

Upregulation of microRNA-451a improves endothelial cell function in atherosclerosis by direct targeting of macrophage migration inhibitory factor

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Abstract: This study aims to detect the expression of selected circulating microRNAs (miRNA), including miRNA-451a, miRNA-486-5p and miR-10b-5p, and their prospective roles as biomarkers in patients with atherosclerosis. For this purpose, levels of miRNAs were detected by real-time quantitative polymerase chain reaction (RT-qPCR) in case (N = 30) and healthy control (N = 30) groups. Receiver operating characteristic (ROC) curve analysis was carried out to evaluate the diagnostic ability of miRNAs. The correlations of miR-451a with lipid parameters were evaluated using Pearson's correlation coefficients. HUVEC tested by ox-LDL was used as a cellular model of atherosclerosis. Cell viability and apoptosis were detected by methyl thiazolyl tetrazolium (MTT) and flow cytometry (FC) assays. Luciferase assay was used to determine the miRNA binding site on target genes. The results showed that miRNA-451a expression level was significantly lower in patients than controls. ROC curve analysis showed that the areas under the curve (AUC) of plasma miRNA-451a was 0.90. The miRNA-451a expression level exhibited significant negative correlations with cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL). Besides, miRNA-451a specifically binds to the 3'UTR of macrophage migration inhibitory factor (MIF) and mediated the cell proliferation and apoptosis of HUVECs exposed to ox-LDL. Furthermore, overexpression of miRNA-451a promoted the proliferation and alleviated apoptosis of HUVECs exposed to ox-LDL, while this result was attenuated by macrophage migration inhibitory factor (MIF) overexpression. Therefore, miRNA-451a could be considered as a potential biomarker for atherosclerosis and miRNA-451a might contribute to regulating atherosclerosis through targeting MIF.

Introduction

Atherosclerosis is the major cause of coronary artery disease (CAD), cerebral infarction, and peripheral vascular disease (Gupta *et al.*, 2020). In the pathogenesis of atherosclerosis, dysfunction of endothelial cells has been linked as a very early action adhered to by the intrusion of proinflammatory cells, proliferation, and the migration of smooth muscle mass cells (Li *et al.*, 2018). Nowadays, heart disease makes up roughly 30% of all health-related deaths worldwide, making atherosclerotic lesions a common cause of death (Poznyak *et al.*, 2020). Early medical diagnosis and therapy can slow down or halt the worsening of atherosclerosis. It is hence essential to create effective techniques for the detection of atherosclerosis at an early stage.

Endothelial injury plays an important role in the initiation and also the development of coronary atherosclerosis.

It is normally believed that the state of the endothelial cell is influenced by several cardio risk variables and various stimulation (Dong *et al.*, 2017; Huang *et al.*, 2019). A low-density lipoprotein (LDL) is one of the most important risk factors of atherosclerosis. After oxidation, LDL obtains changed right into oxidized LDL (ox-LDL) and participates in atherosclerosis progression (Miller *et al.*, 2010).

The microRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs identified as important post-transcriptional regulators (Solly *et al.*, 2019). Most studies have confirmed that miRNAs are key regulators of the cardiovascular system. They are involved in regulating almost all cell types related to the cardiovascular system, such as endothelial cells (Widlansky *et al.*, 2018), cardiomyocytes (Liu *et al.*, 2019), smooth muscle cells (Karvande *et al.*, 2018), fibroblasts (Krakowsky *et al.*, 2018), etc., so they are associated with the occurrence and development of various cardiovascular diseases and their molecular mechanisms (van Rooij, 2012; Solly *et al.*, 2019; Peters *et al.*, 2020). One recent study has described miRNAs

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differentially expressed between coronary atherosclerotic plaques (CAP) and healthy arteries. miRNA microarray chip analysis showed that miRNA-451a, miRNA-486-5p, and miR-10b-5p were downregulated in CAP (Berkan *et al.*, 2019). However, little data were found on the association of atherosclerosis with ox-LDL, as well as with these miRNAs. And the mechanisms underlying ox-LDL and 451a for atherosclerosis remained less well studied. Therefore, the present study aimed to use RT-qPCR to identify the relative expression levels of these miRNAs in patients with atherosclerosis and healthy people. We also evaluated the effects of these miRNAs on cell proliferation and apoptosis in ox-LDL induced HUVECs.

Materials and Methods

Serum sample collection

A total of 60 patients diagnosed with atherosclerosis and 30 healthy control subjects were enrolled from the Henan provincial hospital for this study. Atherosclerosis group defined as having CAD were documented by angiography. Angiographic Inclusion criteria were: $\geq 50\%$ stenosis of at least one major coronary vessel because of atherosclerosis. Tab. 1 shows the detailed characteristic of the patients. All requirements were met according to the Declaration of Helsinki. Patients were fully informed of the study procedures before they provided their consent. Experiments were conducted according to the Declaration of Helsinki. This study was supported and approved by the Ethics Committee of Henan provincial hospital (2019-11).

Cell culture and transfection

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in Dulbecco's modified Eagle's medium

(DMEM, Sigma, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) under humidified incubator containing 5% CO₂ at 37°C. The cell transfection was performed using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. The miRNA-451a mimic/inhibitor, negative control (NC), and pcDNA3.1/pcDNA3.1-MIF were obtained from RiboBio (Guangzhou, China).

Cell proliferation assay

Cell viability and cytotoxicity assay were measured with CCK8 Kit (Solarbio) according to the instructions of the manufacturers. HUVECs cells were seeded in 96-well plates at a density of 5.0×10^3 cells/well and treated with different concentrations of ox-LDL for 24/48/72 h. At the end of the experiments, CCK-8 solution was added to each culture well and incubated for 30 min at 37°C. It was then measured using a microplate reader (Bio-Rad, USA) at 490 nm.

3'-UTR luciferase reporter assays

HUVECs cells were seeded in 6-well plates at a density of 1×10^5 cells/well and co-transfected with 20 ng luciferase reporter plasmid and the indicated miRNAs (final concentration, 25 nmol/L) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Luciferase assays were performed using the Dual-Luciferase Assay System (Promega). Transfection efficiency was normalized by calculating the Luciferase/*Renilla* activity ratio. All experiments were done in triplicate, and three independent experiments were performed.

Detection of apoptosis by flow cytometry

The cell apoptosis was detected by flow cytometry using an Annexin V-FITC Apoptosis Detection kit (Beyotime, China) following the manufacturer's instructions. Briefly, after

TABLE 1

Primer sequences for quantitative real-time PCR

RT-PCR assays	Primers	Sequences (5'-3')
GAPDH	PCR forward primer	ATGACATCAAGAAGGTGGTGAAGCAGG
	PCR reverse primer	GCGTCAAAGGTGGAGGAGTGGGT
MIF	PCR forward primer	CGTGCCGGACGGGTTCTCT
	PCR reverse primer	CCGCCGATCTTGCCGATGCT
miR-451a	Reverse transcription primer	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAACCTCAG
	PCR forward primer	TGCGGAAACCGTTACCATTACTG
	PCR reverse primer	CCAGTGCAGGGTCCGAGGT
miR-486-5p	Reverse transcription primer	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCTCGGGG
	PCR forward primer	TGCGGTCTGTACTGAGCTGCCC
	PCR reverse primer	CCAGTGCAGGGTCCGAGGT
miR-10b-5p	Reverse transcription primer	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCACAAAT
	PCR forward primer	TGCGGTACCCTGTAGAACCGAAT
	PCR reverse primer	CCAGTGCAGGGTCCGAGGT
U6	Reverse transcription primer	AACGCTTCACGAATTTGCGTG
	PCR forward primer	GCTCGCTTCGGCAGCAC
	PCR reverse primer	AACGCTTCACGAATTTGCGTG

TABLE 2

The clinical characteristics of the study subjects of both the atherosclerosis patients and the control group

Variable	Atherosclerosis group (N = 30)	Control group (N = 30)	p
Males/females	20/10	17/13	<0.05
Age (years)	57.47 ± 14.06	45.73 ± 8.81	<0.05
TC (mmol/L)	4.98 ± 1.11	4.34 ± 0.60	<0.001
TG (mmol/L)	2.28 ± 1.31	1.18 ± 0.35	<0.001
HDL (mmol/L)	1.12 ± 0.28	1.17 ± 0.23	>0.05
LDL (mmol/L)	2.90 ± 1.01	2.15 ± 0.40	<0.001
Diabete mellitus (%)	3.3	6.7	<0.05
Hypertension (%)	13.3	6.7	<0.001
Concomitant medications (%)	93.3		

Note: Data are shown as mean ± SD. TC, total cholesterol; TG, Triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

treatment, cells were trypsinized at 37°C for 5 min and incubated with PI and Annexin V-FITC for 15 min at 37°C in the dark. Then, the rates of apoptosis were analyzed in an Accuri C6 flow cytometer (Becton Dickinson) and determined using FlowJo software.

Detection and quantification of miRNAs by RT-qPCR

The expression level of miRNAs (miRNA-451a, miRNA-486-5p, and miR-10b-5p) was detected by qRT-PCR analysis using an Applied Biosystems (ABI) 7300 system. Briefly, total RNA was extracted from 60 samples using a miRcute miRNA isolation kit (Tiangen, China), according to the manufacturer's instructions. Reverse transcription was performed using miRNA RT Reaction Buffer and miRNA RT Enzyme Mix (Tiangen, China). The sequences of primer pairs are listed in [Tab. 1](#). mRNA and miRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference genes GAPDH and U6, respectively.

Western blotting assay

Protein was extracted from HUVECs in RIPA lysis buffer (Beyotime, Shanghai, China) and separated on 8–10% SDS-PAGE to quantify the level of MIF, Bax, Bcl-2, Caspase 3. Then the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After blocking with 5% milk powder diluted in TBS containing 0.05% Tween 20 (TBST), membranes were with being probed with MIF, Bax, Bcl-2, Caspase 3 (Proteintech), GAPDH (Abcam) for 24 h at 4°C. Afterwards, the proteins were incubated with appropriate secondary antibodyies for 2 h at about 25°C. All Western blots were repeated at least three times. The density of the immunoreactive bands was quantified using ImageJ software. Protein levels were normalized to endogenous control of GAPDH protein.

Statistical analysis

All quantitative data were reported at least three separate experiments performed in triplicate. Statistical significance was conducted by GraphPad Prism 5, and One-way ANOVA followed by Tukey's *post hoc* test, and Student's *t*-test was used for comparisons between groups, and $p < 0.05$ was considered

statistically significant. Multivariate regression analysis and the rank-sum test were also used to analyze and process-related data. The area under the ROC curve (AUC) is the average sensitivity of the biomarker over the range of specificities. It is often used as a summary statistic representing the overall performance of the biomarker. GraphPad Prism 8.0 software was used for ROC curve analyses to evaluate the AUC values. The ROC analysis was performed to evaluate the diagnostic performance of parameters showing significant differences between low-grade and high-grade rectal cancer groups together with their combinations by comparing the AUCs. Pearson correlations were performed to characterize the bivariate relationship between the dependent and independent variables.

Results

Clinical characteristics of the study subjects

The patient's basic information was recorded before the patients were admitted into the hospital. The clinical characteristics of the patients were summarized in [Tab. 2](#). Among them, several variables showed significant differences, including age, TC, TG, LDL, and diabetes and high blood pressure between the two groups.

Differentially expression of miRNAs in patients with atherosclerosis

To explore the expression of miRNAs in the patients with atherosclerosis, the blood of atherosclerosis and control group was selected, and the expression of miRNA-451a, miRNA-486-5p, and miRNA-10b-5p were detected by RT-qPCR. The results showed that circulating miRNA-451a was significantly lower expressed in atherosclerosis than controls ([Fig. 1A](#)), while miRNA-486-5p and miRNA-10b-5p levels did not significantly differ between the two groups. To further explore the diagnostic potential of circulating miRNAs, we determined the discriminatory power between the groups by ROC analysis, using the RT-PCR results from the validation study. As shown in [Fig. 1B](#), the ROC curve analysis of miRNA-451a exhibited strong differentiation power between patients with atherosclerosis and healthy people, evidenced by an AUC

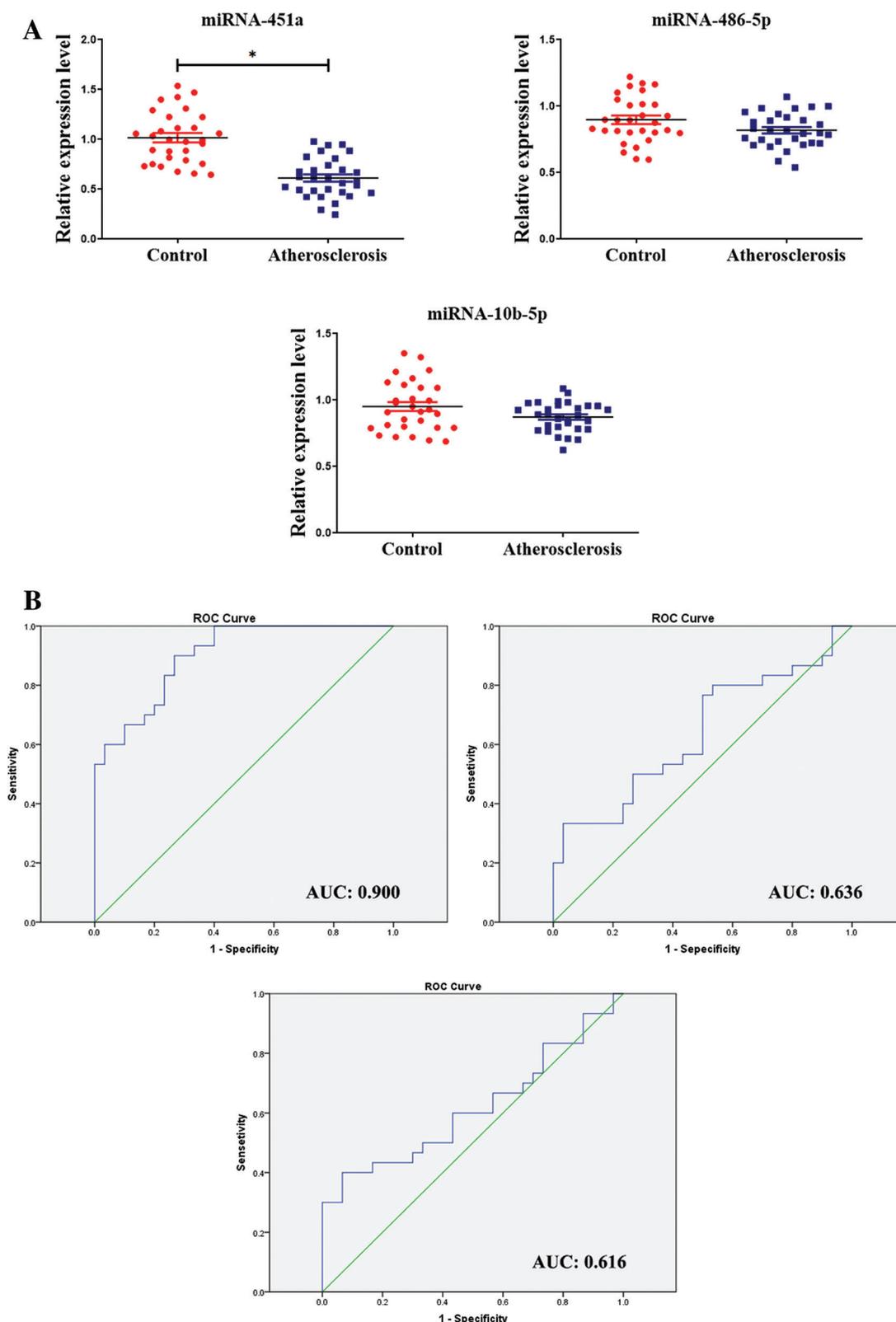


FIGURE 1. The expression level of circulating miRNAs in patients with atherosclerosis.

(A) The expression levels of miRNA-451a, miRNA-486-5p, and miRNA-10b-5p in the serum of atherosclerosis patients. (B) ROC curve for miRNA-451a, miRNA-486-5p, and miRNA-10b-5p between control and atherosclerosis. Results are presented as mean \pm SEM of three independent experiments. * $p < 0.05$.

of 0.900 (95%CI, 0.827–0.973; $p < 0.001$). The AUC of miRNA-486-5p or miRNA-10b-5p in atherosclerosis patients was 0.636 (95%CI, 0.494–0.778; $p = 0.071$), 0.616 (95%CI, 0.470–0.761; $p = 0.124$). Our results indicate that

miRNA-451a could be eligible candidate biomarkers for the diagnosis of atherosclerosis. Besides, correlations of miR-145a with the lipid parameters were determined using Pearson's correlation analyses (Tab. 3). The results

TABLE 3

Pearson correlation analysis of the association between miR-145a and lipid parameters

	miR-145a
TC (mmol/L)	$r = -0.386$ $p < 0.001$
TG (mmol/L)	$r = -0.498$ $p < 0.001$
LDL (mmol/L)	$r = -0.542$ $p < 0.001$

demonstrated that the miR-145a expression level exhibited negative correlations with TC.

Identification of MIF as a target of miRNA-451a in HUVECs

It has been well known that miRNA exerts its function via binding to the 3'-UTR of target genes through partial sequence homology. We sought to identify the direct target of miRNA-451a involved in atherosclerosis. We used the most commonly used bioinformatics prediction tools (TargetScan, starBase,

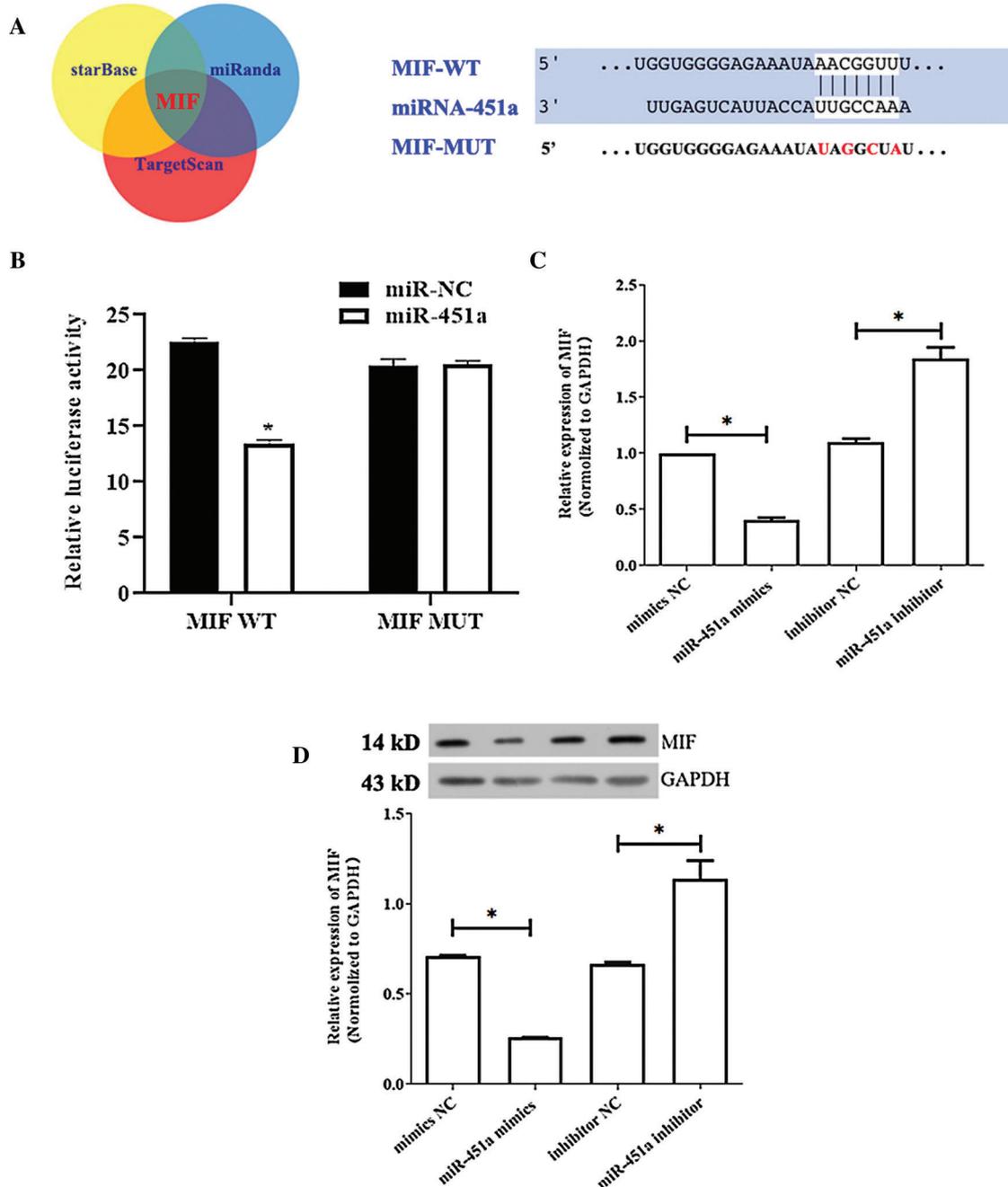


FIGURE 2. miRNA-451a directly targets MIF in HUVECs.

(A) Bioinformatics analysis of the predicted interaction of miRNA-451a and MIF. (B) Luciferase activity was determined by luciferase reporter assay in HUVECs cells co-transfected with MIF-WT or MIF-MUT, and miRNA-451a mimics, miRNA-451a inhibitor, or respective controls. (C) The mRNA and (D) protein levels of MIF in the HUVEC cell by qRT-PCR and western blot, respectively. Results are presented as mean ± SEM of three independent experiments. * $p < 0.05$.

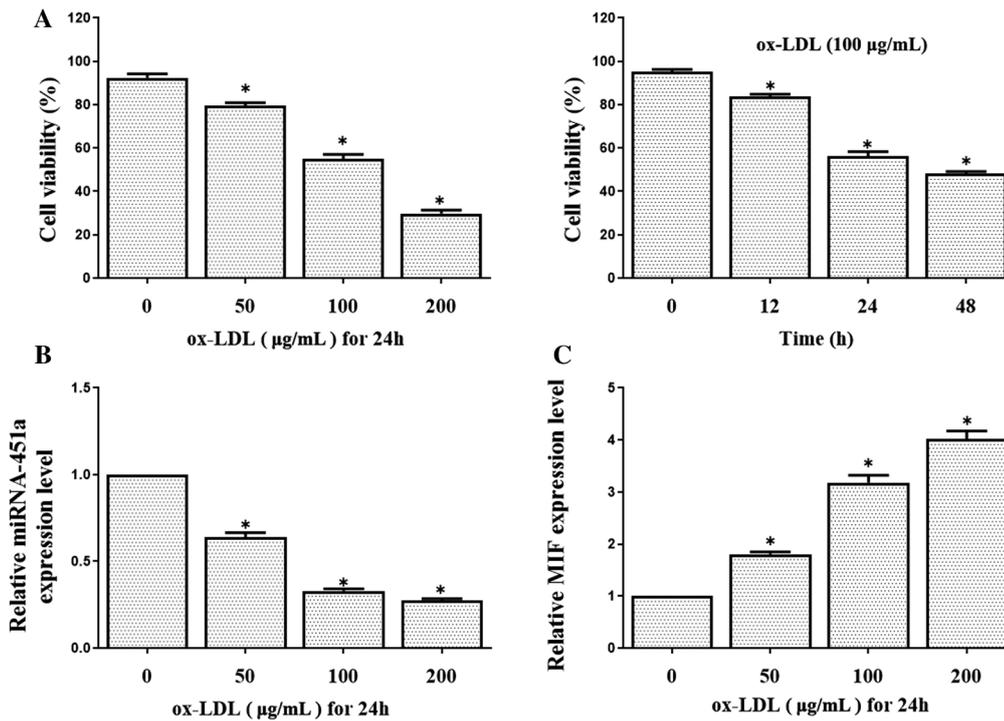


FIGURE 3. (A) HUVECs were exposed to various concentrations of ox-LDL (0, 50, 100, 200 µg/mL) for different periods (12, 24, 48 h). Inhibitory effects of ox-LDL on HUVECs viability percentage were analyzed by CCK-8. (B) The expression of miRNA-451a was analyzed by RT-qPCR. (C) The expression level of MIF was detected by qRT-PCR. Results are presented as mean \pm SEM of three independent experiments. * $p < 0.05$.

and miRanda) to identify the target. Among the top list, one of the potential targets was MIF, an inflammatory cytokine that also exerts chemokine-like function in cell proliferation and organ development (Bernhagen *et al.*, 2007). To verify this possibility, we employed a luciferase gene report system fused downstream to a segment of the MIF 3'-UTR containing either the WT putative miRNA-451a-binding sequence (MIF-3'-UTR-WT) or a point MUT putative miRNA-451a-binding sequence (MIF-3'-UTR-MUT) (Fig. 2A). 48 h after co-transfection, luciferase reporter activity was significantly reduced by miRNA-451a mimic, indicating that MIF is a downstream target gene of miRNA-451a (Fig. 2B). Furthermore, transfection of miRNA-451a mimics into HUVEC cells downregulated both MIF mRNA and protein levels, as shown by real-time PCR and Western blot analysis (Figs. 2C and 2D). Meanwhile, the transfection of miRNA-451a inhibitor resulted in the upregulation of mRNA and protein levels of MIF (Figs. 2C and 2D). Taken together, these results confirmed that MIF is a direct downstream target of miRNA-451a.

miRNA-451a regulates proliferation and apoptosis through targeting on MIF treated with ox-LDL in HUVECs

We consequently conducted further analysis to explore whether miR-451a regulated MIF expression in ox-LDL-treated HUVECs. HUVEC cell survival rates were examined by the CCK8 assay after treatment with increasing concentrations of ox-LDL (0, 25, 50, and 100 µg/mL) at different time periods (12, 24, or 48 h). Ox-LDL caused HUVECs death as compared with the control group in a dose and time-dependent manner. Cell viability decreased to $54.91 \pm 2.23\%$ after treated with 100 µg/mL ox-LDL (Fig. 3A). Therefore, the ox-LDL concentration of 100 µg/mL was used for further experiments. Results showed that miRNA-451a expression was decreased in HUVEC after stimulation of ox-LDL in a

concentration-dependent manner (Fig. 3B). Simultaneously, the mRNA level of MIF increased after ox-LDL treatment (Fig. 3C). In the pathogenesis of atherosclerosis, ECs dysfunction has been implicated as an early step, and the following HUVECs proliferation and apoptosis are particularly important associated with atherosclerotic progression. We observed that the overexpression of miRNA-451a significantly promoted cell proliferation in ox-LDL-treated cells compared with the ox-LDL+mimics NC group, whereas the effect was reversed by co-transfection with miRNA-451a mimics and pcDNA3.1-MIF (Fig. 4A).

Moreover, qRT-PCR results indicated that MIF mRNA levels in HUVEC under ox-LDL treatment could be significantly reduced by miRNA-451a overexpressing, which was largely restored by MIF overexpressing plasmid transfection (Fig. 4B). Also, in ox-LDL-treated HUVECs, the increased apoptotic rate, upregulated Bax, and cleaved-caspase-3 protein expressions, as well as decreased protein Bcl-2 protein, were ameliorated by miRNA-451a mimics, but these changes were eliminated with co-transfection with miRNA-451a mimics and pcDNA3.1-MIF in ox-LDL-treated HUVEC cells (Figs. 4C and 4D). Thus, miRNA-451a upregulation could stimulate HUVECs proliferation and apoptosis by directly targeting MIF.

Discussion

Appropriate statistical treatment of the data is essential. Heart disease is a consequence of epigenetic and genetic interactions. In the last few years, emerging evidence has indicated that miRNAs participated in the progression of various diseases, and studies had proved that some miRNAs are involved in different biological functions of atherosclerosis (Hartmann *et al.*, 2016; Lu *et al.*, 2018). Serum miRNAs have been

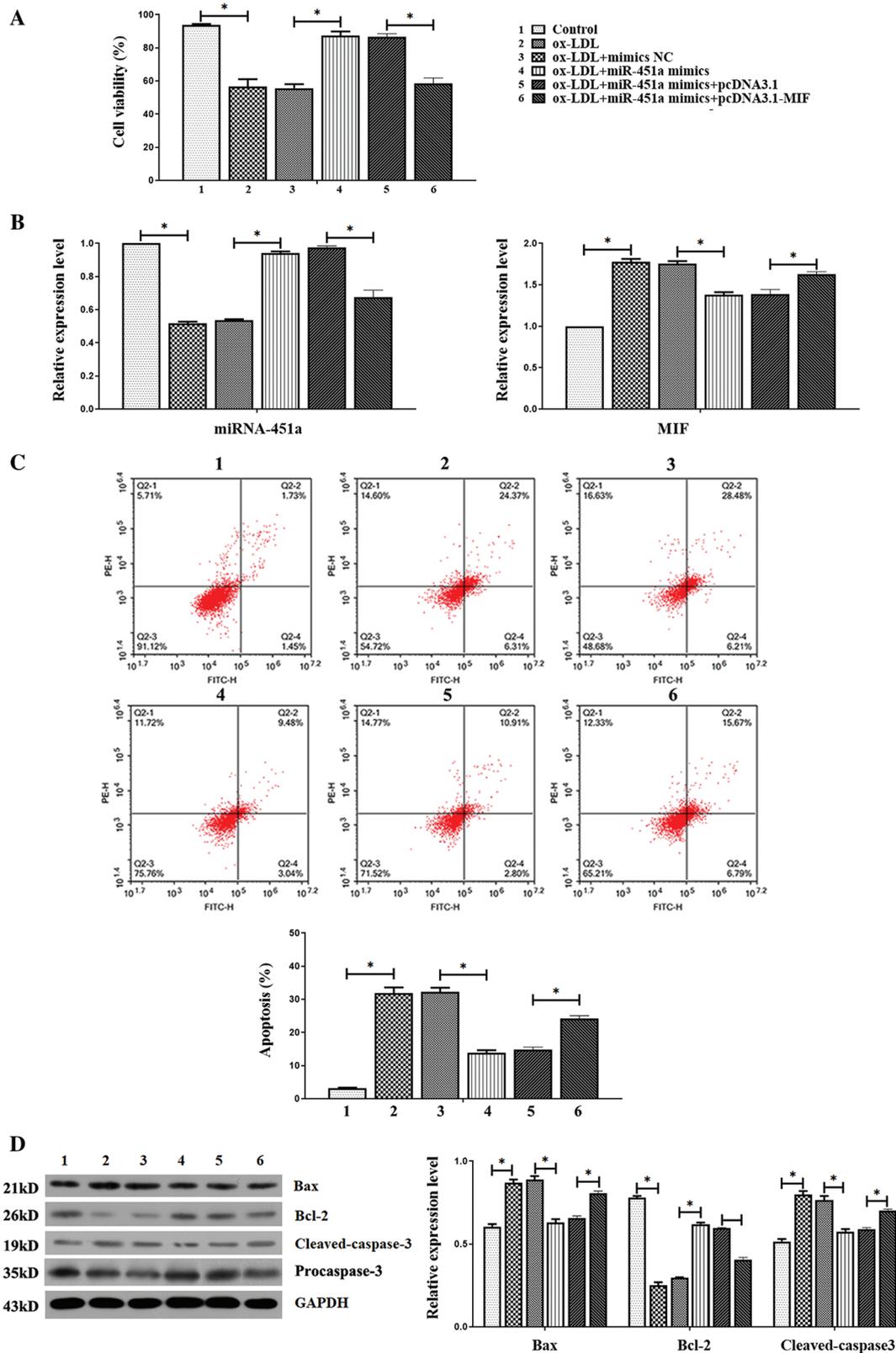


FIGURE 4. The up-regulation of MIF reverses the effect of miRNA-451a overexpression on proliferation and apoptosis in HUVEC stimulated by ox-LDL.

(A) CCK8 assay for cell proliferation of HUVECs co-transfected with miRNA-451a mimics and pcDNA3.1-MIF. (B) The expression of miRNA-451a and MIF was examined by RT-qPCR. (C) Cell apoptosis in HUVEC cells co-transfected with miRNA-451a mimics, and pcDNA3.1-MIF were measured by flow cytometry. (D) The protein expression of Bax, Bcl-2, and caspase 3 was tested by western blot assay. Results are presented as mean \pm SEM of three independent experiments. * $p < 0.05$.

considered a promising novel biomarker for CAD (Du *et al.*, 2020a; Gupta *et al.*, 2020). The results of this study uphold the basic hypothesis that miRNAs are present in serum and represent useful clinical biomarkers. Our study tested whether serum levels of three miRNAs (miRNA-451a, miRNA-486-5p, and miR-10b-5p) were different among subjects of different severities of cerebrovascular disease. We found that atherosclerosis subjects had significantly lower miRNA-451a levels than healthy controls, while the miRNA-486-5p and miR-10b-5p levels did not show a significant difference.

miRNA-451a is located in chromosome 17q11.2 and is widely dysregulated in numerous human malignancies (Bus *et al.*, 2016; Sun and Jiang, 2018). Currently, the function of miRNA-451a in cardiovascular diseases has not been well identified. Most studies provided evidence of the effects of miRNA-451a, showing its capability to inhibit cell proliferation and induce apoptosis in numerous cancer cell lines. Previous studies show that miRNA-451a aggravates lipotoxicity and cardiac hypertrophy in high fat diet-induced diabetic cardiomyopathy (Yamada *et al.*, 2013). miR-451 was very closely related to nonalcoholic fatty liver illness (NAFLD), which led to the rising danger of heart diseases, metabolic diseases, and type 2 diabetes (Zeng *et al.*, 2018). Besides, epidemiological studies had also shown that the expression level of miR-451 was positively correlated with coronary heart disease (CHD) (Ren *et al.*, 2013). We have demonstrated that the serum levels of miRNA-451a can serve as risk or diagnostic markers for AS. Given that circulating miRNAs are a potential key feature of atherosclerosis-associated dysfunction of gene regulatory networks (Zernecke *et al.*, 2009; Zhang *et al.*, 2017), our study focused on the potential cellular properties regulated by miRNA-451a. The MIF-encoded mRNA contains a 3'-UTR element that is partially complementary to miRNA-451a, indicating that the miRNAs might directly target the related sites.

The ox-LDL plays a more important role in the genesis and progression of atherosclerosis. Some studies have shown that the adhesion molecules of endothelial cells increased under the stimulation of ox LDL, which promoted the adhesion between leukocytes and vascular endothelium and induced atherosclerosis (Trpkovic *et al.*, 2015). It has been confirmed that ox-LDL causes the activation of the endothelial disorder (Parthasarathy *et al.*, 2010). Our studies used ox-LDL-induced HUVEC cells to establish an *in vitro* model of atherosclerosis and investigated the role of miRNA-451a.

Macrophage migration inhibitory factor (MIF), an inflammatory cytokine, is widely expressed in various cell types, including endothelial cells (Zhang *et al.*, 2019). Previous studies show that MIF is a key modulator that modulates cardiovascular disease, such as acute coronary syndrome (Du *et al.*, 2020b), ST-elevation myocardial infarction (Zhao *et al.*, 2019), ischemic heart disease (Xiao *et al.*, 2019), and atherosclerosis (Uchida *et al.*, 2019). Recent studies have demonstrated that atherosclerosis development requires a dialogue between vascular smooth muscle cells (VSMCs), endothelial cells, and immune cells (Ramel *et al.*, 2019). In the current study, MIF expression was found to be

significantly higher in ox-LDL caused HUVECs. MIF overexpression also reversed the effects of miRNA-451a overexpressing on proliferation and apoptosis of HUVEC under ox-LDL treatment. Thus, miR-451a/MIF may play potential roles in the regulation of immune cells and VSMCs in relation to atherosclerosis. These findings support that miR-451 can target MIF and highlight its importance in the pathogenesis of atherosclerosis. A limitation of this study is the lack of an independent validation cohort to confirm the association between miR-451a and atherosclerosis. Moreover, we did not detect the miRNA expression level in peripheral blood cells as serum miRNAs are not necessarily reflecting the intracellular ones.

Conclusion

Our study suggested that miRNA-451a, which is down-regulated in atherosclerosis, might be a novel diagnostic marker for atherosclerosis patients. Furthermore, we discovered that miRNA-451a upregulation could stimulate HUVECs proliferation and apoptosis by directly targeting MIF.

Availability of Data and Materials: The data is available upon the request from the corresponding author

Author Contribution: Study conception and design: Hongxia Hu, Ping Guo, Qian Zhao; data collection: Hongxia Hu, Ping Guo, Qian Zhao, Haoran Li, Hualei Liu, Caihong Ma; analysis and interpretation of results: Hongxia Hu1, Ping Guo, Qian Zhao, Haoran Li, Hualei Liu, Caihong Ma; draft manuscript preparation: Hongxia Hu. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: This study was supported and approved by the Ethics Committee of Henan provincial hospital (2019-11) at 9 November 2020.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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