over 90%. Afterwards, the scratches in cell monolayers were made with a sterile 200 μL micropipette tip, and the exfoliated cells were gently washed away with sterile PBS. Then the cells underwent a follou-up culture in serum-free media for 24 hours. The wound areas at 0 h and 24 h were captured with an optical microscope (Leica, Germany) and the wound widths were measured.

Western blot assay

The total protein in VSMCs was extracted by using the RIPA buffer (Solarbio, China) containing proteinase inhibitor cocktail and quantified by the use of the BCA protein assay kit (Pierce, USA). Then after, 30 μg protein was taken out from each experimental group to be separated by 12% SDS-PAGE gels and electronically transferred onto a PVDF membrane (Millipore, MA, USA). The PVDF membrane was then blocked with 5% non-fat milk in PBST (PBS containing 0.1% Tween20) for 2 h at room temperature and applied for incubation with diluted primary anti-TFPI2 antibody (Cat#: ab186747, Abcam, UK) or anti-GAPDH antibody (Cat#: ab9485, Abcam, UK) overnight at 4°C. After washing with PBST again, the membrane was subjected to incubation with HRP conjugated Goat Anti-Rabbit secondary antibody (Cat#: ab205718, Abcam, UK) for 1.5 h at room temperature. At last, the blots were visualized by using the ECL kit (Pierce, USA) and quantitified with Image J .

Dual-luciferase assay

According to the predictive results of TargetScan on the binding sequences between miR-513-5p and TFPI2, the 3'UTR of TFPI2 mRNA containing wild type or mutated binding sequencesof miR-513-5p were amplified and cloned into the pGL3 luciferase reporter plasmid. Then the constructed wild type (WT) and mutant (MUT) plasmid were transiently transfected into VSMCs together with miR-513-5p mimics or its negative control (mimic-NC) by using Lipofectamine 3000 reagent (Invitrogen, USA). 48-hour post-transfection, the cells were collected and lysed to be subjected to luciferase activity measurement by using Secrete-Pair Dual Luminescence Assay Kits (GeneCopoeia,

USA).

Statistical analysis

All the data were obtained from three biological repeats and presented with mean ± standard deviation (SD). The two-tailed unparied Student's t-test and one-way ANOVA with Turkey's post-hoc test were applied for the comparison between two groups and among three and more groups, respectively. The data were statistically analyzed with GraphPad Prism 8.0 (GraphPad Software, USA), andthe statistical significance was considered when $P< 0.05$.

Results

The identification of TFPI2 and miR-513a-5p in AS

By analyzing GSE132651 data (6 samples from patients with normal coronary endothelial function and 13 samples from those with abnormal coronary endothelial function (30), we identified 6 significantly differentially expressed genes that may participate in AS: TFPI2, ANGPT2, FABP4, ADGRF5, IL1RL1 and SULF1. Among them, TFPI2 is the most significantly downregulated in AS (logFC=-1.90, Table 3). To identify the miRNA regulating TFPI2, we first used targetscan algorithm to predict the potential regulatory miRNAs of TFPI2 and obtained 515 candidates. Then, we analysed the differentially expressed miRNAs in AS by analyzing GSE137580. 25 miRNAs were identified. We intersected the two datasets, and found 3 overlapped miRNAs: miR-513a-5p, miR-630, and miR-937-5p (Supplementary Figure 1). We noticed that

miR-513a-5p was once reported to mediate apoptosis of HUVEC cells (31), suggesting that it might promote AS development. Nonetheless, a thorough study on the effects of miR-513a-5p on vascular phenotypes can be further studied. In addition, TFPI2 was reported to affect vascular smooth muscle cells' proliferation and migration (phenotypes that are related to atherosclerosis development) (17), and to be methylated in carotid atherosclerotic plaques (32). However, miR-513a-5p-TFPI2 has not been studied in AS before. We herein selected the two genes to study, intending to unravel a novel mechanism of AS.

MiR-513a-5p is highly expressed in the serum of AS patients and ox-LDL treated VSMCs

To declare the accociation of miR-513a-5p with AS, we first examined miR-513a-5p expression in the serum of AS patients and healthy donors using RT-qPCR. The results observed that miR-513a-5p presented nearly 5-fold higher expression in the serum of AS patients than that of healthy donors (Figure 1A). To further investigate the effect of miR-513a-5p on AS progression, we then try to construct an AS-like cell model mainly by treating VSMCs with ox-LDL according to previous reports (17, 33). The VSMCs were then exposed to a series of concentrations (0~200 μg/mL) of ox-LDL for 48 h or 72 h to screen out the optimal treatment dosage and time for AS-like cell model construction. The results of CCK8 assay showed that the VSMCs had highest cell viabiltiy when stressed with 100 μg/mL ox-LDL either for 48 h or 72 h (Figure 1B). Thus, 100 μg/mL ox-LDL for 48 h might be the most suitable treatment strategy for AS cell model

construction. This notion was supported by the subsequent wound healing assay, which demonstrated that the migration capacity of VSMCs was remarkably enhanced (~2 fold) by treating with 100 μg/mL ox-LDL for 48 h (Figure 1C). More importantly, we discovered that ox-LDL treatment remarkably induced miR-513a-5p expression in VSMCs, with nearly 3.5-fold higher level than that in control cells (Figure 1D). Based on this, we further desighed miR-513a-5p inhibitor and transiently transfected them into ox-LDL treated VSMCs to observe its effect on VSMCs function. The transfection efficiency was then determined by RT-qPCR, and the resutls revealed that miR-513a-5p inhibitor led to an over 50% decrease of miR-513a-5p in ox-LDL treated VSMCs compared to the corresponding NC group (Figure 1E), indicating the high transfection efficiency.

Silencing miR-513a-5p restrains the proliferation and migartion of ox-LDL stressed VSMCs

The CCK8 assay and wound healing assay were then performed in ox-LDL stressed VSMCs with the transfection of miR-513a-5p inhibitor to assess the cell viability and migration ability. As expected, the viability of VSMCs was strikingly enhanced by treating with ox-LDL, while this promotive effect was effectively reversed once miR-513a-5p was suppressed (Figure 2A). Similarly, the resutls of wound healing assay documented that ox-LDL treatment induced over 2-fold enhancement in the migration rate of VSMCs, and tranfecting miR-513a-5p inhibitor remarkably restrained (~20%) the elevation of migration rate in ox-LDL stressed VSMCs (Figure 2B). Furthermore,

through western blot analysis, we found that the protein level of proliferating cell nuclear antigen (PCNA), a molecular maker representing cell proliferation capacity, was upregulated by 1.4-fold in ox-LDL stressed VSMCs) compared with control group. At the same time, the inhibition of miR-513a-5p in ox-LDL treated VSMCs induced a 20% decrease of PCNA in contrast to the corresponding NC group (Figure 2C). These data indicated that miR-513a-5p might contribute to AS development through promoting VSMCs proliferation and migration.

MiR-513a-5p can target and negatively regulate TFPI2 and in VSMCs

According to the data in bioinformatical analysis, miR-513a-5p emerged as a miRNA candidate with the capacity of targeting TFPI2. Thus, we further predicted the blinding sites of miR-513a-5p in the 3'-UTR of TFPI2 with TargetScan, and the results did show a potential sequence mediating the interaction between miR-513a-5p and the 3'-UTR of TFPI2 (Figure 3A). The dual-luciferase assay was then performed to validate the target relationship between these two molecules. The results observed that miR-513a-5p mimic and wild-type TFPI2 3'UTR co-transfection reduced the luciferase activity of VSMCs by almost 60%. However, miR-513a-5p mimic and mutant TFPI2 3'UTR co-transfection showed no consequnce on the luciferase activity in VSMCs cells (Figure 3B). Correspondingly, the AS patients showed an over 50% decrease of TFPI2 expression in the serum compared to healthy controls (Figure 3C). The Pearson's correlation analysis further revealed a significantly negative correlation between the expression of TFPI2 and miR-513a-5p in the serum of AS patients (Figure 3D). These

data declared that TFPI2 was a target gene of miR-513a-5p and negatively correlated with miT-513a-5p in AS.

MiR-513a-5p mediates the proliferation and migration of ox-LDL treated VSMCs via targeting TFPI2

To determine whether miR-513a-5p exert its function in AS through regulating TFPI2, we then transiently transfected TFPI2 siRNA and/or miR-513a-5p inhibitor into ox-LDL treated VSMCs. RT-qPCR analysis was followingly performed to determine the expression of TFPI2 in VSMCs after transfection, the results of which showed more than 4-fold higher expression of TFPI2 in ox-LDL treatment group than that in control group. In ox-LDL+si-TFPI2 group, the TFPI mRNA level was sharply decreased (~60%) in comparison with the ox-LDL+NC group, while this reduction was reversed by co-transfection of miR-513a-5p inhibitor (Figure 4A). The subsequent western blot analysis exhibited an similar results with RT-qPCR, showing elevation of TFPI2 in ox-LDL group, reduction in ox-LDL+si-TFPI2 group, and rise again in ox-LDL+si-TFPI2+miR-513a-5p inhibitor group (Figure 4B). In the further CCK8 assay, we found that silencing TFPI2 in the ox-LDL treated VSMCs resulted in a strongly increased cell viability. However, it was observed that the promotive effect caused by silencing TFPI2 was completely reversed by simutaneously inhibiting mIT-513a-5p (Figure 4C). Similar resutls was observed in the wound healing assay, the ox-LDL stressed VSMCs with the transfection of si-TFPI2 presened a 1.3-fold increase in cell migration rate compared to the corresponding NC group. However, in the

co-transfection group, the migration rate of VSMCs was reset to level in ox-LDL+NC group (Figure 4D). Moreover, the results of western blot assay displayed that the PCNA protein level was significantly upregulated (~1.5 fold) by silencing TFPI2. Meanwhile, co-transfection of miR-5143a-5p inhibitor could effectively alleviate this promotive effect caused by TFPI2 silence, indicating the crucial regualtion of miR-513a-5p targeted TFPI2 in the proliferation of ox-LDL stressed VSMCs (Figure 4E). These observations collectively suggested that TFPI2 played a suppresive role in the proliferation and migration of VSMCs, which could be directly mediated by miR-513a-5p.

Discussion

In this study, we first screened out three dysregulated miRNAs in AS cell or mice model that might target TFPI2 via bioninformatic analysis of GEO and TargetScan datasets. Among them, miR-513a-5p was discorvered with overtly high expression in the serum of AS patients. To determine the mediation of miR-513a-5p to AS progression, we generated an AS-like cell model by treating VSMCs with ox-LDL. Our further findings in this model revealed that the miR-513a-5p expression level was upregulated by ox-LDL, and transcriptional inhibition of miR-513a-5p restrained VSMCs viability, proliferation and migration. Furthermore, we proved that miR-513a-5p could bind to 3'UTR of TFPI2 mRNA to the inhibit TFPI2 expression, thus rescue the consequence of TFPI2 on the cell viability, proliferation and migration of ox-LDL tressed VSMCs.

In the past couple of decades, it has been well established that the switch of VSMCs from a quiescent contractile phenotype to a proliferative synthetic phenotype proliferation is a fundamental event during AS progression, which induces VSMCs substantially proliferate and migrate from the media to the intima, thus hastens the intimal hyperplasia and atherosclerotic plaque formation (1, 6, 7). Given the critical role of VSMCs in AS pathogenensis, emerging studies have focused on developing VSMCs based AS-like cell model to explore the molecules that control VSMCs dysfuction. Oxidized low density lipoprotein (ox-LDL) is a detrimental factor abundant in the early stage of atherosclerosis and has been unveiled to serve as the curcial stimulator for VSMCs phenotype switching and subsequent apoptosis (34, 35). In this context, ox-LDL is frequently utlized for the construction of AS-like cell model (36-38). Here, we also employed ox-LDL to treat VSMCs and found that ox-LDL had a dose-dependent effect on VSMCs proliferation, and treatment with 100 ug/mL ox-LDL could effetively promote the VSMCs proliferation and migration. These results was completely accordant with the preivous reports (17, 38), furhter confirming the practicability of ox-LDL stressed VSMCs in studying the molecular basis of VSMCs dysfuction and AS etiology.

The function of TFPI2 in AS development has been gradually described in the recent years. In 2001, Herman et al. primarily demonstrated that TFPI-2 was able to inhibit the activation of MMPs produced by VSMCs to prevent the ECM from degradation, thus impeding the plaque rupture and furhter AS occurrance (15). It was then revealed that

fluid shear stress induced TFPI-2 upregulation weakened the proliferation while hastened the apoptosis of VSMC, indicating its potential role in VSMC dysfunction during AS development (39). Moreover, an in vivo study evidenced that conditional knockout of TFPI 2 in vascular endothelial cells presented a promotvie effect on atherosclerotic plaque development (16). In this study, we took advantage of VSMCs based AS-like cell model to validate that TFPI-2 was downregulated once VSMCs were tressed with ox-LDL, and silencing TFPI-2 enhanced the proliferation and migration of VSMCs on the basis of ox-LDL treatment. These observations was completely in accordance with the previous study (17), underlining the vital regulation in VSMCs dysfunction and AS progression.

Emerging evidences have demonstrated that miRNAs take important parts in the etiology of AS through mediating function of multiple cells such as ethothelial cells, VSMCs and macrophages (24). As a newly indentified miRNA, the biological function of miR-513a-5p has been gradually demonstrated. Specifically, miR-513a-5p was proved to regulate tumor cell proliferation, migration and apoptosis in several cancer types (40-42). In addtition, miR-513a-5p was observed to mediates TNF-α and LPS induced endothelial cells apoptosis, showing its potential in controling VSMCs function and AS development (31). Here, we made a cutting-edge exploration to prove that miR-513a-5p was able to further promote VSMCs proliferation and migration on the basis of ox-LDL treatment. Moreover, we revealed that miR-513a-5p could directly target TFPI2 and compromise the effect of TFPI2 on VSMCs phenotypes.

In spite of these observations, many quenstions remain to be further figrue out. For example, all the results were obtained from *in vitro* cell model, which might not reflect the exact function of the molecules in vivo. Therefore, one of the further work needs to focus on validating the regulatory role of miR-513a-5p in an AS animmal model, such as *Apoe* KO mice. In addition, the downstream signaling pathway of miR-513a-5p/TFPI2 axis is not elcucidated in the present study, which is also the one of the important part in our future research.

In summary, our study unveiled that miR-513a-5p could act as an atherogenic factor to promote the proliferation and migration of ox-LDL treated VSMCs, which was mainly achieved by suppressing TFPI2. These findings extend our understanding on AS etiology and might provide a novel insight for AS diagnosis and therapy.

Declarations

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Authors' contributions

FY designed the study and methods, conducted literature analysis, and revised the manuscript. CW conducted the study, collected, and analyzed the data, collected materials and resources, and prepared manuscript. All authors read and approved the final manuscript.

Conflict of interest

Authors state no conflict of interest.

Ethics approval

The present study was approved by the Ethics Committee of the Sixth Hospital of Wuhan (Wuhan, China). The clinical tissue samples were collected and processed by following the ethical standards of the Declaration of Helsinki.

Informed consent

Informed consent has been obtained from all individuals included in this study.

Consent to publish statement

Consent for publication was obtained from the participants.

Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figure legends

Figure 1. The expression of miR-513a-5p was abberently upregulated both in the serum of AS patients and ox-LDL stressed VSMCs. (A) The expression of miR-513a-5p in the serum of AS patients and heathy donors was measured by RT-qPCR. **P<0.001 versus normal group (healthy donor), n=40. (B) The cell viability in VSMCs treated with various dosages (0, 25, 50, 75, 100, 150, 200 μg/mL) of ox-LDL for 48 h and 72 h was measured by CCK-8 assay. *P<0.05, **P<0.001 versus 0 mg/mL at 72 h group; $^{\#}$P<0.05, $^{\#\#}$P<0.001 versus 0 mg/mL at 48 h group. (C) The migration capacity of VSMCs treated with 100 μg/mL ox-LDL for 48 h was assessed by wound healing assay. **P<0.001 versus CON group. (D) The expression of miR-513a-5p in VSMCs treated with 100 μg/mL ox-LDL for 48 h was measured by RT-qPCR. **P<0.001 versus CON group. (E) The expression of miR-513a-5p in VSMCs cells treated with 100 μg/mL ox-LDL and/or miR-513a-5p inhibitor was measured by RT-qPCR. CON, the cells without any treatment; Inhbitor, miR-513a-5p inhibitor; Inhibitor-NC, inhibitor corresponding negative control. *P<0.05, **P<0.001 versus CON group, $^{\#\#}$P<0.001 versus ox-LDL+inhibitor-NC group.

Figure 2. The inhibition of miR-513a-5p induced decrease of proliferation and migration in ox-LDL treated VSMCs. (A) The cell viability of VSMCs cells treated with 100 μg/mL ox-LDL and/or miR-513a-5p inhibitor was measured by CCK-8 assay. The culture time for measurement included 0 h, 24 h, 48 h, 72 h. (B) The migration capacity of VSMCs cells treated with 100 μg/mL ox-LDL and/or miR-513a-5p inhibitor

was measured by wound healing assay. (C) The protein level of PCNA in VSMCs cells treated with 100 μg/mL ox-LDL and/or miR-513a-5p inhibitor was examined by western blot. CON, the cells without any treatment; ox-LDL+NC, the cells treated with 100 μg/mL ox-LDL and transfected with inhibitor negative control; ox-LDL+inhbitor, the cells treated with 100 μg/mL ox-LDL and transfected with miR-513a-5p inhibitor. *P<0.05, **P<0.001 versus CON group; $^{\#}$P<0.05, $^{\#\#}$P<0.001 versus ox-LDL+inhibitor-NC group.

Figure 3. TFPI2 was targeted by miR-513a-5p in VSMCs. (A) The binding site of miR-513a-5p in the 3'UTR of TFPI2 was predicted by TargetScan. (B) The target relationship between miR-513a-5p and TFPI2 was validated by dual luciferase reporter gene assay. 3'UTR, pGL3 plasmid containing wild type 3'-UTR of TFPI2; MUT, pGL3 plasmid containing mutant 3'-UTR of TFPI2. miRNA, miR-513a-5p mimic; NC, miRNA mimic negative control. **P<0.001 versus 3'UTR+NC group. (C) The expression of TFPI2 in the serum of AS patients and heathy donors was measured by RT-qPCR. **P<0.001 versus normal group (healthy donor), n=40. (D) The correlation between TFPI2 and miR-513a-5p expression in the serum of AS patients was determined by Pearson's correlation test.

Figure 4. The regulatory effect of TFPI2 on the proliferation and migration of ox-LDL treated VSMCs could be reversed by miR-513a-5p. (A) The TFPI2 mRNA level was measured by RT-qPCR in VSMCs treated with ox-LDL, TFPI2 siRNA (si-TFPI2) and miR-513a-5p inhibitor. (B) The TFPI2 protein level was measured by

western blot in VSMCs treated with ox-LDL, TFPI2 siRNA (si-TFPI2) and miR-513a-5p inhibitor. (C) The cell viability of VSMCs treated with ox-LDL, TFPI2 siRNA (si-TFPI2) and miR-513a-5p inhibitor (inhibitor) was measured by CCK8 assay. (D) The migration capacity of VSMCs treated with ox-LDL, TFPI2 siRNA (si-TFPI2) and miR-513a-5p inhibitor (inhibitor) was measured by wound healing assay. (E) The protein level of PCNA was measured by western blot in VSMCs treated with ox-LDL, TFPI2 siRNA and miR-513a-5p inhibitor. CON, the cells without any treatment; ox-LDL+NC, the cells treated with 100 μg/mL ox-LDL and transfected with siRNA negative control and inhibitor negative control; ox-LDL+si, the cells treated with 100 μg/mL ox-LDL and transfected with TFPI2 siRNA; ox-LDL+si+inhibitor, the cells treated with 100 μg/mL ox-LDL and transfected with TFPI2 siRNA and miR-513a-5p inhibitor. *P<0.05, **P<0.001 versus CON group; $^{\#}$P<0.05, $^{\#\#}$P<0.001 versus ox-LDL+NC group. $^{\$}$P<0.05, $^{\$\$}$P<0.001 versus ox-LDL+Si group

Supplementary Figure 1. The identification of possible key participants in AS. A. The intersection between the predicted target miRNAs of TFPI2 by targetscan algorithm (http://targetscan.org/vert_72/) and the differentially expressed miRNAs in AS by analyzing GSE137580 data using the criteria of adjusted P<0.05, |logFC|>=1.

Figures

Figure 1

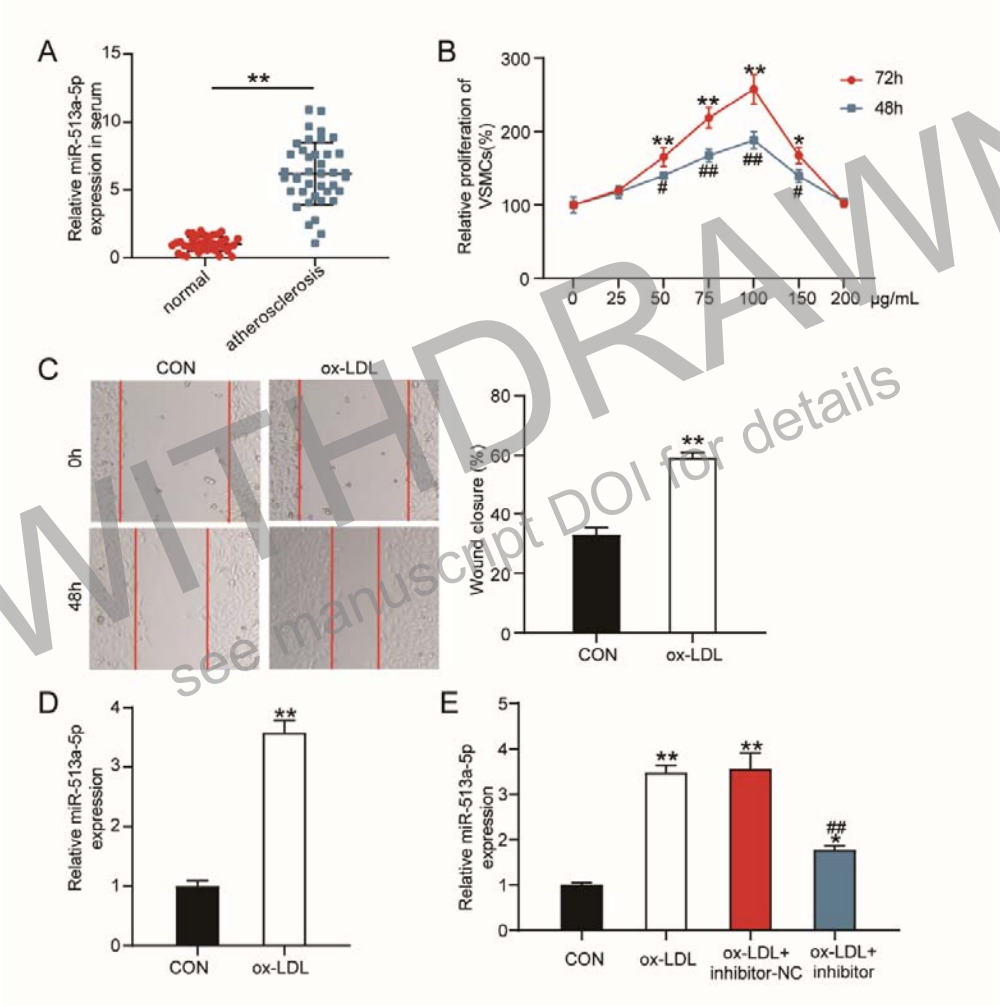


Figure 2

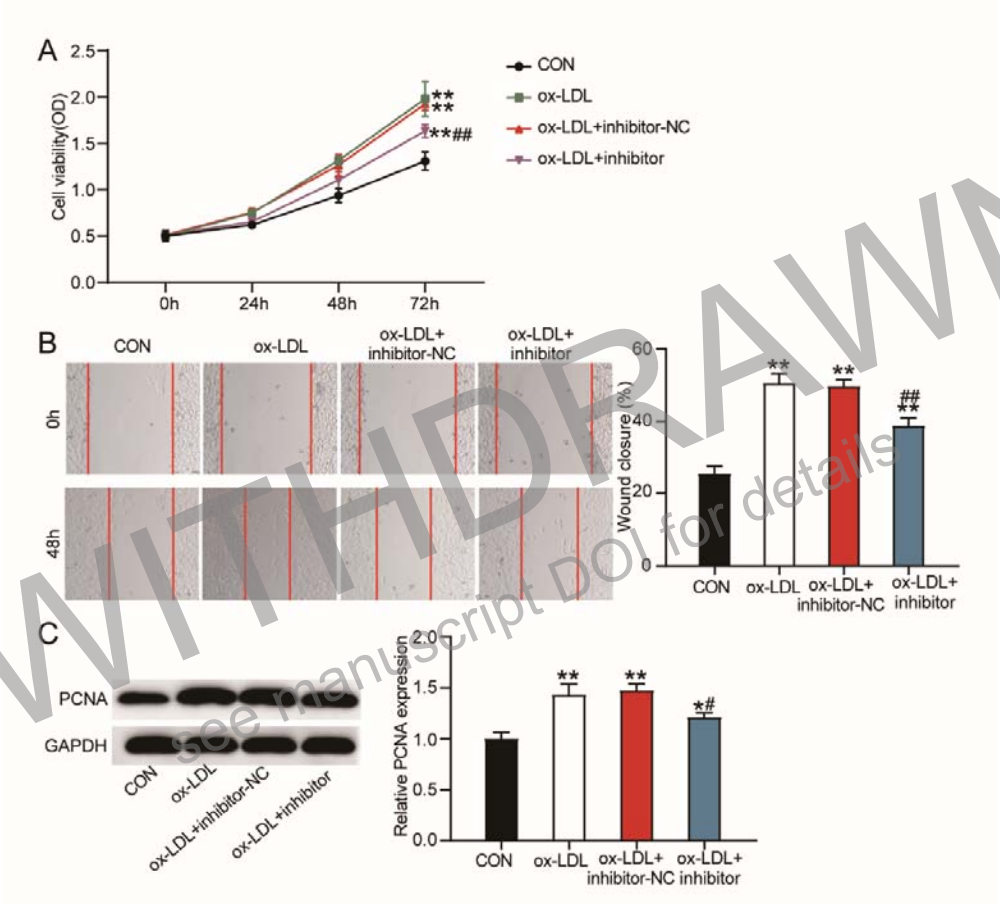


Figure 3

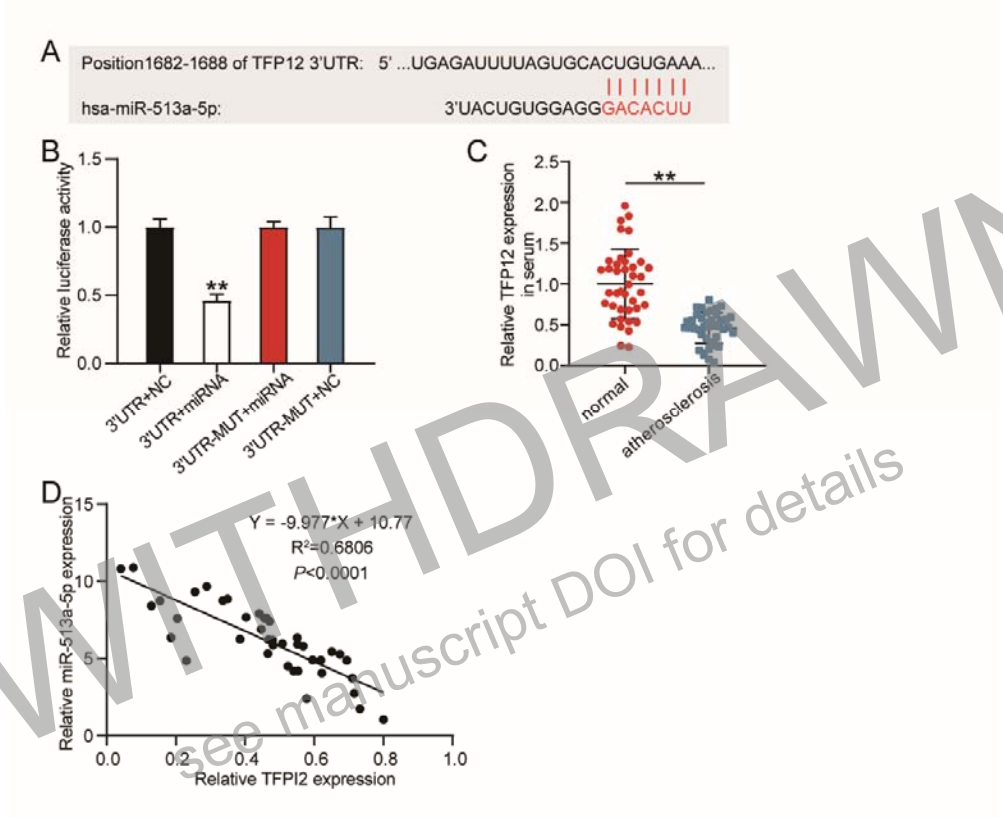
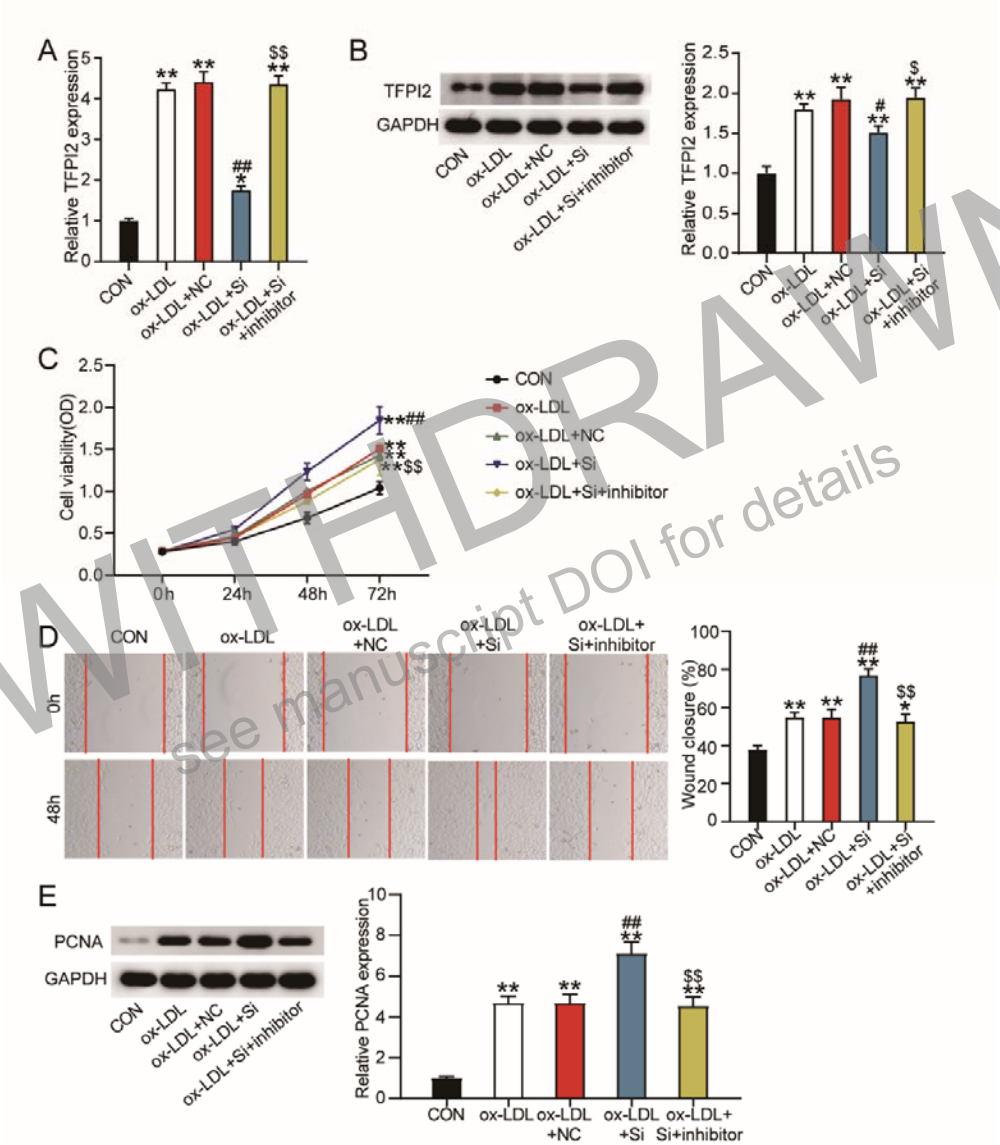


Figure 4



Tables

Table 1. Sequences for cell transfection

Name	Sequences (5'-3')
si-TFPI2	AGCCCAUACAAGUAGCUUCAUCUGG
miR-513-5p mimic	UUCACAGGGAGGUGUCAUGG
miR-513-5p inhibitor	CCAUGACACCUCCCUGUGAA
si-NC	UUGUACUACACAAAAGUACUG
mimic-NC	UUCUCCGAACGUGUCACGUTT
inhibitor-NC	CAGUACUUUUGUGUAGUACAA

Table 2. The primer sequences for RT-qPCR

GENE	Primer sequences (5'-3')
TFPI2	Forward: CTTGCGACGATGCTTGCTG
	Reverse: ACACCCACCGGAAAAGAATTT
miR-513a-5p	Forward: GGAGGGTTCACAGGGAGGT
	Reverse: GTGCGTGTCGTGGAGTCG
GAPDH	Forward: GTCAAGGCTGAGAACGGGAA
	Reverse: AAATGAGCCCCAGCCTTCTC
U6	Forward: CTCGCTTCGGCAGCACATATACT
	Reverse: ACGCTTCACGAATTTGCGTGTC

Table 3. DEGs analysis of GSE132651.

Probe ID	P value	logFC	Gene symbol	Gene title
209278_s_at	0.033476	-1.9027693	TFPI2	tissue factor pathway inhibitor 2
205572_at	0.0017336	-1.8569663	ANGPT2	angiopoietin 2
203980_at	0.0180486	-1.7440375	FABP4	fatty acid binding protein 4
212950_at	0.0109683	-1.520014	ADGRF5	adhesion G protein-coupled receptor F5
207526_s_at	0.0060358	1.5810909	IL1RL1	interleukin 1 receptor like 1
212354_at	0.0368797	1.8545557	SULF1	sulfatase 1