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MiR-152 reduces human umbilical vein endothelial cell proliferation and migration by targeting ADAM17

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ABSTRACT

As a cleavage enzyme of precursor TNF-α, the high expression level of ADAM17 in endothelial cells is an important factor in atherosclerosis. In this study, we demonstrate that ADAM17 is the target of miR-152. We found that miR-152 could reduce TNF precursor cleavage and inhibit cell proliferation and migration by targeting ADAM17 in human umbilical vein endothelial cells (HUVECs). Furthermore, the expression pattern of miR-152 and corresponding target ADAM17 was opposite in HUVECs under hypoxic conditions. The levels of circulating miR-152 in AS patient sera were lower than those detected in the sera of normal individuals. Our results indicate that miR-152 may be involved in the development of human atherosclerosis and could be used as diagnostic biomarker or therapeutic target in atherosclerosis.

1. Introduction

Atherosclerosis (AS) is a common chronic disease contributing to high mortality and morbidity all over the world. As a disease of polygenetic inheritance, AS shows significant genetic heterogeneity, and the incidence presents regionalist and ethnic specificity. The course of atherosclerosis is regulated by multiple levels of response mechanisms and the etiology is highly complex [1]. Research has shown that inflammation, endothelial cell dysfunction and abnormal lipid metabolism are critically involved in the pathogenesis [2].

The disintegrin–metalloproteinases (ADAMs) family is a group of recently identified cell-surface glycoproteins. Members of the ADAMs family (ADAM 8, 9, 10, 12, 15, 17, 19 and 28) are expressed widely in epithelial cells, smooth muscle cells and leukocytes [3,4]. As a well-known ectodomain sheddase, ADAM17 plays an important role in vascular physiological and pathological processes through regulating membrane-anchored protein maturation [5]. Recent studies have shown that patients with myocardial infarction have increased expression of ADAM17 in plaques [6] and microparticles of human atherosclerotic plaques contain active ADAM17 that is capable of cleaving inflammatory factors from endothelial cells [7]. These inflammatory factors, including transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular cell adhesion molecule (VCAM) may lead to proliferation and migration of vascular smooth muscle cells, intimal hyperplasia and induction of mononuclear and macrophage inflammatory infiltration or conversion into foam cells. This complex process ultimately results in atherosclerotic plaque formation and luminal stenosis [8–10].

MicroRNAs are a class of evolutionarily conserved non-coding RNAs consisting of 19–22 nucleotides which play a pivotal role in the initiation and progression of vascular disorders by modulating the expression of molecules involved in vascular tone, inflammation and remodeling, thus inducing endothelial dysfunction [11,12]. It also has been shown that microRNAs involved in the regulation of members of the ADAMs family [13–16].

Circulating microRNAs are present in circulating blood, which function as negative regulators of gene expression. It has been reported that vascular endothelial cells produce circulating microRNAs, such as miR-126 and miR-143/145 and that mediate autoregulation or regulate the function of other cells [17,18]. These

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observations suggested that miRNAs, both inside cells or in serum, are involved in the development of vascular diseases such as AS.

In this study, we demonstrated for the first time that miR-152 reduced TNF precursor cleavage and inhibited the proliferation and migration of human umbilical vein endothelial cells (HUVEC) by targeting ADAM17. The expression pattern of miR-152 and corresponding target ADAM17 showed a negative correlation in HUVEC in hypoxia or normoxia. We also found that the levels of circulating miR-152 in AS patient sera were much lower than those detected the sera of normal individuals. Our results indicated that miR-152 may be involved in the development of human atherosclerosis.

2. Materials and methods

2.1. Serum specimens and cell culture

Serum samples obtained from normal individuals and atherosclerosis patients ($n = 10$ per group) were acquired from General Hospital of Chinese People's Liberation Army (Beijing, China). All patients had hyperlipidemia and were diagnosed with coronary artery disease by coronary angiography and serum lipid tests. The appearance of atherosclerotic plaques was confirmed in all patients by ultrasound examination of the carotid artery. Coronary angiography showed varying degrees of stenosis in coronary artery branches, including left anterior descending, circumflex, or right coronary artery branch. All patients had unstable angina and or myocardial infarction and were prepared to undergo coronary artery bypass grafting. Serum samples were collected preoperatively. All of the serum specimens were obtained after consent was given by patients.

Human umbilical vein endothelial cell lines (HUVEC, ATCC# CRL-1730) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone). The cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.2. DNA and RNA transfection

MiR-152 mimics were obtained from RiboBio (Guangzhou, China): 5′-TCAGTGCATGACAGAACTTGG-3′; and miR-152 inhibitors were obtained from GenePharma Company (Shanghai, China): 5′-CCAAGUUCUGUCAUGCACUGA-3′. ADAM17 siRNA were synthesized by GenePharma (Shanghai, China): sense 5′-CCAUGAAGAACACGUGUAATT-3′, antisense 5′-UUACACGUGUUCUUCAUGGTT-3′. Random scrambled RNA from GenePharma was used as negative controls (NC).

The pGL-3-control vector and the pRL-TK vector were kindly provided by Dr. Zheng Xiaofei (Beijing Institute of Radiation Medicine, China). Plasmids pCMV-myc and plasmids pCMV-ADAM17-myc were purchased from OriGene Company (OriGene, USA). All transfections were carried out using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol.

2.3. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA (including miRNA) was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A M-MuLV reverse transcriptase (Promega, USA) was used to create a cDNA library. Quantitative PCR was performed on an Mx3000p (Stratagene, La Jolla, CA, USA) using GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions. The C_T values were determined through the Mx3000p software (version 4.10) using the amplification-based threshold determination and adaptive baseline analysis options. β-Actin mRNA or U6 levels were used for normalization. Primer sequences used in this study were as follows:

β-Actin: forward 5′-GGCATCGTGATGGACTCCG-3′, reverse 5′-GCTGGAAGGTGGACAGCGA-3′;

ADAM17: forward 5′-GTGGATGGTAAAAACGAAAGCG-3′, reverse 5′-GGCTAGAACCCTAGAGTCAGG-3′;

U6: forward 5′-CGCTTCGGCAGCACATATACTA-3′, reverse 5′-CGCTTCACGAATTTGCGTGTCA-3′;

hsa-miR-16: forward 5′-TAGCAGCACGTAAATATTGGCG-3′, reverse 5′-GCGAGCACAGAATTAATACGAC-3′;

hsa-miR-145: forward 5′-GTCCAGTTTTCCCAGGAATCCCT-3′, reverse 5′-GCGAGCACAGAATTAATACGAC-3′;

hsa-miR-152: forward 5′-TCAGTGCATGACAGAACTTGG-3′, reverse 5′-GCGAGCACAGAATTAATACGAC-3′;

hsa-miR-224: forward 5′-CAAGTCACTAGTGGTTCCGTT-3′, reverse 5′-GCGAGCACAGAATTAATACGAC-3′.

2.4. Western blots

Rabbit monoclonal anti-ADAM17 was purchased from Epitomics (Epitomics, USA). Mouse monoclonal anti-TNF-α was purchased from ProteinTech (Proteintech, USA). Mouse polyclonal anti-β-actin (CoWin Biotech, China) was used as an internal control antibody. Proteins were extracted from cells with SDS lysis buffer (BioRad, USA) and separated by SDS–PAGE. Subsequently, protein samples were transferred to polyvinylidene difluoride (PVDF, Millipore, USA) membranes, which were probed with primary antibodies at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected by SuperSignal® west pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA).

2.5. Dual-luciferase reporter gene assay

Luciferase reporter plasmids were generated by insertion of target fragments into the multiple cloning site (*Xba*I and *Nde*I) downstream of the Renilla luciferase reporter gene in the pGL-3-control vector. The sequence from 2989–2995 (5′-GCACUGA-3′) in the human ADAM17 mRNA (NM_003183.4) was designated the miR-152 binding site. The 660-nt fragment of ADAM17 3′UTR (2659–3318, NM_003183.4) was generated using the following primers: forward: 5′-gctctagaTTTAGTTCTCAGCTCTTC-3′; reverse: 5′-ggaattccatatgCATGTATAGCCCTCACTGTA-3′. 5′ modified primers carrying restriction sites for either *Xba*I or *Nde*I were used for amplification (sequence in lowercase). The miR-152 seed-site in the 3′UTR of ADAM17 was mutated to remove complete miR-152 binding site. Mutagenesis of the miR-152 binding site in the ADAM17 3′UTR was carried out using a previously reported method [19] with the following primers: forward: 5′-TTGTGAAGACTGGGAAGCAGATGCTGGTCA-3′; reverse: 5′-TGACCAGCATCTGCTTCCCAGTCTTCACAA-3′. All constructs were sequenced by Sangon Biotech (Shanghai, China).

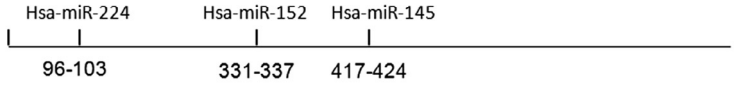
HUVEC were seeded 5×10^4 cells per well in 24-well plates and transfected with 100 ng constructed luciferase reporters and 10 ng pRL-TK as an internal control for transfection efficiency with 20 pmol miRNA mimics or negative control RNA. Firefly and Renilla luminescence was measured 48 h after transfection using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Measurements of luminescence were performed on the luminometer (Glomax 20/20, Promega).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The culture media of HUVEC were collected and centrifuged at 12,000 rpm for 10 min, and the clarified supernatants were

A

Human ADAM17 3'UTR (895bp)



miRNAs	seed match	site-type contri- bution	3' pairing contri- bution	local AU contri- bution	position contri- bution	TA contri- bution	SPS contri- bution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Hsa-miR-145	8mer	-0.247	0.003	-0.032	-0.009	0.014	-0.003	-0.27	92	1.541	0.53
Hsa-miR-152	7mer-1A	-0.074	0.001	-0.015	-0.014	0.003	-0.05	-0.15	61	3.259	0.82
Hsa-miR-224	8mer	-0.247	0.003	0.019	-0.09	0.009	0.017	-0.29	96	1.546	N/A

B

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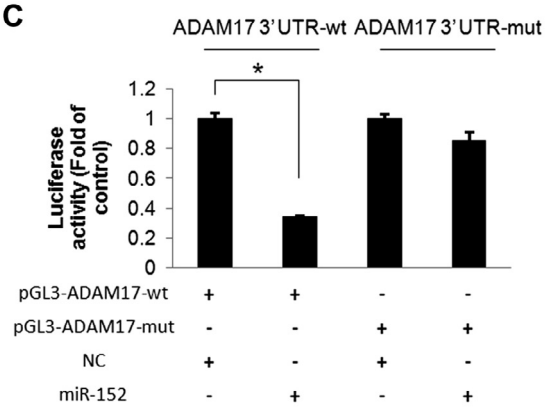
Human ADAM17 3'UTR   5'  -  UUAUAUUGAUAACAGCACUGA  -  3'
                                      ||||||          mfe: -22.7 kcal/mol
Hsa-miR-152          3'  -  GGUUCAAGACAGUACGUGACU  -  5'
```

	ADAM17 3'UTR	miR-152
Human	UUAUAUUGAUAACA**GCACUGA**CUAGGGAAAUGAU	U**CAGUGC**ACUACAGAACUUUGU
Chimpanzee	UUAUAUUGAUAACA**GCACUGA**CUAGGGAAAUGAU	U**CAGUGC**AUGACAGAACUUGG
Rhesus	UUAUAUUGAUAACA**GCACUGA**CUAGGGAAAUGAU	U**CAGUGC**AUGACAGAACUUGG
Mouse	------UGGUGACAGUGCUG-UU----AAGUCUU	U**CAGUGC**AUGACAGAACUUGG
Rat	------UGGUGACA**GCACUGA**UU----AAGCCUU	U**CAGUGC**AUGACAGAACUUGG
Dog	UU-UAUCGAGAACA**GCACUGA**UUAAGGAGAUGAU	U**CAGUGC**AUGACAGAACUUGG
Cow	UUACAUUGAGAACA**GCACUGA**UUAGGGAGAUGAU	U**CAGUGC**AUGACAGAACUUGGG

C



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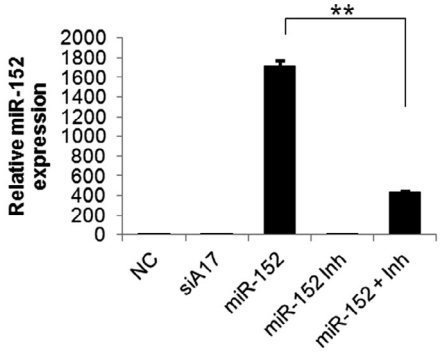

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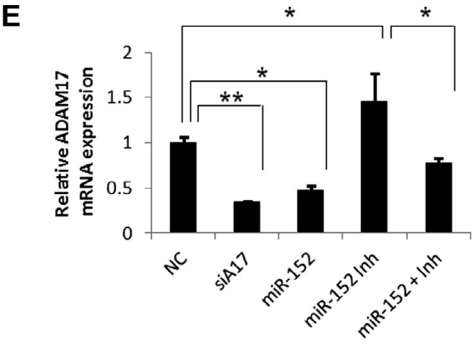

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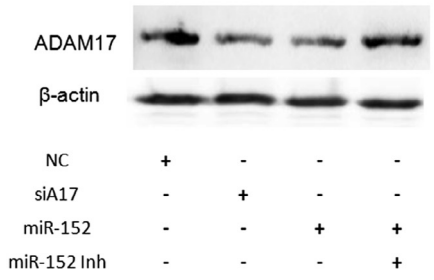

Fig. 1. ADAM17 is a direct target gene of miR-152 in HUVEC. (A) Predicted miRNAs target sites in human ADAM17 3′UTR with TargetScan 6.2 software. All the data analysis and contributes scores are cited from the software. (B) Predicted duplex formation between miR-152 and the targeted ADAM17 3′UTR, the predicted minimum free energy is calculated by RNAhybrid 2.2 software. The target site of miR-152 in ADAM17 3′UTR and miR-152 is conserved among mammalian species. (C) Insertion of ADAM17 3′UTR target sequence in the luciferase reporter vector led to the decreased luciferase activity in the presence of miR-152 in HUVEC 48 h after co-transfection. miR-152 is short for miR-152 mimics, NC is short for negative control RNA. Values represent means of three independent experiments. (D) Real-time PCR analysis of miR-152 level in HUVEC transfected with ADAM17 siRNA (siA17), miR-152 mimics and miR-152 inhibitors (miR-152 Inh). The expression level of miR-152 was normalized by using U6 as an internal control and the expression level of ADAM17 mRNA was normalized by using β-actin as an internal control. Values represented means of three independent experiments. (E) Real-time PCR was employed to analyze the expression level of ADAM17 in previously transfected HUVEC. The expression level of miR-152 was normalized by using U6 as an internal control and the expression level of ADAM17 mRNA was normalized by using β-actin as an internal control. Values represented means of three independent experiments. (F) Western blot analysis of protein extracts of HUVEC transfected with miR-152 versus a negative control RNA. MiR-152 overexpression decreases ADAM17 protein levels; β-actin was used as a loading control. $^{*}P < 0.05$ and $^{**}P < 0.01$ are considered statistically significant.

collected. TNF-α secretion levels were determined in collected supernatants by ELISA according to manufacturer's instructions (Sino Biological Inc., China).

2.7. Hypoxic treatment

For hypoxic treatment, HUVEC were exposed to hypoxic conditions in a hypoxic glove box (Coy Laboratory Products, USA). O_2 level in the box was maintained at 1% and temperature at 37 °C. Samples were collected for further analysis after 24 h incubation.

2.8. MTS assay

HUVEC proliferation was determined by CellTiter 96® AQueous One Solution Assay (Promega). Equal numbers of 2×10^3 cells per well were seeded in 96-well plates. After incubation under different conditions for various periods of time, 20 μl 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphe nyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS; Promega) was added to each well. The plates were further cultured for 1 h. Absorbance was measured at 490 nm with a microplate spectrophotometer (Bio-Tek, USA).

2.9. Wound-healing cell migration assay

HUVEC (1×10^5 cells per well) were grown to 80% confluence in a 24-well plate before wounding. The wound was made by scraping a conventional pipette tip across the monolayer. Cell migration was induced by DMEM supplemented with 10% FBS. The image of the wound was captured by microscopy (Olympus, Japan) immediately after wounding at 24 h intervals.

2.10. Transwell chamber migration assay

HUVEC (5×10^4 cells per well) were placed in serum-free DMEM in 24-well Transwell inserts (8-μm pore size; Corning Costar, USA), while the bottom chambers contained serum-supplemented medium. The migration of cells was allowed to proceed for 48 h at 37 °C. Cells that migrated to the bottom of the insert were fixed with methanol at −20 °C for 10 min and stained by 0.1% crystal violet for 12 h and counted under the microscope (Olympus).

2.11. Statistics

All of the experiments were performed three times independently. Statistical significance of representative data was analyzed by unpaired Student's *t* test, *P*-values less than 0.05 ($P < 0.05$) were considered significant (*) and *P*-values less than 0.01 ($P < 0.01$) were considered highly significant (**).

3. Results and discussion

3.1. ADAM17 is a direct target gene of miR-152 in HUVEC

Recent studies have shown that patients with AS show increased expression of ADAM17 in plaques, indicating that ADAM17 is an important target in AS pathogenesis. MiR-152 was predicted to target ADAM17 in bioinformatics analysis using TargetScan 6.2 software (Fig. 1A). MiR-152 and the predicted target site of ADAM17 are conserved among different mammalian species except for mouse (Fig. 1B). To test this prediction, we cloned the wild-type and the mutated region of the miR-152 seed-site in the 3′UTR of ADAM17 into luciferase reporter plasmids. Dual luciferase reporter gene assay showed that insertion of the ADAM17 3′UTR target sequence in the luciferase reporter vector led to decreased luciferase activity in the presence of miR-152 in HUVEC.

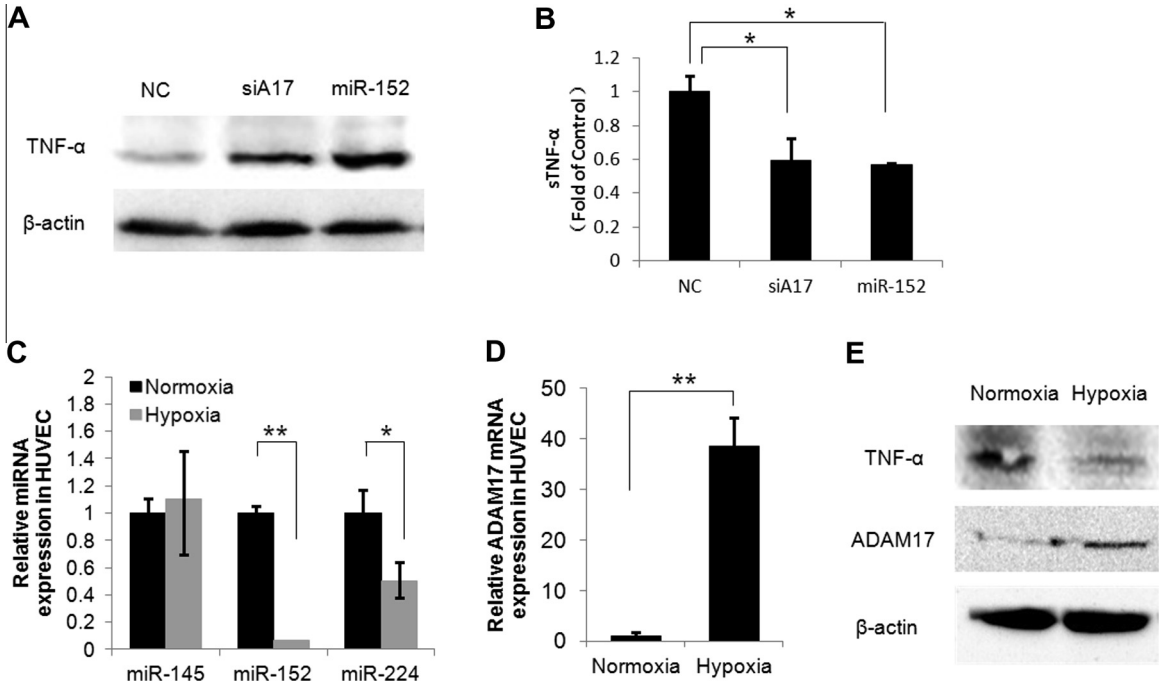

Fig. 2. ADAM17 is a functional target gene of miR-152 in HUVEC. (A) TNF-α protein expression in ADAM17 siRNA or miR-152 mimics transfected HUVEC. The level of ADAM17 and TNF-α were analyzed by Western blot analyses; β-actin was used as a loading control. (B) HUVEC were transfected with negative control RNA, ADAM17 siRNA or miR-152 mimics for 48 h. Culture medium of cells were collected for measuring sTNF-α concentration using ELISA. Values represented means of three independent experiments. (C) Effect of hypoxia on miR-145, miR-152 and miR-224 expression were analyzed by real-time PCR. U6 served as an internal control. (D) The expression level of ADAM17 mRNA was normalized by using β-actin as an internal control. (E) Western blot analysis of protein extracts of HUVEC under normoxia and hypoxia; β-actin was used as a loading control. *$P < 0.05$ and **$P < 0.01$ are considered statistically significant.

This effect was not observed for the miR-152 seed-site mutant (Fig. 1C), indicating that ADAM17 is a direct target gene of miR-152.

To verify the role of miR-152 in regulating ADAM17 expression, we performed real-time PCR and Western blot assays in HUVEC. Results showed that the mRNA and protein levels of ADAM17 were decreased in cells transfected with either ADAM17 siRNA or an miR-152 mimics compared with the levels detected in the control groups (Fig. 1D–F). It is suggested that miR-152 could regulate the gene expression of ADAM17 at both the mRNA and protein levels.

3.2. ADAM17 is a functional target gene of miR-152 in HUVEC

As ADAM17 is a well-known ectodomain sheddase, we then tested whether miR-152 treatment could reduce TNF precursor cleavage in endothelial cells. After ADAM17 siRNA and miR-152 transfection, results showed that miR-152 indeed reduced TNF precursor cleavage in HUVEC (Fig. 2A and B).

To evaluate the function of miR-152 targeting ADAM17 in physiological situation, HUVECs were cultured under hypoxic condition. The results showed that hypoxic treatment have reduced miR-152 expression and increased ADAM17 expression and TNF-α precursor cleavage in HUVEC when compared to normoxia (Fig. 2C–E). That is, the expression pattern of miR-152 and corresponding target ADAM17 was changed oppositely in HUVEC under hypoxic condition, which further confirmed that ADAM17 was the functional target of miR-152 in HUVEC.

3.3. MiR-152 inhibits HUVEC proliferation and migration

To investigate the function of miR-152 in HUVEC, MTS, wound-healing and Transwell chamber migration assays were carried out. Results showed that, compared with control cells, HUVEC transfected with the miR-152 mimics showed reduced proliferation levels similar to those of ADAM17 siRNA-transfected cells (Fig. 3A), while miR-152 inhibitors-transfected cells had the opposite effect. The results of wound-healing and Transwell chamber migration assays also showed that HUVEC transfected with the miR-152 mimics showed a significant decrease in migration ability (Fig. 3C and D). HUVEC proliferation and migration could be partially rescued by treatment with ADAM17 overexpression (Fig. 3A–C). Interestingly, we found overexpression of miR-152 had no effect on apoptosis of HUVEC in vitro (data not shown). Our results demonstrated that miR-152 could affect the HUVEC cell fate which plays important roles in the development of human atherosclerosis.

3.4. MiR-152 levels in clinical atherosclerosis patient serum samples

In atherosclerosis, microRNAs plays a crucial role in inflammation progress as previous reported and they could be detected in

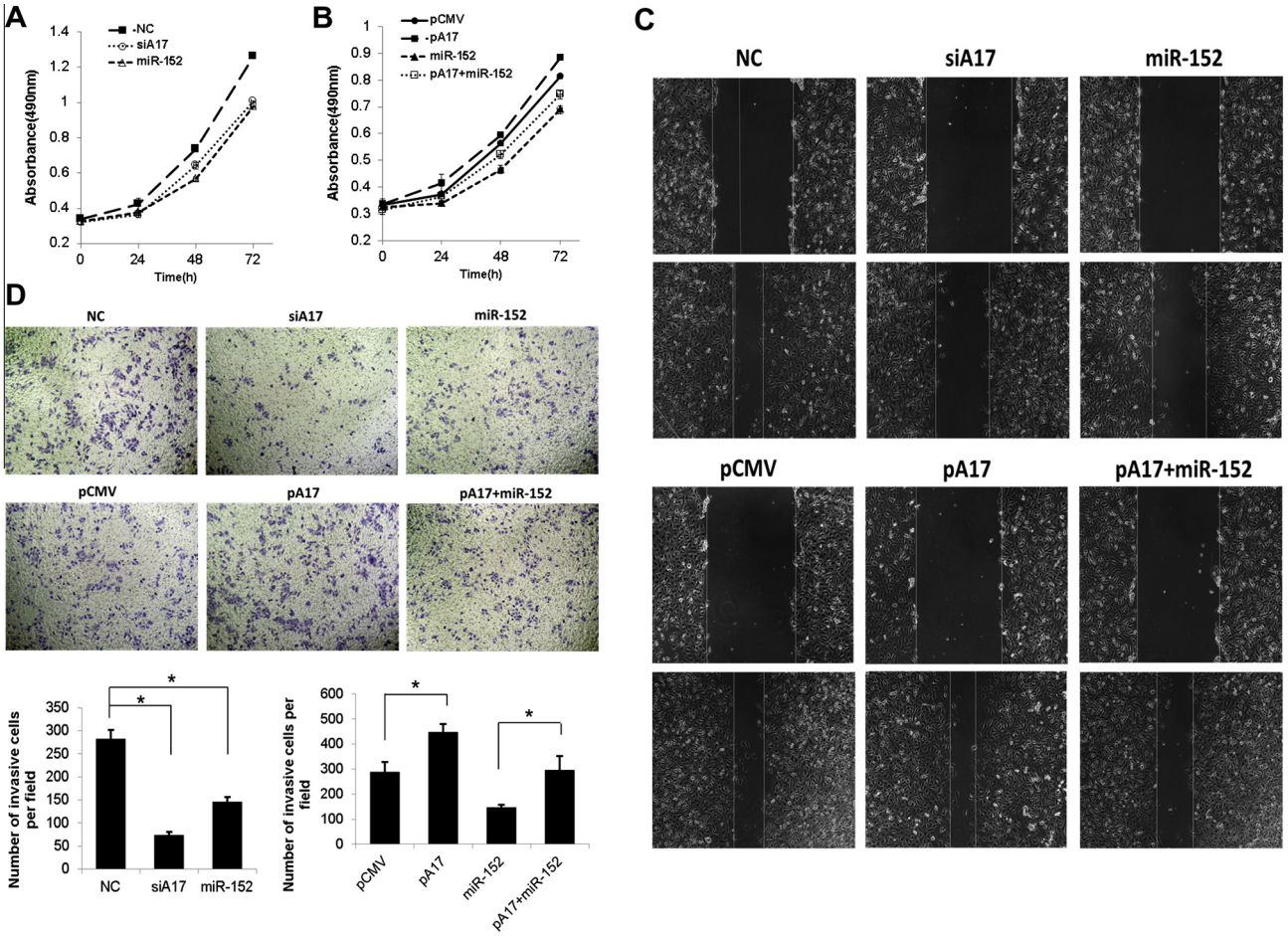

Fig. 3. MiR-152 inhibits HUVEC cell proliferation and migration. (A) MTS assay of HUVEC transfected with negative control RNA, ADAM17 siRNA, miR-152 mimics. (B) MTS assay of HUVEC transfected with pCMV-myc (pCMV), pCMV-ADAM17-myc (pA17) and miR-152 mimics. (C) Wound-healing assay of HUVEC transfected with negative control RNA, ADAM17 siRNA, miR-152 mimics, pCMV-myc and pCMV-ADAM17-myc. (D) Transwell chamber migration assay of HUVEC transfected with negative control RNA, ADAM17 siRNA, miR-152 mimics, pCMV-myc and pCMV-ADAM17-myc. Values represented means of three independent experiments. $^{*}P < 0.05$ are considered statistically significant.

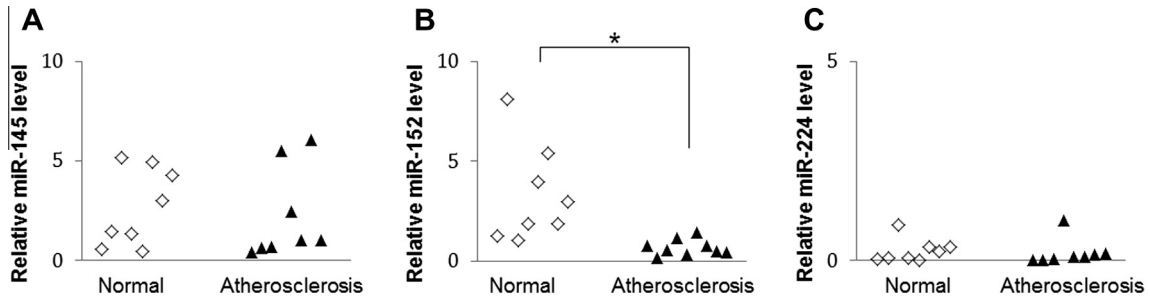

Fig. 4. Serum miR-152 levels in clinically diagnosed atherosclerosis patients and normal individuals. The expression of miR-145 (A), miR-152 (B) and miR-224 (C) in the sera of atherosclerosis patients and normal individuals was measured by qRT-PCR. The expression level of miRNAs was normalized by using miR-16 as an internal control. $^{*}P < 0.05$ are considered statistically significant.

patient serum sensitively and rapidly through real-time PCR analysis. To investigate the involvement of miRNAs in pharmacological effects of atherosclerosis, we performed miRNA expression analysis in clinical atherosclerosis patient serum samples. Analysis of serum miR-152 revealed lower levels in AS patients compared with those detected in normal individuals (Fig. 4B). However, there were no significant differences in the serum levels of other predicted or reported miRNAs target ADAM17, such as miR-145 and miR-224 (Fig. 4A and C). This data suggested that miR-152 could be exert important function in AS; however, this requires confirmation by the analysis of greater numbers of serum samples and investigation of the detailed mechanisms.

4. Discussion

It is reported previously that miR-145 targets ADAM17 in renal cancer cells [13]. It is now known that miRNA targeting depend on cell type and the nature of the environmental stimulus that triggers targets dynamic changing [20]. Our results showed a more perfect negative correlation of the expression pattern of miR-152 and corresponding target ADAM17 than that of other predicted or reported miRNAs [21,22] in HUVEC under native state or hypoxia condition. It is indicated that ADAM17 is the special functional target of miR-152 in HUVEC.

MicroRNAs have been considered promising candidates for diagnostic biomarkers because of the strong correlation between microRNA expression patterns and disease status demonstrated not only in animal models but also in patients. A lot of studies suggest that serum microRNAs are reliable indicators of disease status. We found that the levels of circulating miR-152 in AS patient serum samples were much lower than that detected in normal healthy serum samples, which indicated that miR-152 may be involved in the development of human atherosclerosis and could be used as diagnostic biomarker.

In conclusion, we demonstrated for the first time that miR-152 could reduce TNF precursor cleavage and inhibit cell proliferation and migration by targeting ADAM17 in HUVEC. The expression pattern of miR-152 and corresponding target ADAM17 was opposite in HUVEC under hypoxic condition. The levels of circulating miR-152 in AS patient sera were much lower than those detected in the sera of normal individuals Our results indicated that miR-152 may be involved in the development of human atherosclerosis and could be used as diagnostic biomarker or therapeutic target in atherosclerosis.

Acknowledgments

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