

MiR-106b-5p Inhibits Tumor Necrosis Factor- α -induced Apoptosis by Targeting Phosphatase and Tensin Homolog Deleted on Chromosome 10 in Vascular Endothelial Cells

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Abstract

Background: Apoptosis of endothelial cells (ECs) plays a key role in the development of atherosclerosis and there are also evidence indicated that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a viable target in therapeutic approaches to prevent vascular ECs apoptosis. Aberrant miR-106b-5p expression has been reported in the plasma of patients with unstable atherosclerotic plaques. However, the role and underlying mechanism of miR-106b-5p in the genesis of atherosclerosis have not been addressed. In this study, we explored the anti-apoptotic role of miR-106b-5p by regulating PTEN expression in vascular ECs.

Methods: Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to detect the expression levels of miR-106b-5p in human atherosclerotic plaques and normal vascular tissues. Human umbilical vein endothelial cells (HUVEC) were transfected with miR-106b-5p mimic or negative control mimic, and apoptosis was induced by serum starvation and tumor necrosis factor- α (TNF- α) treat. Western blotting and real-time RT-PCR experiments were used to detect PTEN expression levels and TNF- α -induced apoptosis was evaluated by the activation of caspase-3 and cell DNA fragmentation levels in HUVEC.

Results: The expression of miR-106b-5p was significantly downregulated in plaques than in normal vascular tissues. TNF- α significantly downregulated miR-106b-5p expression levels and upregulated activation of caspase-3 and cell DNA fragmentation levels in HUVEC. Overexpression of miR-106b-5p with miR-106b-5p mimic inhibited PTEN expression and TNF- α -induced apoptosis in HUVEC. Luciferase reporter assays confirmed that miR-106b-5p binds to PTEN mRNA 3' untranslated region site.

Conclusion: MiR-106b-5p could inhibit the expression of PTEN in vascular ECs, which could block TNF- α -induced activation of caspase-3, thus prevent ECs apoptosis in atherosclerosis diseases.

Key words: Apoptosis; Atherosclerosis; MicroRNAs; Phosphatase and Tensin Homolog Deleted on Chromosome 10

INTRODUCTION

Atherosclerosis, a systemic inflammatory disease, is the major cause of life-threatening events such as myocardial infarction and stroke.^[1] Activation of the cellular suicide signaling pathways leading to apoptosis of vascular endothelial cells (ECs) may be an initial step in the pathogenesis of atherosclerosis.^[2] ECs apoptosis can break the integrity and barrier function of endothelium and compromise the ability of endothelium to regulate lipid homeostasis, immunity, and inflammation, causing atherogenesis.^[3] In addition, ECs apoptosis is responsible for vulnerable plaque formation,^[4] which ultimately leads to acute plaque rupture and artery occlusion.^[5]

MicroRNAs (miRNAs) are endogenous, ~22-nucleotide noncoding RNAs that regulate the expression of human genes and play important roles in pathophysiological processes.^[6] The expression of specific miRNAs has been identified as critical regulators of cardiovascular system,^[7] and accumulating evidence has implicated a subset of

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miRNAs as essential regulators of atherosclerosis by targeting important factors or key pathways.^[8] However, studies on their participation in the genesis of vascular ECs apoptosis remain limited.^[8]

Aberrant miR-106b-5p expression has been reported in the plasma of patients with unstable atherosclerotic plaques,^[9] and upregulated miR-106b-5p in plasma may be derived from the systemically activated/apoptotic ECs.^[9,10] However, the role and underlying mechanism of miR-106b-5p in atherosclerosis have not been addressed. Bioinformatics analysis showed that phosphatase and tensin homolog deleted on chromosome 10 (PTEN), commonly known as a tumor suppressor gene which encodes a phosphoinositide phosphatase that opposes the phosphatidylinositol 3-kinase (PI3K)-Akt pathway,^[11,12] is a candidate target of miR-106b-5p. Moreover, there is evidence indicated that PTEN is also expressed in vascular ECs and may serve as a viable target in therapeutic approaches to prevent vascular ECs apoptosis.^[13,14] Thus, in the present study, we explored the anti-apoptotic role of miR-106b-5p by regulating PTEN expression in vascular ECs.

METHODS

Harvesting of human atherosclerotic plaques and normal vascular tissue

Endarterectomy specimens constituting the intima and inner media of carotid atherosclerotic regions were obtained from the patients underwent carotid endarterectomy (CEA) ($n = 9$). Only the bifurcation carotid segments were cut from these CEA tissues. Patients undergoing coronary artery bypass grafting (CABG) surgery procedure ($n = 9$) were included as controls and the whole thickness vascular rings of normal vascular tissue internal thoracic artery (ITA) were obtained. The protocols were approved by the Ethics Review Board of Peking University People's Hospital (Clinical Trials Registry No. 2011-92, Beijing, China). Informed consent was obtained from each participant.

Human umbilical vein endothelial cells culture and transfection with microRNA mimic or negative control mimic

Human umbilical vein endothelial cells (HUVEC) were obtained from the School of Basic Medical Sciences, Peking University. HUVEC were cultured in a 5% CO₂, 37°C water-saturated atmosphere. For all experiments, HUVEC between passages 4 to 6 were used. HUVEC were cultured in endothelial cell medium (ECM) (Sciencell, USA) supplemented with 5% fetal bovine serum (Sciencell), 1% EC growth supplement (Sciencell), penicillin (100 units/ml), and streptomycin (100 mg/ml). HUVEC plated in 6-well plates were cultured and transfected with miRNA mimic or negative control mimic (NC-m) (Life Technology, USA). Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, USA) was used for transfection according to the manufacturer's instructions.

MiR-106b-5p mimic, NC-m was transfected at a final concentration of 30 pmol/ml.

Apoptosis induction in endothelial cells

For induction of apoptosis in HUVEC, cells were synchronized by serum starvation and then treated with recombinant human tumor necrosis factor- α (TNF- α) (50 ng/ml; PeproTech, USA) for 3 h, which has been shown to induce ECs apoptosis.^[14,15] Apoptosis of HUVEC was quantified by DNA fragmentation levels, by means of a photometric enzyme-linked immunosorbent assay (Cell Death Detection ELISA plus kit, Roche, Switzerland) according to the manufacturer's protocol and was also determined by the activation of caspase-3.

Real-time reverse transcription polymerase chain reaction

RNA was isolated from tissues or HUVEC using miRNeasy Mini Kit (Qiagen, Germany). MiR-106b-5p expression was analyzed in 30 ng RNA by real-time reverse transcription polymerase chain reaction (PCR) with TaqMan miRNA reverse transcription and miRNA assay kits (Life Technology) according to the instructions of the manufacturer. Target miRNA expressions were normalized between different samples based on the values of U6 and were expressed as $2^{-\text{CT}[\text{miRNA}]-\text{CT}[\text{U6}]}$. The expression data of target miRNAs are presented in logarithmic form.

The mRNA expression levels were also acquired from the value of the threshold cycle (Ct). GAPDH mRNA levels were used as an internal control. The primers used were as the following: PTEN-S: 5'-CAA GAT GAT GTT TGA AAC TAT TCC AAT G-3'; PTEN-AS: 5'-CCT TTA GCT GGC AGA CCA CAA-3'; GAPDH-S: 5'-GGA AGG TGA AGG TCG GAG TCA-3'; and GAPDH-AS: 5'-GTC ATT GAT GGC AAC AAT ATC CACT-3'.

Western blotting analysis

Transfected HUVEC were lysed and centrifuged at 12,000 \times g for 10 min at 4°C. The supernatants were collected, and protein concentration was determined with bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). The 30–50 μ g proteins were separated by 10.0–12.5% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore, USA). The membranes were probed against PTEN (Santa Cruz, USA), or cleaved caspase-3 (Cell Signaling Technology, USA), and reprobated against GAPDH (Santa Cruz) as a loading control.

Plasmid construct and dual luciferase reporter assay

The putative binding sites of miR-106b-5p in the 3' untranslated region (3'-UTR) of PTEN were predicted by three target prediction algorithms, including TargetScan, miRanda/mirSVR, and PicTar. Firefly luciferase reporter plasmid containing PTEN 3'-UTR (PTEN-luci-WT) or mutated PTEN 3'-UTR (PTEN-luci-MUT) was constructed. Human PTEN 3'-UTR sequences were obtained by PCR amplification. Primer pairs were designed to amplify a segment containing the predicted miR-106b-5p target site

within the PTEN 3'-UTR (PTEN-WT-F: 5'-CTA GAA ATT AGG ATT AAT AAA GAT GGC ACT TTC CCG TTT TAT TCC AGTT-3' and PTEN-WT-R: 5'-TTT AAT CCT AAT TAT TTC TAC CGT GAA AGG GCA AAA TAA GGT CAA GATC-3'). PCR products were cloned into separate pGL3 Luciferase Reporter Vectors (Promega, USA). The correct orientation of the insert was determined by sequencing. To generate mutations in PTEN 3'-UTR, seven nucleotides binding to the seed region of miR-106b-5p were mutated by PCR amplification (PTEN-MUT-F: 5'-CTA GAA ATT AGG ATT AAT AAA GAT GTT TGC ACC CCG TTT TAT TCC AGTT-3' and PTEN-MUT-R: 5'-TTT AAT CCT AAT TAT TTC TAC AAA CGT GGG GCA AAA TAA GGT CAA GATC-3'). Sequencing was performed to verify the mutations of the seven nucleotides.

HUVEC plated in 24-well plate were cotransfected with NC or miR-106b-5p mimic (final concentration: 60 pmol/ml, Life Technology) and firefly luciferase reporter plasmid (final concentration: 300 ng/ml) along with renilla luciferase control plasmid (final concentration: 10 ng/ml; Promega) using lipofectamine 2000 (final concentration: 4 mg/ml, Promega). After 24 h, luciferase activity was measured according to the manufacturer's instructions (Dual Luciferase Assay System, Promega). Each measured firefly luciferase activity was normalized by the renilla luciferase activity in the same well.

Statistical analysis

Quantitative data are presented as the mean \pm standard error (SE). Comparisons between groups were performed with Student's *t*-test or Mann-Whitney *U*-test for continuous variables and with the Fischer exact test for categorical variables. All tests were two-sided. A significance level of $P < 0.05$ was considered statistically significant. SPSS 13.0 (SPSS Inc., USA) was used for all statistical analyses.

RESULTS

MiR-106b-5p expression downregulated in human atherosclerotic plaques and tumor necrosis factor- α -treated human umbilical vein endothelial cells

To explore whether miR-106-5p expression was altered in atherosclerotic plaques, miR-106-5p expression levels of human carotid atherosclerotic plaques (CEA specimens, $n = 9$) and normal vascular tissue (ITA, $n = 9$) were compared. The clinical characteristics of the study population for patients underwent CEA and CABG are summarized in Table 1. The expression level of miR-106b-5p was significantly downregulated in atherosclerotic plaques compared to normal vascular tissues ($P < 0.01$).

Next, we examined the expression level of miR-106b-5p in apoptotic ECs. TNF- α is known to induce apoptosis in ECs.^[14-16] To induce apoptosis, HUVEC were serum-starved in the presence of TNF- α with a concentration of 50 ng/ml (TNF- α group, $n = 3$) or

Table 1: Clinical characteristics of the study population for patients underwent carotid endarterectomy and coronary artery bypass surgery procedure

Items	CABG cases ($n = 9$)	CEA patients ($n = 9$)	<i>P</i>
Baseline data			
Gender (male/female), <i>n</i>	4/5	6/3	0.637
Age (years), mean \pm SE	55 \pm 11	57 \pm 6	0.641
SBP (mmHg), mean \pm SE	124 \pm 11	131 \pm 14	0.261
DBP (mmHg), mean \pm SE	75 \pm 5	79 \pm 10	0.334
BMI (kg/m ²), mean \pm SE	25 \pm 4	26 \pm 5	0.455
LVEF (%), mean \pm SE	0.69 \pm 0.04	0.69 \pm 0.06	0.655
HR (beats/min), mean \pm SE	71 \pm 11	67 \pm 10	0.490
High sensitivity-CRP (mg/L)	8.5	11.3	0.224
PLT ($\times 10^{12}$ /L), mean \pm SE	246.4 \pm 76.5	215.7 \pm 44.6	0.330
Glucose (mmol/L), mean \pm SE	5.19 \pm 0.96	4.88 \pm 0.62	0.450
Lipid profile (mmol/L), mean \pm SE			
LDL cholesterol	2.56 \pm 0.95	2.65 \pm 0.72	0.842
HDL cholesterol	1.11 \pm 0.25	0.99 \pm 0.32	0.431
TC	4.12 \pm 1.11	4.06 \pm 0.80	0.897
Risk factors, <i>n</i>			
Cigarette smoking	3	3	>0.99
Hypertension	6	7	>0.99
Diabetes mellitus	0	3	0.206
Hyperlipemia	6	6	>0.99
Drug administration, <i>n</i>			
Statin	3	4	>0.99
CCB	3	3	>0.99
Beta-blocker	4	3	>0.99
Aspirin	0	4	0.082
Clopidogrel	0	3	0.206
ACEI	0	2	0.471
ARB	1	0	>0.99

All *P* values represent comparisons between CEA patients and CABG cases. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LVEF: Left ventricular ejection fraction; HR: Heart rate; PLT: Platelet; CRP: C-reactive protein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TC: Total cholesterol; CCB: Calcium channel blocker; ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker; CEA: Carotid endarterectomy; CABG: Coronary artery bypass grafting; SE: Standard error.

vehicle (control group, $n = 3$) for 3 h.^[14] Treating with TNF- α significantly downregulated the expression level of miR-106b-5p in HUVEC ($P < 0.05$).

Overexpression of miR-106b-5p inhibits phosphatase and tensin homolog deleted on chromosome 10 expression in human umbilical vein endothelial cell

To detect whether miR-106b-5p was involved in the regulation of PTEN expression in ECs, we directly overexpressed miR-106b-5p by transfecting HUVEC with miR-106b-5p mimic or NC-m. RNA or protein lysates were collected at 24 h or 36 h after miRNA mimics transfection, respectively. The significant ($P < 0.001$) 1000-fold elevated expression levels of miR-106b-5p in HUVEC were confirmed at all-time points (data not shown). Overexpression of miR-106b-5p decreased PTEN

mRNA levels by 42–53% [Figure 1a] and protein levels by 32–43% [Figure 1b] as shown by real-time PCR and western blotting, respectively.

Phosphatase and tensin homolog deleted on chromosome 10 is a direct target of miR-106b-5p

To confirm whether PTEN is a direct target of miR-106b-5p, we cloned the putative miR-106b-5p binding site on PTEN 3'-UTR or its mutational sequences into luciferase reporter plasmid and cotransfected it with miR-106b-5p mimics into HUVEC [Figure 2a]. We found that overexpression of miR-106b-5p decreased the expression of luciferase reporter gene to approximately 56% in HUVEC [$P < 0.05$, Figure 2b], which contained the wild-type binding site of miR-106b-5p. However, when the putative binding site of miR-106b-5p was mutated, miR-106b-5p mediated inhibition of luciferase gene expression was absolutely rescued in HUVEC [$P < 0.05$, Figure 2b]. These results demonstrated that PTEN was a direct target of miR-106b-5p.

MiR-106b-5p suppresses tumor necrosis factor- α -induced apoptosis in human umbilical vein endothelial cell

We next investigated the effects of miR-106b-5p on TNF- α -induced apoptosis. Caspase-3 is a key mediator of apoptosis, and cleavage of this enzyme to its active form correlates with the onset of apoptosis.^[14] To determine whether the effect of miR-106b-5p on PTEN expression correlated with TNF- α -mediated apoptosis in ECs, we evaluated caspase-3 cleavage and DNA fragmentation levels in HUVEC transfected with miR-106b-5p mimic.

HUVEC were preincubated with either miR-106b-5p mimic or NC-m for 24 h and were then induced to undergo apoptosis as described above. We found that overexpression of miR-106b-5p could partially block TNF- α -induced up-regulation of caspase-3 cleavage [$P < 0.05$, Figure 3a] and significantly suppress DNA fragmentation levels in HUVEC [$P < 0.001$, Figure 3b].

DISCUSSION

Atherosclerosis is the leading cause of death and disability worldwide.^[1] It is well known that the maintenance of a structural and functional inner vascular ECs surface is of particular importance and ECs apoptosis is a crucial event in the initiation and progression of atherosclerotic lesions.^[2] To date, more than 1900 human miRNAs have been identified.^[17] MiRNAs govern complex physiopathologic processes by modulating the expression of mRNA targets that often have important functions.^[6] Therefore, gene therapies based on manipulating miRNAs levels, such as miRNA mimics and anti-miRNAs, are now being developed to overexpress protective miRNAs or repress pathological miRNAs, respectively.^[7] Although a subset of miRNAs have been identified as important regulators of atherosclerosis,^[8] studies on their participation in ECs apoptosis in atherosclerosis have been limited.

Our previous studies showed that the expression level of miR-106b-5p was upregulated in the plasma of patients with unstable atherosclerotic plaques,^[9] which might be derived from microparticles released by the

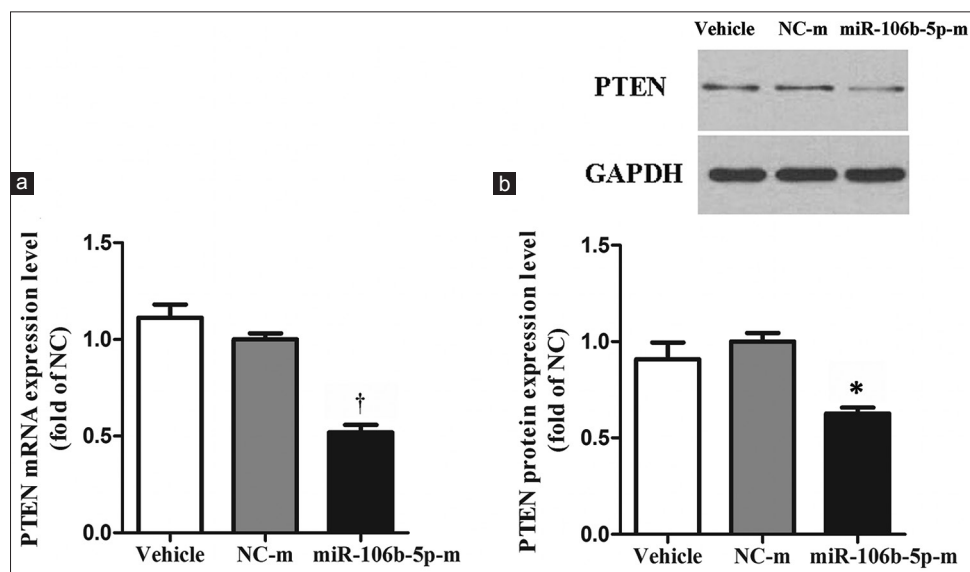


Figure 1: The effects of miR-106b-5p overexpression on the expression of phosphatase and tensin homolog deleted on chromosome 10 mRNA and protein in human umbilical vein endothelial cells transfected with miR-106b-5p mimic (miR-106b-5p-m). RNA or protein lysates were collected respectively at 24 h or 36 h after microRNA mimics transfection. (a) Phosphatase and tensin homolog deleted on chromosome 10 mRNA expression was measured by real-time PCR and normalized with respect to GAPDH. The normalized data were expressed as fold changes relative to the data of the cells transfected with scrambled negative control mimic. (b) Phosphatase and tensin homolog deleted on chromosome 10 protein expression was measured by western blotting with GAPDH used as a loading control. Densitometry was performed and normalized with respect to GAPDH expression level. The normalized data were expressed as fold changes relative to the data of cells transfected with negative control mimic. Data represent the mean \pm standard error ($n = 3$, * $P < 0.01$ vs. negative control mimic group, $^{\dagger}P < 0.001$ vs. negative control mimic group).

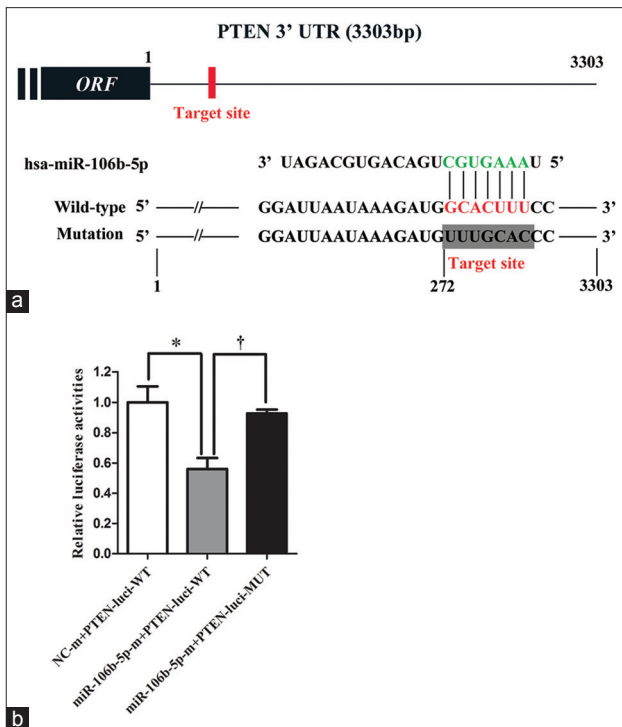


Figure 2: Luciferase reporter assays of miR-106b-5p binding sites on human phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region. The putative binding sites of miR-106b-5p in the 3' untranslated region of phosphatase and tensin homolog deleted on chromosome 10 were predicted by three target prediction algorithms, including TargetScan, miRanda/mirSVR, and PicTar. (a) The scheme of constructed firefly luciferase reporter plasmid containing phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region (phosphatase and tensin homolog deleted on chromosome 10-luci-WT) and mutated phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region (phosphatase and tensin homolog deleted on chromosome 10-luci-MUT). Phosphatase and tensin homolog deleted on chromosome 10-luci-WT plasmid contained human phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region sequences and green letters were the seed sequence, phosphatase, and tensin homolog deleted on chromosome 10-luci-MUT plasmid contained human phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region sequences with mutated miR-106b-5p-binding site (gray boxes). (b) Human umbilical vein endothelial cells were transfected with either wild-type phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region (phosphatase and tensin homolog deleted on chromosome 10-luci-WT) or mutant phosphatase and tensin homolog deleted on chromosome 10 3' untranslated region (phosphatase and tensin homolog deleted on chromosome 10-luci-MUT), along with miR-106b-5p mimic (miR-106b-5p-m). Luciferase activities were normalized to renilla activities. The normalized data were expressed as fold changes relative to the data of cells co-transfected with negative control mimic and phosphatase and tensin homolog deleted on chromosome 10-luci-WT plasmid. Data represent the mean \pm standard error ($n = 3$, * $P < 0.05$, † $P < 0.05$).

systemically activated/apoptotic ECs.^[9,10] Besides, by predicting the possible target genes of miR-106b-5p with bioinformatics analysis, we found that miR-106b-5p may involve in several aspects of atherosclerosis, such as inflammation, angiogenesis, apoptosis, and

extracellular matrix (ECM) degradation.^[9] However, the exact mechanism(s) underlying miR-106b-5p in the atherosclerosis remain(s) poorly understood. In this report, we found that miR-106b-5p was substantially reduced in CEA tissues, which were known under condition of significant ECs apoptosis.^[18] In addition, treatment with TNF- α , which was commonly found as a pro-apoptosis cytokine in atherosclerotic lesions,^[3] could downregulate miR-106b-5p expression in cultured vascular ECs. These results suggest a mechanistic link between miR-106b-5p and ECs apoptosis in atherosclerosis.

Moreover, we have demonstrated that miR-106b-5p can modulate ECs apoptosis through direct targeting PTEN. PTEN was identified originally as a tumor suppressor protein which could inhibit proliferation and survival of tumor cells by suppressing PI3K/Akt signaling.^[11,12] Several studies showed that PTEN is also expressed in vascular ECs and could serve as an important target for therapeutic pro-angiogenesis in ischemic heart and vascular diseases.^[13,14] In our study, we found that forced expression of miR-106b-5p decreased PTEN expression at both mRNA and protein levels, and miR-106b-5p directly interacts with the seed region of PTEN 3'-UTR. In addition, overexpression of miR-106b-5p could abolish the pro-apoptotic effect of TNF- α as evidenced by DNA fragmentation assay and caspase-3 cleavage levels. In accordance with previous studies,^[15,16,19] after TNF- α stimulation, apoptosis induction and caspase-3 activation were observed. Overexpression of miR-106b-5p partially reduced the pro-apoptotic effect of TNF- α in ECs. Thus, our data showed that repression of PTEN-associated functions with overexpression of miR-106b-5p might become a promising molecular targeted therapy in many biological processes, such as ECs apoptosis and neovascularization.

One of the limitations of the current study is lacking of the evidence from animal experiments. Thus, carotid plaques from patients with acute ischemic symptoms of stroke or TIA were used to explore the role of miR-106b-5p on the apoptosis of ECs in this study, and the expression levels of miR-106b-5p were found downregulated in carotid plaques. Future studies on animals would further demonstrate whether manipulation of miR-106b-5p expression levels could regulate the expression of PTEN in focal atherosclerotic plaques.

Taken together, our study reveals a novel role of miR-106b-5p in vascular ECs apoptosis and indicates a therapeutic potential of miR-106b-5p for atherosclerosis associated with apoptotic cell death. MiR-106b-5p inhibited the expression of PTEN gene, thereby controlling PI3K/Akt pathway activity and apoptosis of vascular ECs. This anti-apoptotic effect may importantly contribute to ECs homeostasis and protect the integrity of the endothelium. Further investigations are needed to examine the protective function of miR-106b-5p against atherosclerosis *in vivo*.

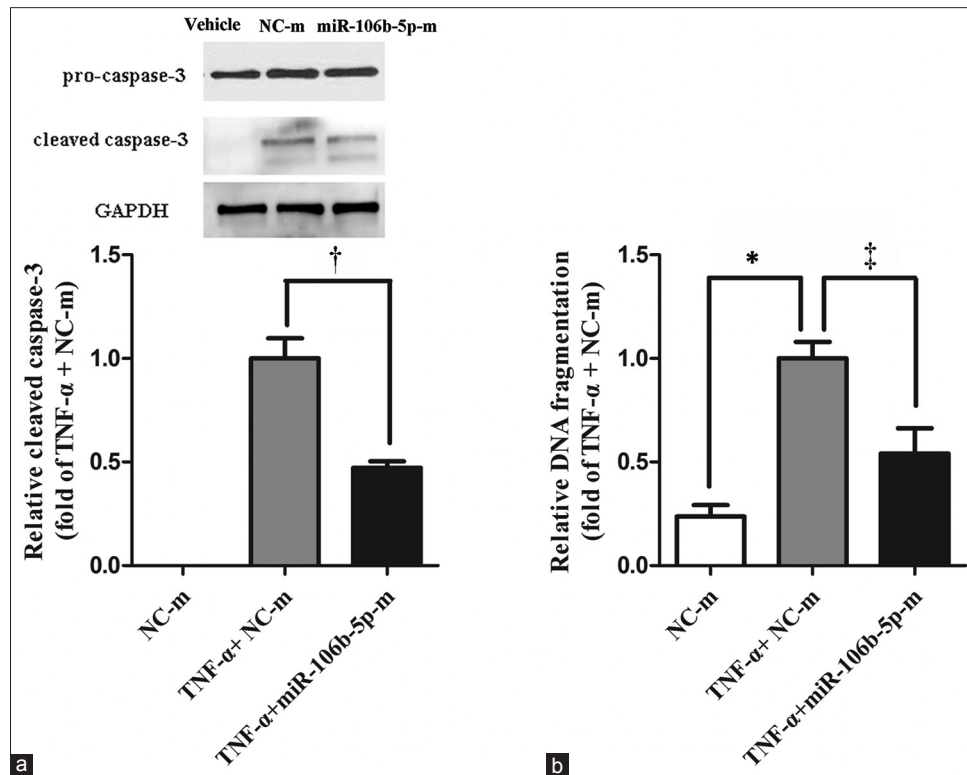


Figure 3: The effects of miR-106b overexpression on caspase-3 cleavage and DNA fragmentation levels in human umbilical vein endothelial cells treated with tumor necrosis factor- α . Human umbilical vein endothelial cells were preincubated with either miR-106b-5p mimic (miR-106b-5p-m) or scrambled negative control mimic for 24 h, and were then serum-starved in the presence of tumor necrosis factor- α (50 ng/ml) for 3 h to induce apoptosis. (a) Caspase-3 expression levels were measured by western blotting. Representative blots and quantitative data evaluated by densitometry are shown. (b) Cell apoptosis was also evaluated by means of DNA fragmentation as described in materials and methods. The normalized data were expressed as fold changes relative to the data of cells treated with tumor necrosis factor- α and transfected with negative control mimic. Data represent the mean \pm standard error ($n = 3$, * $P < 0.001$, † $P < 0.05$, ‡ $P < 0.001$ vs. tumor necrosis factor- α + negative control mimic group).

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Conflicts of interest

There are no conflicts of interest.

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