Original Article
MicroRNA-9500 attenuates TNF-induced endothelial cells inflammatory damage through inhibition of NF-κB signaling pathway

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Abstract: Background: TNF induces inflammation in endothelial cells (ECs) but the mechanism at post-transcriptional levels is not fully understood. The purpose of this study is to elucidate the post-transcriptional factors regulating TNF-induced injury in HUVECs. Methods: To confirm the predicted miR-9500 is matched with AKT-1, 3'UTR luciferase activity of AKT-1 was used to assess. Then, HUVECs were exposed to TNF in the presence or absence of miR-9500, its mimics or inhibitors. The NF-κB signaling pathway is involved in TNF-induced chronic EC inflammation was investigated. Results: Luciferase reporter analysis showed that miR-9500 over-expression leads to decreased activity of luciferase gene fused with AKT1 3'-UTR as well as reduced AKT-1 expression in human HUVECs. Therefore, AKT-1 is direct targets of miR-9500. TNF resulted in markedly increased the up-expression of MMP-9, sICAM-1, and intercellular adhesion molecule 1 (ICAM-1) in HUVECS, whereas miR-9500 reduced expression of MMP-9, ICAM-1, and sICAM-1. Furthermore, miR-9500 substantially attenuated TNF-induced up-regulation expression of AKT-1 and NF-κB in HUVECs, respectively. But transfection with inhibitors of miR-9500, antagomiR-9500, overtly accentuated TNF-induced up-expression level of AKT-1 and NF-κB in HUVECs. MiR-9500 exerts effects on cell proliferation or viability of HUVECS, and accentuates caspase-dependent apoptosis. Conclusions: We identified miR-9500, which specifically bind to AKT1 mRNA 3'-UTR. MiR-9500 is a crucial mediator of endothelial inflammatory damage, and regulating expression of ICAM-1, sICAM-1, and MMP-9 at post-transcriptional levels, protecting against endothelial inflammatory damage through inhibiting the NF-κB signaling pathway. Moreover, it exerts effects on controlling viability and apoptosis of HUVECS. Our findings suggest that targeting miR-9500, being involved in chronic EC inflammation, is a promising strategy for the prevention and treatment of chronic inflammation associated diseases, including non-healing wound.

Keywords: microRNA, TNF, endothelial cell, inflammation, AKT, NF-κB

Introduction

In pathological conditions, the relationship between angiogenesis and inflammation is well known accepted [1]. As a result, the two courses expand coordinately together. There are many chronic inflammatory diseases are relying on them [2]. During inflammation and angiogenesis, a number of factors regulate gene expression at transcriptional level or post-translational levels [3].

The miRNAs (MicroRNAs) are a group of tiny molecules and noncoding RNA, 22 to 25 nucleotides in length that function on regulation of gene expression at post-transcriptional level [4]. MicroRNAs (miRNAs) control gene expression by pairing with incompletely matching aim sites of the 3' untranslated regions (UTRs) of mRNA, and cause translational repression and/or mRNA destabilization, thereby down-regulating the expression of the targeted gene. The growing literatures hold up the vital function of miRNAs on expression regulation at post-transcriptional level. Moreover, the regulated expression of genes involve in numerous biological progressions, especially for the different pathogenesis disorders (including inflammatory disorders) [5]. A variety of miRNAs are regarded as

symbolize of a new category of inflammation mediators, too [6-8].

Vascular ECs (endothelial cells) are a target of proinflammatory cytokines, play a crucial role in immune responses. The pro-inflammatory cytokines induce de novo expression of adhesion molecule, which are endothelial cell surface proteins that bind leukocytes [9]. But, the miRNA-dependent mechanisms of stimuli-mediated inflammation on ECs are unclear. Without a doubt, several researches had indicated these adhesion molecules derived by endothelium were up-regulated expression in many advanced vascular dysfunction and the vascular inflammation models in experiment [10]. In the current work, we investigated the effects of a novel miRNA, miR9500, on mediating the inflammatory of ECs induced by pro-inflammatory cytokine TNF.

To assess the role of miR-9500 in human ECs, we first identify whether miR-9500 sequences is in the AKT-1 mRNA, and then evaluated the levels expression levels of miR-9500 in TNF-treated HUVECs, accompanied with outstanding activation of the adhesion molecule on cell surface. Our work firstly demonstrated that AKT-1 is one of the targets of miR-9500. The up-expression of AKT-1 has been induced followed with the activation of NF-κB caused by TNF. It revealed that AKT-1 could control survival of cell by regulating activate NF-κB and mediating the expression of BcL-xL as well as inhibit cell apoptosis by activating the NF-κB subunit, RelA/p65. We, therefore, investigated the effect of miR-9500 on regulating activation of NF-κB induced by TNF in HUVECs. In particular, the cell behavior effects of miR-9500 have been associated with the regulation of AKT-1 and NF-κB signaling pathway.

Materials and methods

Culture of cell

Cells were gained from discarded human tissues or peripheral blood of identified donors under protocols approved by our Investigation Committee. HUVECs were isolated and serially cultured, as described previously [5].

Reporter gene assays

cDNAs encoding the entire 3'UTR of AKT-1 (300 kb) mRNAs were predicted and amplified from total RNA of HUVECs using Xho I and Not I link-er/primers, and then cloned into the vector pGL4 (the luciferase reporter vector) included the gene could expressed firefly and renilla luciferase. AKT-1 3'UTRs was cloned in reverse orientation as controls lacking the miRNA target sequence [9]. Additionally, the complementary region to the seed region of miR-9500 sequence in position 955-961 and 1284-1290 of human AKT-1 3'UTR, TCTTGCC, was mixed up with CTGCCTT. These constructs were all identified with COS-7 cells (gift of Dr. Feng Liu, Chinese Academy of Sciences), which were transfected with the reporter construct and the indicated miRNA mimics or its negative control sequences by Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The activity of Renilla luciferase was normalized with the corresponding control of Dual-Glo Luciferase Assay System. The mutant construct of the AKT1-3'-UTR was created with Site-Mutation kit (Promega, Madison, WI, USA). Then, either NC or miR-9500 and the plasmid were co-transfected into cells. The pGL4-control vector was used for normalization as a control. The analysis of luciferase activity of cells was detected with analyzer VICTOR (PerkinElmer, Foster City, CA, USA) by Dual-Luciferase Reporter Assay System kit of Promega.

Extract total RNA and clone novel miRNA

Total RNA of cells was obtained using method with TRIzol (Sigma, CA, USA), based on the protocol for manufacturer. Total RNAs were isolated with mirVana RNA isolation kit then get rid of the RNA smaller than 200 nt. The miR-9500 was cloned into the open code frame of vector with DynaExpress miRNA Cloning Kit based on its instructions for manufacturer, and then modified it.

The mimics of miR-9500 and transfection

Transfection with mimics of miR-9500 were performed using Lipofectamine 3000 (Sigma, CA, USA), according to the protocol of manufacturer. The mimics of miR-9500 were showed as follows: sense 5'-AAGGGAAGAUGGUGACCAC-UU-3' and antisense 5'-AAGUGGUCACCAUCUU-CCCUU-3'. The inhibitors of miR-9500 were showed as follow: 5'-GUGGUCACCAUCUUCCC-UU-3'. Moreover, the negative control (NC) was showed as follows: sense 5'-ACGUGACACGUU-CGGAGAAUU-3' and antisense 5'-AAUUCUCCGA-ACGUGUCACGU-3', which was not homologous with the genome sequences of human. The qRT-PCR was used to identify the dose effect of miR-9500.

Table 1. Predicted consequential pairing of target region in AKT1 and miR-9500

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Te type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 955-961 of AKT1 3'UTR hsa-miR-9500	5'...AAUAUAAUAUGGAACCUUCCCUC... ||||||| 3'...CACCAGUGGUAGAAGGGAA	7mer-m8	-0.14	86	-0.14	0	N/A
Position 1284-1290 of AKT1 3'UTR hsa-miR-9500	5'...UGGGAUCCACCUCUUCUUCCCUU... |||| ||||||| 3'...CACCAGUGGUA--GAAGGGAA	7mer-m8	-0.11	79	-0.00	0	N/A

The qRT-PCR analysis

We had identified the expression pattern of miR-9500 in HUVECs according to the TaqMan miRNA assays with its specific primers. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data. The qRT-PCR was performed with SYBR Green kit (Sigma, USA) and the Real-Time PCR Detection System (Bio-Rad, Berkeley, CA, USA). The primer sequences were showed as follows: AKT-1 forward 5'-TCCCGAGGCCAAGTCCTT-3' and reverse 5'-AAGGACTTGGCCTCGGGA-3' and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward 5'-CTCATGACCACAGTCCATGCC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'.

Western blotting

Cells were lysed, and then total proteins were extracted with RIPA lysis buffer. Total proteins were analyzed with electrophoresis method using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel, and then transferred to a PVDF (polyvinylidene fluoride) membrane with 0.45 μm pore size (Roche, NJ, USA). The membranes were blocked with 5% skim milk at room temperature and then washed three times with TBST. The membranes were probed with primary antibodies: p-AKT, AKT1 (1:2000 dilution, Santa Cruz, CA, USA), MMP-9, IACM-1, NF-κB (1:3000 dilution, Cell Signaling, Danvers, MA, USA), or β-actin (1:3000 dilution, Cell Signaling, Danvers, MA, USA), at 4°C, overnight. Then there were incubated at room temperature with proper secondary antibodies (1:5000 dilutions, Santa Cruz, CA, USA).

Cell viability or proliferation assay, and determination of apoptosis

The CCK-8 assay kit for detection of cell viability was purchased from DOJINDO Laboratories. The absorbance of viability was analyzed in pretreated cells in 96-well plate for 16 hrs. The multi-well plate reader was used to measure the absorb value of incubated cells with CCK-8

solution for another 1 hr at 37°C. The assay for proliferation was analyzed with XTT in Raji cells. The micro-titer plate reader was used to determine the 450 nm XTT absorb value of pretreated cells in 96-well plate. The apoptotic cells were determined with FACS technique base on Annexin-V and 7-AAD dual staining or Caspase 3/7 activity.

Statistical analyses

Statistical differences between groups were analyzed with two-tailed paired Student t-test. Data of qRT-PCR and luciferase reporter assays were expressed relative to the control in each experiment, and 95% confidence intervals were calculated. Continuous variables with normally distributed were represented as SD (mean ± standard deviation). Abnormally distributed data between groups were analysis using Kruskal-Wallis ANOVA. All statistical analyses were performed with SPSS software version 18.0. $P<0.05$ was considered with statistically significant.

Results

Identification of miRNA sequences in AKT1 mRNA

To identify the target gene of miR-9500, the online software at http://targetscan.org/ was used. One member of serine/threonine protein kinases subfamily, AKT-1 was forecasted as the one of potential target gene regulated by miR-9500. To investigate whether miRNA 9500 regulates AKT-1 in HUVECs, we first predict the miRNAs target AKT-1. Then, it was predicted within the 3'-UTR of presumed miR-9500-binding sites (**Table 1**). Furthermore, to identify whether miR-9500 directly combines with AKT-1 3'UTRs, the dual luciferase reporter assay was performed in the 3'UTR of AKT-1. The results of it demonstrated that the relative luciferase activities of AKT1 3'UTR decreased markedly in HUVECs treated with miR-9500
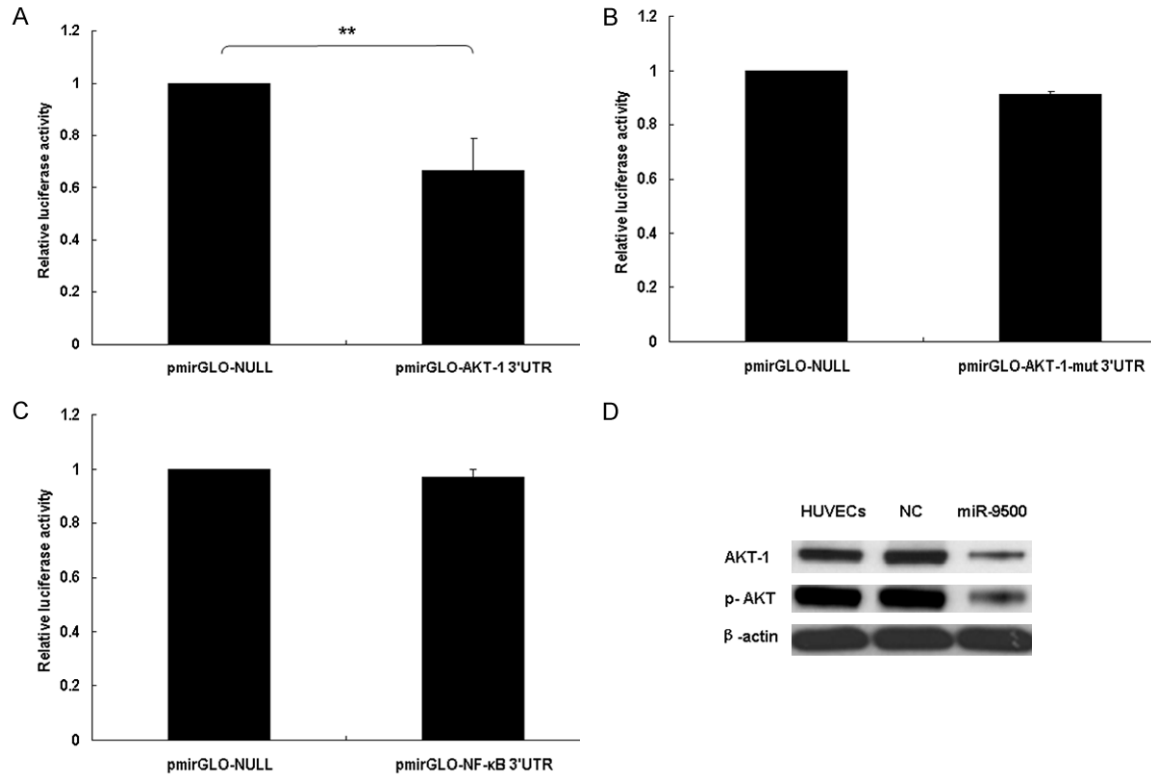
Figure 1. MiR-9500 inhibit expression of AKT-1 in HUVECs. A. The relative 3'UTR luciferase activities of AKT-1 signifi-cantly decreased in miR-9500 transfected HUVECs compared to control (*P*=0.001). B. The relative 3'UTR luciferase activities of mutant-AKT-1 decreased lightly in miR-9500 transfected HUVECs compared to control. These results confirmed that AKT-1 mRNAs are direct targets of miR-9500. C. The relative 3'UTR luciferase activities of NF-κB decreased lightly in miR-9500 transfected HUVECs compared to control. Theresult confirmed that AKT-1 mRNAs are not direct targets of miR-9500. D. Western blot analyses showed that the miR-9500 control the expression of AKT-1 and p-AKT in HUVECs. The data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01.

compared to control (**Figure 1A**) (*P*=0.001). The results of dual luciferase reporter assay in HU-VECs indicated that miR-9500 could directly bind to the 3'-UTR of AKT-1. For determining the presumed binding site of AKT-1, we created a mutant construct of AKT-1 (mutant-AKT-1) with site-mutation kit in its 3'-UTR. As shown in **Figure 1A**, the luciferase activity of the AKT-1 was significantly regulated by miR-9500, whe-reas luciferase activity was not generated from the mutant-AKT-1 construct (**Figure 1B**). It dem-onstrated that miR-9500 may regulate AKT-1 expression by targeting its 3'-UTR, and AKT-1 mRNAs are direct targets of miR-9500. More-over, as observed in other dual luciferase reporter assay, miR-9500 failed to directly bind to the 3'-UTR of NF-κB (**Figure 1C**).

In addition, to value the issue if miR-9500 regu-lates the expression of AKT-1 in HUVECs, west-ern blotting was used for quantitative analysis at protein level. As shown in **Figure 1D**, after

being transfected 48 hrs, miR-9500 signifi-cantly down-regulated the protein expression of AKT-1 compared with the control groups (negative control, NC). It demonstrated that the protein expression levels of AKT-1 were regu-lated by miR-9500. Moreover, we also con-firmed that the expression levels of phosphory-lated-AKT (p-AKT) were reduced in the miRNA-transfected cells (**Figure 1D**). All together, these results suggest that miR-9500 directly regu-lates the protein expression of AKT-1 by inter-acting with its 3'-UTR.

MiR-9500 mediated inhibition of TNF-induced inflammation response of endothelial cells

As above, we found that miR-9500 inhibit ex-pression of AKT-1 in HUVECs. To investigate the role of miR-9500 on the inflammatory response in endothelium cells, we explored the effect of miR-9500 on activation of ECs in culture stimu-lated with TNF. In our work, the cultured endo-
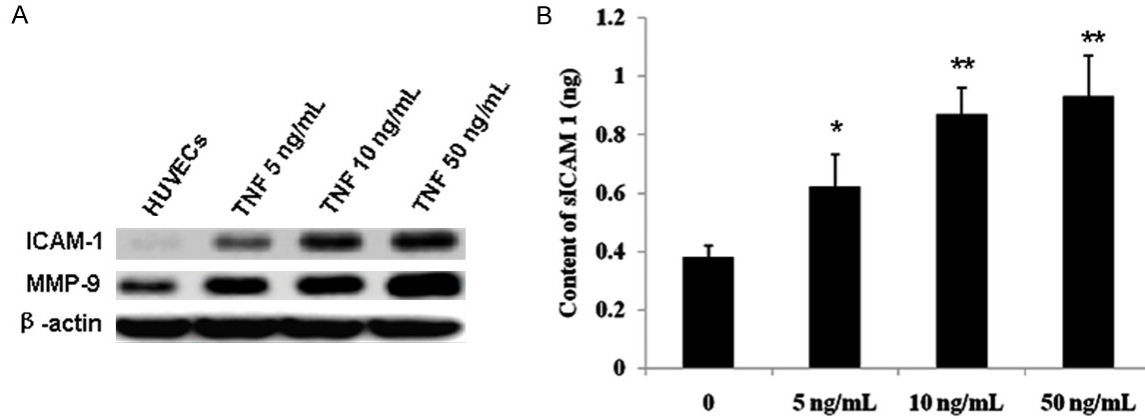
Figure 2. TNF induced inflammation process of endothelial cells. In accordance with previous observations, in HUVECs treated with different concentrations (5, 10 and 50 ng/mL) of TNF for 4 hrs, the expression of ICAM-1 and MMP-9 all significantly increased (A). As well as the soluble ICAM (sICAM 1) level increased (B) in endothelial cells. The data are presented as means ± SD from three independent experiments. *$P<0.05$, **$P<0.01$.
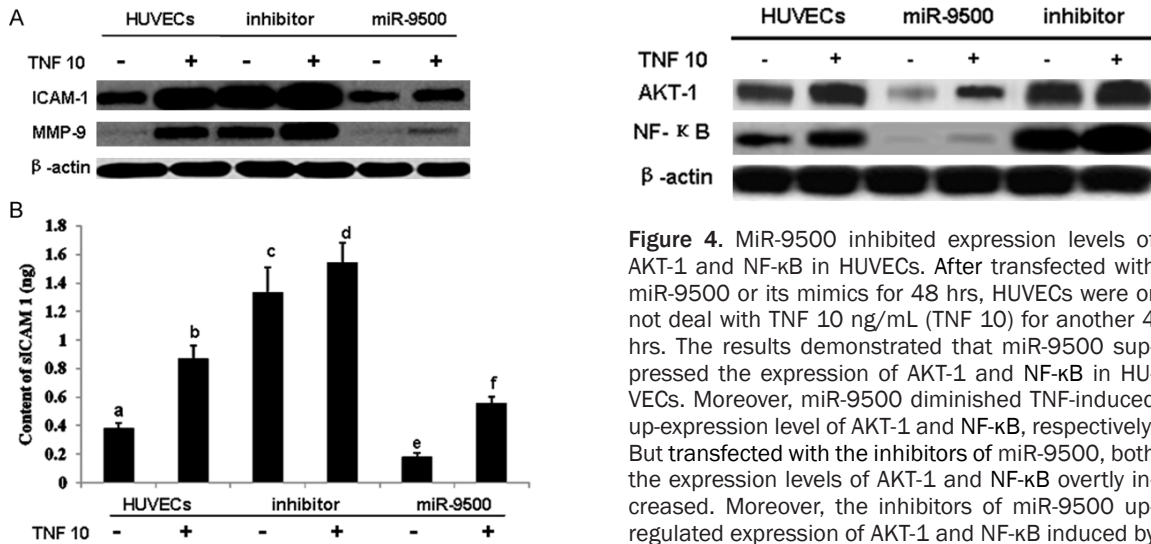

Figure 3. MiR-9500 mediated inhibition of TNF-induced inflammation in endothelial cells. In accordance with previous observations, the expression of ICAM-1 and MMP-9 significantly increased in endothelial cells induced by different concentrations (5, 10 and 50 ng/mL) of TNF (A), As well as the soluble ICAM (sICAM-1) level in endothelial cells (B). (b vs. a, $P<0.01$; c and d vs. a, $P<0.01$; d vs. b, $P<0.01$; e vs. a, $P<0.01$; f vs. a, $P<0.05$; f vs. b, $P<0.01$; c vs. e, $P<0.01$; d vs. f, $P<0.01$).

thelial cells, HUVECs was applied as a suitable model as in many studies. TNF is widely used to investigate the inflammatory response in both primary cell cultures and immortalized cell lines *in vitro*. The PI3K/AKT signaling pathway is well known to be important on regulating the expression of TNF-induced adhesion molecular. Consequently, TNF-induced endothelial ac-

Figure 4. MiR-9500 inhibited expression levels of AKT-1 and NF-κB in HUVECs. After transfected with miR-9500 or its mimics for 48 hrs, HUVECs were or not deal with TNF 10 ng/mL (TNF 10) for another 4 hrs. The results demonstrated that miR-9500 suppressed the expression of AKT-1 and NF-κB in HUVECs. Moreover, miR-9500 diminished TNF-induced up-expression level of AKT-1 and NF-κB, respectively. But transfected with the inhibitors of miR-9500, both the expression levels of AKT-1 and NF-κB overtly increased. Moreover, the inhibitors of miR-9500 up-regulated expression of AKT-1 and NF-κB induced by TNF.

tivation was assessed by analyzing the expression of ICAM-1, sICAM-1, and MMP-9.

The results of western blot analysis indicated the high baseline protein expression levels of ICAM-1, MMP-9, and sICAM-1 in HUVECs induced by TNF. As expected, followed with the treatment of TNF at different concentrations (5, 10 and 50 ng/mL) for an additional 4 hrs, the expression level of ICAM-1 drastically increased 1.5-fold, 2.5-fold, and 2.8-fold, respectively. It is in dose-dependent response when compared to control cells (**Figure 2A**) ($P<0.05$). As well as the increase of MMP-9 was showed in **Figure 2A**. The results of ELISA analysis showed that a 4 hrs exposure to TNF in HUVECs, the soluble ICAM (sICAM-1) level in supernatant of
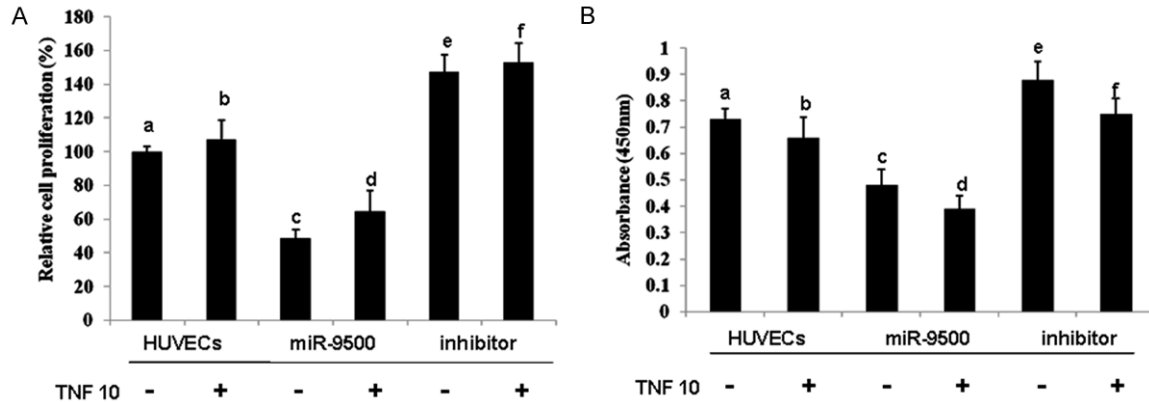
Figure 5. MiR-9500 suppressed proliferation and viability of HUVECs. A. Following HUVECs were transfected with miR-9500 for 48 hrs, the relative proliferation rate of HUVECs significantly reduced compared with control. Moreover, the inhibitors of miR-9500 significantly enhanced relative proliferation rate of HUVECs. B. Besides, miR-9500 suppressed obviously viability of HUVECs, and the inhibitors of miR-9500 significantly enhanced viability of HUVECs. The data are presented as means ± SD from three independent experiments. *$P<0.05$, **$P<0.01$. A. b vs. a, $P<0.05$; c and d vs. a, $P<0.05$; d vs. b, $P<0.01$; e and f vs. a, $P<0.05$; f vs. b, $P<0.05$; c vs. e, $P<0.01$; d vs. f, $P<0.01$. B. b vs. a, $P<0.01$; c and d vs. a, $P<0.01$; d vs. b, $P<0.01$; e and f vs. a, $P<0.01$; c vs. e, $P<0.01$; d vs. f, $P<0.01$.

HUVECs is obviously increased compared to control cells (**Figure 2B**). The raise of TNF-induced expression level of ICAM-1, sICAM-1, and MMP-9 was dose-dependent, and the most effective concentration of TNF was 50 ng/ml.

Moreover, after transfected with miR-9500 or its inhibitors for 48 hrs, HUVECs were followed by treating with above concentrations of TNF for an additional 4 hrs. The consequences indicated that miR-9500 (0.2 nM) in HUVECs could suppressed both basic and TNF-induced expression of ICAM-1, MMP-9 (**Figure 3A**) and sICAM-1 (**Figure 3B**). It is noteworthy that the mimics of miR-9500 correspondingly had the same activities (data not shown). It demonstrated that miR-9500 could significantly prohibit the expression level of sICAM1, ICAM1 and MMP-9 in HUVECs, especially for treatment with TNF.

MiR-9500 inhibited expression levels of AKT-1 and NF-κB in TNF-exposured HUVECs

It is well known that TNF induces "canonical" NF-κB pathway signaling in ECs [11]. The transcription factor NF-κB has an essential role in inflammation in ECs [12]. It has been reported that NF-κB could be activated through inhibition of the PI3K-AKT pathway. The present work was conducted to shed light on illustrating upstream mechanisms of AKT and NF-κB activation related to miRNA regulation. We further studied the specificity effects of miR-9500 on PI3K-AKT and NF-kB signaling pathway, respectively.

We here found that the NF-κB pathway was activated by inflammation stimuli, tumor necrosis factor-α (TNF), which enhanced the expression levels of AKT-1. After transfected with miR-9500 or its mimics for 48 hrs, and then HUVECs was deal with TNF 10 ng/mL (TNF 10) for another 4 hrs. The results demonstrated that miR-9500 suppressed the expression level of AKT-1 and NF-κB in HUVECs. Moreover, miR-9500 diminished TNF-induced expression level of AKT-1 and suppressed TNF-induced expression of NF-κB in HUVECs, respectively. To further test the specificity of miR-9500 for AKT/NF-κB/MMP-9 signaling pathway, antisense oligonucleotides against miR-9500 were used as miR-9500 inhibitors. By blocking endogenous miR-9500 in HUVECs, transfected with the inhibitors of miR-9500, both the inhibit effects of miR-9500 on AKT-1 and NF-κB were overtly reversed. Moreover, the inhibitors of miR-9500 up-regulated expression of AKT-1 and NF-κB induced by TNF (**Figure 4**).

MiR-9500 represses proliferation and viability of HUVECs

A suite of functional research on potential biological consequences induced by miR-9500 had been performed in HUVECs. The CCK-8 assay was used to analyze the effect of miR-9500 on cell viability and proliferation of HUVECs. TNF did not influence cell proliferation or viability of HUVECs, as well as HUVECs treated with miR-9500 or its inhibitors (**Figure 5**). Following HUVECs were transfected with miR-
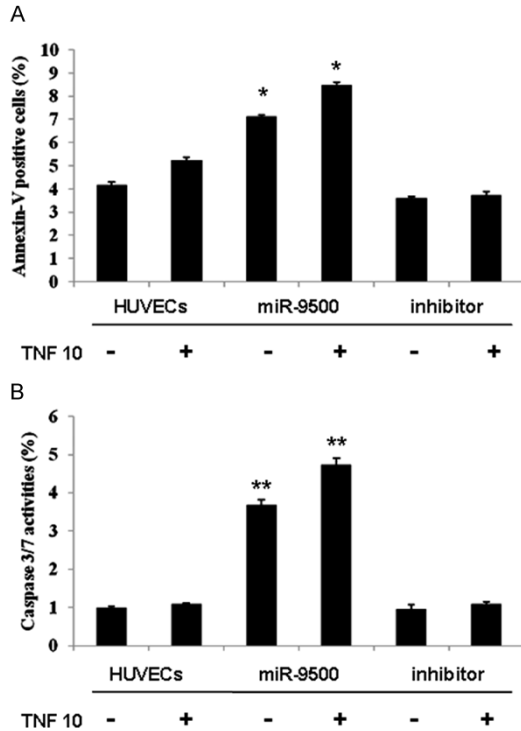
A

B

Figure 6. MiR-9500 promoted apoptosis of HU-VECs. A. The results of Annexin V-FITC/7-AAD staining and flow cytometric analysis demonstrated that miR-9500 accentuated apoptosis of HUVECs (both early and late phase, *P*<0.01) compared with control groups. But, inhibitors of miR-9500 suppressed TNF-induced apoptosis of HUVECs. B. Moreover, miR-9500 in HUVECs also significantly enhanced caspase 3/7 activities. Conversely, inhibitors of miR-9500 suppressed these activities. The data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01. A. c and d vs. a, P<0.05; e and f vs. a, P<0.05. B. c and d vs. a, P<0.01; e and f vs. a, P<0.01.

9500 for 48 hrs, the relative proliferation rate of HUVECs significantly reduced compared with control (**Figure 5A**). Moreover, the inhibitors of miR-9500 significantly enhanced relative proliferation rate of HUVECs (**Figure 5A**). Besides, miR-9500 suppressed obviously viability of HUVECs, and the inhibitors of miR-9500 significantly enhanced viability of HUVECs (**Figure 5B**).

MiR-9500 promoted apoptosis of HUVECs

Since miR-9500 inhibits the expression of AKT, and regulate cell survival and cell apoptosis. We investigated whether miR-9500 shows angiopreventive properties through inducing apoptosis. The results of Annexin V-FITC/7-AAD staining and flow cytometric analysis demon-

strated that miR-9500 accentuated apoptosis of HUVECs (both early and late phase, *P*<0.01) compared with control groups (**Figure 6A**). But, inhibitors of miR-9500 suppressed TNF-induced apoptosis of HUVECs (**Figure 6A**). Moreover, miR-9500 in HUVECs also significantly enhanced caspase 3/7 activities. Conversely, inhibitors of miR-9500 suppressed these activities (**Figure 6B**). These results suggested that a caspase-dependent apoptotic mechanism is presented, and be involved in miR-9500-induced HUVECs death.

Discussion

Inflammation-associated angiogenesis has been implicated in many human diseases, and inhibition of angiogenesis is vital for treatment of diseases. In the initiation of inflammatory reactions, the basic cellular incident is related to activation of endothelial cells at the site of inflammation [13]. The mechanism for transcriptional control of inflammatory gene expression has been explored. Although it is well known that several different mechanisms of angiogenesis, it is still unclear about how miRNA modulate the inflammation response-mediated angiogenesis. In the present work, we ascertained the novel miRNA, miR-9500, and searched after its effects and meanings in inflammatory process. A number of factors such as microRNAs play an important role in regulation of gene expression, which was involved in inflammatory ECs [14], but the microRNAs-mediated regulation of AKT-1 activity during inflammation is poorly understood. As shown in the current study, our bioinformatics analysis by using online software (http://targetscan. orgr/) confirmed that 3'-UTR site of AKT-1 mRNA is complimentary to miR-9500. To further verify whether miR-9500 straight combine with 3'UTRs of AKT-1, a vector embracing the 3'-UTR of the AKT-1 gene has been constructed, including the sequence of it was from 955 to 961 nt. The results of luciferase reporter analysis indicated that the relative activity of AKT-1 3'UTR significantly decreased in HUVECS transfected with miR-9500. It confirmed that the expression of AKT-1 was modulated by miR-9500 (**Figure 1A**) (*P*=0.001). Following over-expression of miR-9500 in HUVECs compared to NC, western blot analyses verified that protein expression levels of AKT-1 and p-AKT down-regulated in HUVEC induced by miR-9500 (**Figure 1D**). These results confirmed that miR-

9500 could directly bind with the 3'-UTR of the AKT-1 and negatively regulates its expression in HUVECs. Therefore, this provide new therapy target for treatment of pathological angiogenesis.

TNF exposure induces wider region of inflammatory reactions both *in vivo* and *in vitro* [15]. As a pro-inflammatory cytokines, TNF induce release and expression of a great deal of chemokines or adhesion molecules [16]. In multiple cells, TNF exposure commonly induced expression of inflammatory marker, for instance, MMP-9 and ICAM-1 [17, 18]. On the surface of ECs, adhesion molecules expression induced by TNF, resulted in the migration and adhesion of monocytes to the space of subendothelial, named TNF triggered EC-monocyte interaction [19].

ICAM-1 (Intercellular adhesion molecule-1) is a glycoprotein located at cell surface of endothelial cells mediating various interactions between cells at the sites of inflammation. There are two forms of ICAM-1, the membrane-bound form (mICAM-1) and the soluble form (soluble intracellular adhesion molecular 1, sICAM-1). ICAM-1 and its secreted proteins, sICAM-1, play a key role in the TNF triggered inflammatory process [20]. Previous researches have illustrated that TNF can alter both the expression of sICAM-1 and mICAM-1 in ECs, and then lead to inflammatory and immunomodulatory consequences. As the vascular inflammatory markers in various inflammatory diseases, both ICAM-1 and sICAM-1 mediated the interaction between endothelial cells and monocytes, which is a multi-step process including firm adhesion and transmigration of monocytes into arterial intima [21]. Both ICAM-1 and sICAM-1 enhanced endothelial dysfuction and up-regulated the expression of intracellular inflammatory mediators. Moreover, they were involved in several of severe diseases, for instance, cardiovascular disease, trauma, and cancer diseases [22]. The secretion of sICAM-1 is based on the over-expression of MMP-9 induced by TNF-α in ECs. Because up-regulation of MMP-9 is associated with the release of sICAM-1 on cells challenged with TNF, for example, TNF-induced MMP-9 expression contributes to enhancing soluble ICAM-1 production [23]. Therefore, ICAM-1, sICAM-1, and MMP-9 were determined for its expression levels in our study.

In this present study, firstly our results demonstrated that the high baseline protein expression levels of ICAM-1, MMP-9, and sICAM-1 in HUVECs induced by TNF. As expected, followed by treatment with TNF (5, 10 and 50 ng/mL) at different concentrations for an additional 4 hrs, all of the expression of ICAM-1 and sICAM-1, MMP-9 drastically increased, respectively. The raise of TNF-induced expression level of ICAM-1, sICAM-1, and MMP-9 was dose-dependent (**Figure 2**). The findings implied that TNF, in HUVECs, play a vital role in development of inflammatory process. Besides, we have successfully established the ICAM-1, MMP-9, and sICAM-1 over-expression model in HUVECs induced by TNF *in vitro* [18]. To determine whether miR-9500 mediated TNF-induced intracellular inflammatory in HUVECs, we investigated the effect of over-expression miR-9500 in TNF-treated HUVECs *in vitro*. TNF-induced expression levels of ICAM-1, MMP-9, and sICAM-1 were significantly blocked by miRNA-9500; conversely, accentuated by transfection with the inhibitors of miR-9500. It demonstrated that miR-9500 exerts effects on inhibition of the inflammatory process in HUVECs at post-transcription level.

TNF could regulate its downstream molecules, such as transcription factors AKT or NF-κB, and then control several cellular pathophysiological progressions. In angiogenesis, the crucial role of AKT has been emphasized in many studies [16]. In ECs, AKT could be activated by a great deal of stimuli, and regulates multiple steps of angiogenesis, such as invasion, migration and survival of endothelial cell. We found that miR-9500 down-regulated AKT-1 expression in HUVECs but did not reduce the promoter activity and mRNA stability. Moreover, transfected with the inhibitor of miR-9500, both the expression levels of AKT-1 and NF-κB overtly increased, and the inhibitors suppressed TNF-induced enhance of AKT-1 (**Figure 4**). Moreover, miR-9500 diminished TNF-induced expression level of AKT-1 and NF-κB, respectively. It suggested that AKT-1 was a direct target of miR-9500.

To induce both the activation of AKT and NF-κB, TNF, the stimulation rapidly brings about phosphorylation of NF-κB p65. In acute inflammatory responses, AKT phosphorylation and inhibition of PI3K-AKT pathway act as upstream signaling of NF-κB activation [24]. However, little was known about the miRNA-dependent regu-

lation mechanisms of silencing NF-κB expression in ECs. The present study was performed to illustrate upstream mechanisms of AKT and NF-κB activation related to miRNA regulation. We verified the specificity effects of miR-9500 on PI3K-AKT and NF-κB signaling pathway, respectively. We found that the NF-κB pathway was activated by inflammation stimuli, TNF, which enhanced the protein expression levels of AKT-1. Our results demonstrated that miR-9500 suppressed the expression of AKT-1 and NF-κB in HUVECs. Moreover, miR-9500 diminished TNF-induced up-expression level of AKT-1 and NF-κB, respectively. But transfected with the inhibitors of miR-9500, both the expression levels of AKT-1 and NF-κB overtly increased. It demonstrated that the inhibitors of miR-9500 enhance expression of AKT-1 and NF-κB induced by TNF (**Figure 4**).

However, TNF-mediated over-expression of ICAM-1, sICAM-1, and MMP-9 were inhibited by miR-9500, suggesting that miR-9500 is NF-κB-responsive miRNAs in controlling expression of ICAM-1 and MMP-9 and secretion sICAM-1, and then lead to inhibition of angiogenesis. Moreover, the up-expression of AKT-1 and NF-κB induced by inhibitors of miR-9500. These results suggested that inflammation induced by TNF was inhibited by miRNA-9500, which targeting AKT-1 and exerting effects on NF-κB. The previous study suggested that TNF stimulation increases NF-κB p65 DNA-binding activity in the nucleus, therefore it is probably that miR-9500 may modulate NF-κB p65 phosphorylation by modulating AKT-1 activity. These data provide evidence that miR-9500 is a negative regulator of AKT-1 and NF-κB expression under inflammatory conditions, indicating that miR-9500 is a potential therapeutic target for preventing TNF-mediated vascular inflammation and endothelial dysfunction.

For evaluating the potential biological roles elicited by miR-9500, a series of cell functional assays had been carried out. Both NF-κB and AKT-1 could regulate cell apoptosis and proliferation. For example, NF-κB regulates the expression of a great deal of antiapoptotic genes, as well as MMP-9, chemokines, cell cycle regulatory genes cyclin D1, adhesion molecules, and inflammatory cytokines, *etc*. AKT affect cell survival and apoptosis by activating NF-κB-mediated expression of Bcl-xL, and inhibit cell apoptosis through stimulate activation of RelA/

p65 subunit of NF-κB. Down-regulate expression level of p-AKT caused by knockdown AKT-1 fairly affects cell proliferation. By suppressing the expression of p-AKT and AKT-1, an S-phase cell cycle arrest is brought about because of down-regulation of cell cycle regulatory proteins. In this study, we focused on apoptosis and proliferation of HUVECs associated to miR-9500. The CCK-8 assay was used to analyze the effect of miR-9500 on cell viability and proliferation in HUVECs. We found that TNF did not influence cell proliferation or viability of HUVECs. But the relative proliferation rate induced by miR-9500 in HUVECs significantly reduced compared with control. Moreover, the inhibitors of miR-9500 significantly enhanced relative proliferation rate of HUVECs. Besides, miR-9500 suppressed obviously viability of HUVECs, and the inhibitors of miR-9500 significantly enhanced viability of HUVECs (**Figure 5**).

Actually, miR-9500 accentuated TNF-induced apoptosis of HUVECs (both early and late phase) compared to control. Conversely, miR-9500 inhibitors suppressed TNF-induced apoptosis of HUVECs. MiR-9500 in HUVECs also significantly induced the increase of caspase 3/7 activities. Moreover, inhibitors of miR-9500 suppressed these activities (**Figure 6**). These results suggested that a caspase-dependent apoptotic mechanism is presented, and involved in miR-9500-induced cell death. Collectively, our results indicated that miR-9500 has effects for biological function of HUVECs, such as cell proliferation, cell apoptosis, and inflammation process. This kind of regulation is likely to be important for fine-tuning of the process.

Conclusions

In summary, we illustrated miR-9500, which specifically bind to AKT-1 mRNA 3'-UTR. MiR-9500 is a crucial mediator of endothelial inflammatory damage, regulating adhesion molecules ICAM-1, sICAM-1, and MMP-9 expression at post-transcriptional levels, protecting against endothelial inflammatory damage by inhibiting the NF-κB signaling pathway, and exerts effects to control HUVECS cell viability and accentuate caspase-dependent apoptosis. Our findings suggest that targeting miR-9500, being involved in chronic EC inflammation, is a promising strategy for the prevention and treatment of chronic inflammation associated diseases, including non-healing wound.

Disclosure of conflict of interest

None.

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