

# MiR-30e and miR-92a are related to atherosclerosis by targeting ABCA1

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**Abstract.** Atherosclerosis is a chronic disease characterized by the accumulation of lipids and fibrous elements in the large arteries, which is the principal cause of coronary artery disease. Dysregulated exosomal microRNA (miRNA) levels in serum have been identified in patients with various diseases, including CAD. In the present study, nine candidate miRNAs were detected in the plasma exosome from 42 patients with coronary atherosclerosis, and a higher expression of miR-30e and miR-92a was identified in patients. Following bioinformatics analysis and confirmation through immunoblotting, it was demonstrated that ATP binding cassette (ABC)A1 is a direct target of miR-30e, and miR-92a. Furthermore, a negative correlation was identified between plasma miR-30e and ABCA1, or miR-30e and cholesterol. Thus, the results of the present study suggest that the miR-30e level in exosomes from serum may have the potential to be a novel diagnostic biomarker for coronary atherosclerosis.

## Introduction

Atherosclerosis, a chronic disease characterized by the accumulation of lipids and fibrous elements in the large arteries, is the principal cause of CAD, a leading cause of morbidity and mortality worldwide (1). Plasma high-density lipoprotein (HDL) is thought to be a sterol transporter which is a protective factor against atherosclerosis. Meanwhile, the inverse

correlation between plasma HDL-C and the incidence of atherosclerosis is well established (2,3).

The formation of HDL occurs in the liver and intestine. The interaction between lipid poor apolipoprotein A1 (ApoA1) with the ATP binding cassette A1 (ABCA1) mediates this first step in HDL formation (4). ABCA1 is a member of the ABC family of membrane transporters that promotes phospholipid and cholesterol transfer from cells to poorly lipidated ApoA1. Recently, genetic association study and functional study in mice have indicate an important role of ABCA1 during the pathogenesis of atherosclerosis (5-8).

In cardiovascular pathologies circulating miRNAs have been described as disease-specific biomarkers and various animal models and clinical studies have proven miRNAs suitable for diagnostic purposes in CAD and myocardial infarction (MI) (9,10). Exosomes ranging in size from 40-100 nm in diameter, secreted by cells are proposed to be mechanism through which secreted cells pass signals to targeted cells. Meanwhile, altered exosomal miRNAs in serum has been found existed in the patients with CAD (11,12).

In the present study, we detected 9 candidate miRNAs in the plasma exosome from 42 patients with coronary atherosclerosis. The function of disturbed miRNA was examined by dual-luciferase assay and immunoblotting.

## Materials and methods

**Clinical samples.** This study includes 42 consecutive patients with coronary atherosclerosis and 42 age and sex paired healthy controls. All the participants were collected from Beijing Institute of Heart Lung and Blood Vessel Diseases between September 2014 and November 2015. Clinical diagnosis of coronary atherosclerosis was evaluated by percutaneous coronary angiography, reviewed by two experienced cardiologists. Healthy control subjects, without atherosclerosis, were selected in the same period. Written informed consent was obtained from all participants and this study was approved by the Ethics Committee of Beijing Institute of Heart Lung and Blood Vessel Diseases.

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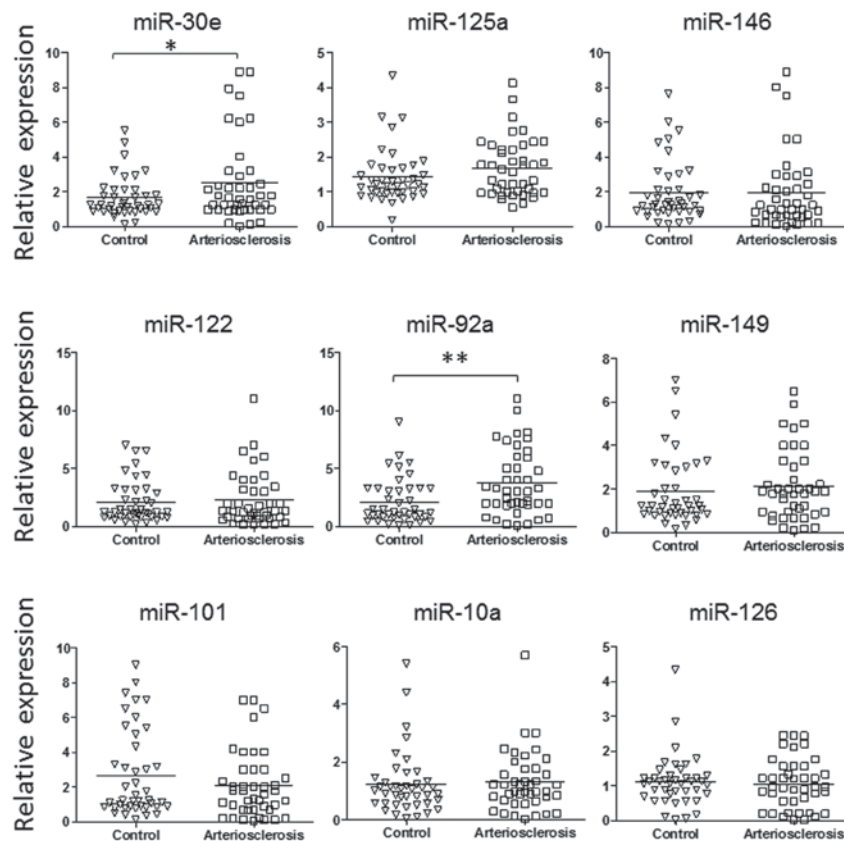


Figure 1. miR-30e and miR-92a were down regulated in patients with coronary atherosclerosis. The peripheral blood samples were collected from 42 patients with coronary atherosclerosis and 42 age and sex paired healthy controls. Exosomes were extracted from plasma using ExoQuick Exosome Precipitation Solution. Total RNA was extracted from plasma exosomes and candidate miRNAs were detected by RT-qPCR. The results were analyzed by student's t-test and  $P < 0.05$  was considered statically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

A total of 10 ml peripheral venous blood was collected from each participant. Portion of the blood samples were processed for total cholesterol, HDL-C and LDL-C detection. Portion of blood sample was processed for plasma separation and exosomes extracted subsequently. The processing of these blood samples was started within 30 min after collection.

**Plasma exosome extraction.** Exosomes were extracted from plasma using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA). Plasma was obtained by centrifugation at  $\times 3,000$  g for 15 min to remove cells and cellular fragments, and subsequent filtration of the supernatant was accomplished through a  $0.45\text{-}\mu\text{m}$  pore polyvinylidene fluoride filter (Millipore, Billerica, MA, USA). Add  $100\text{ }\mu\text{l}$  Thromboplastin D reagent rapidly into  $100\text{ }\mu\text{l}$  plasma sample to mix thoroughly and then incubate at  $37^\circ\text{C}$  for 15 min. Spin at  $10,000$  rpm at RT for 5 min. ExoQuick was added to the supernatants, and exosomes were precipitated by refrigeration at  $4^\circ\text{C}$  for 12 h. Exosome pellets collected by centrifugation at  $\times 1,500$  g for 30 min were dissolved in  $20\text{ }\mu\text{l}$  PBS.

**Cell culture.** HepG2 and HEK293T cells were purchased from China Infrastructure of Cell Line Resources and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA),  $100\text{ IU/ml}$  penicillin

and  $10\text{ mg/ml}$  streptomycin. THP1 cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Hyclone). All cells were maintained at  $37^\circ\text{C}$  under an atmosphere of 5%  $\text{CO}_2$ .

For macrophages differentiation, THP1 cells ( $1 \times 10^6$  cells/ml) were transferred into 100 mm-dishes by the addition of  $100\text{ ng/ml}$  phorbol 12-myristate 13-acetate for a 72-h period.

**RNA isolation and qRT-PCR.** Total RNA was extracted from exosomes or from cell samples by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression level of miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems) together with miRNA-specific TaqMan MGB probes (Applied Biosystems). U6 level was quantified for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times for the detection of miRNAs.

**Dual luciferase assay.** A segment of 956 bp ABCA1 3'UTR segment containing the potential target sites of miR-92a and miR-30a was cloned into downstream of firefly luciferase coding region in pmirGLO plasmid (Promega, Madison, WI,

Table I. Characteristics of cases and controls.

Characteristics	Cases (n=42)	Controls	P-value
Age	63 (42)	63 (42)	1
Sex (male/female)	22/20	22/20	1
Diabetes	7 (42)	5 (42)	0.041
Hypertension (yes/no)	29 (42)	21(42)	0.0035
Total cholesterol (mmol/l)	4.64 (4.24-5.48)	4.01 (3.28-5.23)	<0.001
HDL-C (mmol/l)	1.19 (1.05-1.43)	1.25 (1.03-1.64)	<0.001
LDL-C (mmol/l)	3.09 (2.70-3.45)	2.42 (2.04-3.01)	<0.001

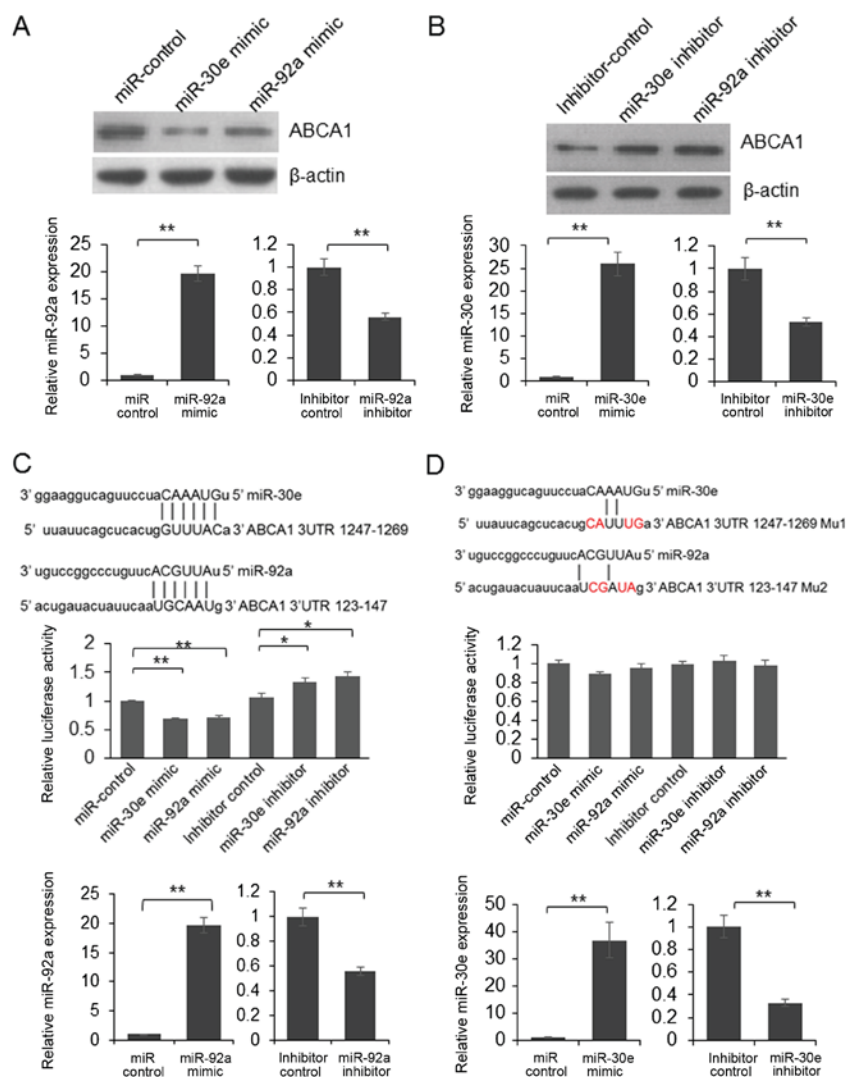


Figure 2. ABCA1 is a direct target of miR-30e and miR-92a. (A, B) immunoblotting. HepG2 cells were transfected with mimic or inhibitor of miR-30e or miR-92a, 48 h after transfection, cells were lysed and expression of ABCA1 were examined by immunoblotting. The expression of  $\beta$ -actin was used as loading control. The relative miR-92a and miR-30e level was determined by qRT-PCR. The results were analyzed by student's t-test and  $P < 0.05$  was considered statistically significant. (C, D) Schematic diagram of the predicted interaction between ABCA1 mRNA and miR-30e or miR-92a. A segment of 956 bp ABCA1 3'UTR segment containing the potential target sites of miR-92a and miR-30a was cloned into downstream of firefly luciferase coding region in pmirGLO plasmid. The reporter vector transfected with miR-92a or -30e mimic or inhibitor into HEK293T cells for 48 h. Dual luciferase assay was used to examine the direct interaction ABCA1 mRNA and miRNAs. The relative miR-92a and miR-30e level was determined by qRT-PCR. The results were analyzed by student's t-test and  $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

USA) to generate luciferase reporter vector. For luciferase reporter assays, HEK293T cells were seeded in 48-well

plates. 20 nM miRNAs mimic or inhibitor and luciferase reporter vector (200 ng/well) were co-transfected by using

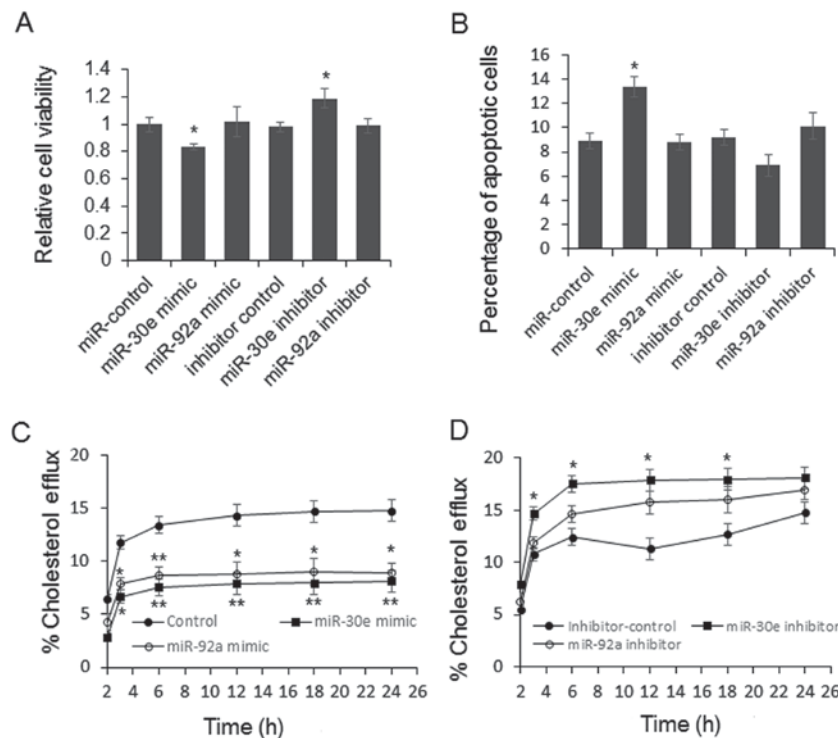


Figure 3. Functional study of miR-30e and miR-92a in THP1 cells. (A) THP1 cells were transfected with miR-30e or miR-92a mimic or inhibitor for 48 h. The cell viability was examined by MTT assay. (B) THP1 cells were transfected with miR-30e or miR-92a mimic or inhibitor for 48 h. The apoptosis cell number was examined by flowcytometry after staining. (C, D) The cholesterol efflux of differentiated THP1 macrophages was examined using cholesterol efflux assay kit following the manufacture's instruction. Results were analyzed by student's t-test and  $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

lipofectamine 2000 (Invitrogen). Two days later, cells were harvested and assayed with the Dual-Luciferase Assay kit (Promega). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

**Western blot analysis.** Protein extracts were boiled in SDS/ $\beta$ -mercaptoethanol sample buffer, and 30  $\mu$ g samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with mouse anti-ABCA1 monoclonal antibody (Abcam, Cambridge, MA, USA) or mouse anti- $\beta$ -actin monoclonal antibody (Santa Cruz Biotechnology Inc.) over night at 4°C. The specific protein-antibody complex was detected by using horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The  $\beta$ -actin signal was used as a loading control.

**Enzyme linked immunosorbent assay (ELISA) for estimating ABCA1 protein.** Serum ABCA1 level was estimated by using sandwich ELISA method and rabbit and mouse anti-ABCA1 antibodies (Abcam). Briefly, the 96-well plates were coated by mouse anti-ABCA1 (1:1,000 diluted) antibody and then incubated with 1:100 diluted serum samples for 2 h at room temperature. After washed by PBST, the plates were incubated using rabbit anti-ABCA1 antibody (1:1,000 diluted) followed

by HRP labeled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). TMB solution (Abcam) was added into each well, and incubated for 15-30 min. After adding equal volume of stopping solution the optical density was read at 450 nm. The relative concentrations were compared using OD value directly.

**Blood biochemical indexes.** Blood samples, from the same participants, were drawn for measurement of serum levels of TC, HDL-C, LDL-C after a 12-h overnight fast. Serum levels of TC (mmol/l), HDL-C (mmol/l), and LDL-C (mmol/l) were determined by colorimetric enzymatic assays with use of an Auto-Analyzer.

**Cell proliferation assay.** THP1 cells were seeded in 96-well plates at low density ( $5 \times 10^3$ ) and then transfected with miR-30e or miR-92a mimic or inhibitor. Twenty microliters MTT (5 mg/ml) (Sigma, St. Louis, MO, USA) were added into each well 48 h after transfection, and the cells were incubated for further 4 h. The absorbance was recorded at A570 nm with a 96-well plate reader after the DMSO addition.

**Apoptosis analysis.** Cell apoptosis was performed using Annexin V-FITC and propidium iodide (PI) staining and analyzed by flow cytometry.

**Cholesterol efflux assessment.** The cholesterol efflux of differentiated THP1 macrophages was examined using cholesterol efflux assay kit (Abcam) following the manufacture's instruction. Briefly, differentiated THP1 cells

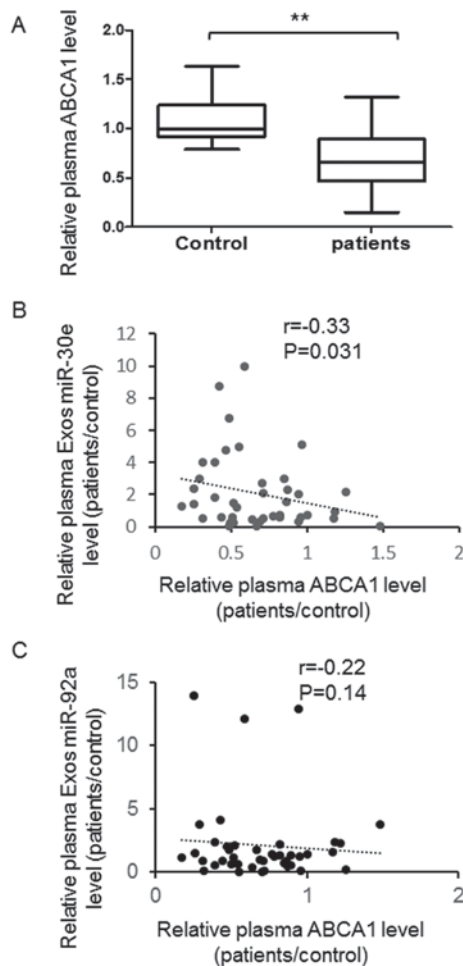


Figure 4. Plasma ABCA1 level decreased and negative correlate with miR-30e. (A) The plasma ABCA1 level was determined by ELISA using double antibody sandwich method and (B and C) the correlation analysis was processed by  $\chi^2$ -analysis. \*\* $P<0.01$ .

were transfected with miR-30e or miR-92a mimic or inhibitor for 6 h and then incubated with Labeling Reagent for 16 h. Wash cells by RPMI1640 medium, the cells were cultured at 37°C. Transfer the supernatant to 96-well plates at different time points to measure the fluorescence (Ex/Em=482/515 nm).

**Statistical analysis.** All the results were analyzed by using SPSS Statistical Package version 16. The data of two groups were analyzed by student's t-test and the correlation analysis was processed by  $\chi^2$ -analysis.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

Atherosclerosis is a chronic inflammatory disease of the vascular wall which leads to cardiovascular pathologies such as myocardial infarction, ischemic stroke and peripheral arterial disease. To find a new marker for atherosclerosis diagnosis and unveil the pathogenesis of atherosclerosis related CAD, we detected the expression of 9 candidate miRNAs expression in the exosome from serum sample of 42 patients with coronary atherosclerosis and age, sex paired healthy controls (Table I). These candidate miRNAs were reported to be related to the pathogenesis of CAD (13-15). As shown in Fig. 1, the level

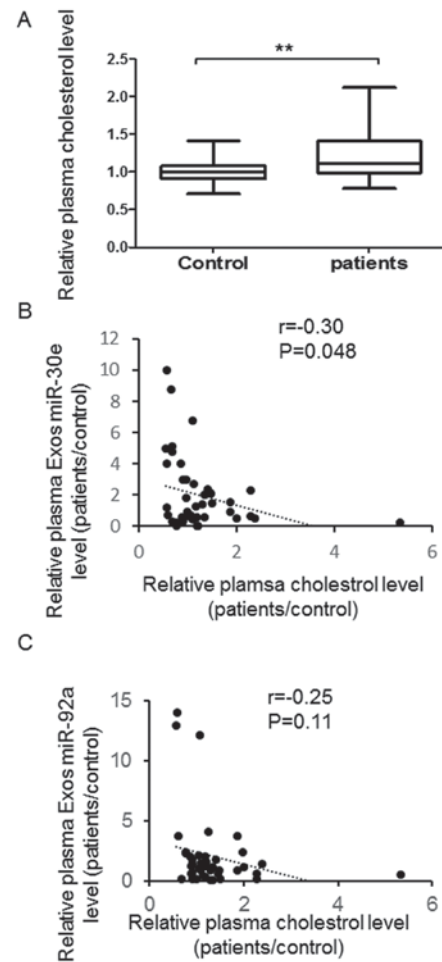


Figure 5. Plasma cholesterol level decreased and was negatively correlated with miR-30e. (A) Plasma cholesterol level was determined by colorimetric enzymatic assays using an Auto-Analyzer and (B and C) correlation analysis was processed by  $\chi^2$ -analysis. \*\* $P<0.01$ .

of miR-30e and miR-92a was significantly upregulated in the plasma exosome of patients with atherosclerosis.

To further understand the biological function of miR-30e and miR-92a, the potential direct targets of miR-30e and miR-92a were predicted using online bioinformatics tool: miRanda (<http://www.microrna.org>). We found that ABCA1 is a potential target of miR-30e and miR-92a. To understand whether the expression of endogenous ABCA1 is repressed by miR-30e and miR-92a, HepG2 cells were transfected with mimic or inhibitor of miR-30e or miR-92a. 48 h after transfection, the cells were lysed and the expression of ABCA1 was examined by immunoblotting. As shown in Fig. 2A, the protein level was reduced in miR-30e or miR-92a mimic transfected cells and up-regulated in the cells transfected with miR-30e or miR-92a inhibitor (Fig. 2B).

To confirm the direct interaction between ABCA1 and miRNAs, we constructed a reporter vector through inserting ABCA1 3'UTR into pmirGLO vector, following the stop codon of firefly luciferase. Subsequently, dual luciferase assay was processed. As shown in Fig. 2C, the relative luciferase activity was decreased significantly in miR-30e or miR-92a mimic transfected cells. Meanwhile, the luciferase activity was up-regulated by inhibitors of miR-30e and miR-92a (Fig. 2C). When 4 nucleotides in the predicted target regions altered, the

luciferase activity was not changed significantly by the mimic of miR-30e or miR-92a (Fig. 2D). These results indicated that miR-30e and miR-92a can repress the expression of luciferase by targeting 3'UTR of ABCA1. These results indicated that ABCA1 is a direct target of miR-30e and miR-92a.

ABCA1, also known as the cholesterol efflux regulatory protein (CERP) is a major regulator of cellular cholesterol and phospholipid homeostasis. Meanwhile, disturbed miRNA level also has the potential of altered cell proliferation and apoptosis. To further explore the function of miR-30e and miR-92a during the pathogenesis of CAD, we first examined the cell viability and apoptosis by MTT assay and flow cytometry. As shown in Fig. 3A, the relative cell viability was significantly repressed by miR-30e and up-regulated by miR-30e inhibitor. Meanwhile, the apoptotic cell number was increased in the cells transfected with miR-30e mimic (Fig. 3B). Subsequent cholesterol efflux assay results indicated that miR-30e and miR-92a mimic can repress the cholesterol efflux significantly (Fig. 3C). miR-30e inhibitor treatment relates to increased cholesterol efflux (Fig. 3D). miR-92a inhibitor up-regulated the cholesterol efflux slightly, but the difference was not significant (Fig. 3D).

To understand the correlation between the expression of miR-30e, miR-92a in the plasma and clinical characters of patients, we detected the plasma level of ABCA1 and cholesterol in the patients and relative controls. As shown in Fig. 4A, a higher plasma ABCA1 exists in the patients with atherosclerosis compared with healthy control. Meanwhile, a significant negative correlation was found between plasma ABCA1 level and plasma exosomal miR-30e level (Fig. 4B). However, no significant correlation was found between plasma ABCA1 level and exosomal miR-92a level (Fig. 4C). Furthermore, plasma cholesterol level was up-regulated in patients with atherosclerosis and negatively correlate with plasma exosomes miR-30e level instead of miR-92a (Fig. 5A-C).

## Discussion

Atherosclerosis is a chronic disease characterized by the accumulation of lipids and fibrous elements in the large arteries which is the principal cause of CAD. Disturbed exosomal miRNAs in serum have been found in patients with a lot of kinds of diseases including CAD. In this study, we detected 9 candidate miRNAs in the plasma exosome from 42 patients with coronary atherosclerosis and found a higher expression of miR-30e and miR-92a in patients. Analyzed by bioinformatics tools and confirmed by immunoblotting, we found that ABCA1 is a direct target of miR-30e and miR-92a. Furthermore, a negative correlation was found between plasma miR-30e and ABCA1, or miR-30e and cholesterol. So miR-30e may have the potential to be a new biomarker for coronary atherosclerosis.

Exosomes are shed by cells under both normal and pathological conditions, and they carry nucleic acids and proteins from their host cells that are indicative of pathophysiological conditions. Meanwhile, under the protection of lipid bilayer, the bio-functional molecules are more stable than that exposed in the biofluids (16). So, they are widely considered to be crucial for biomarker discovery for clinical diagnostics, and that is why we choose miRNAs in exosomes to screen biomarkers

for atherosclerosis. We chose 9 candidate miRNAs that were reported to be related to the pathogenesis of CAD (13-15,17). We find an increased exosomal miR-92a expression in patients with coronary atherosclerosis, which is in line with the report of Niculescu LS *et al* (14). Meanwhile, we report for the first time that overexpressed miR-30e relates to coronary atherosclerosis, which was first reported in a mouse atherosclerosis model (15). However, we could not verify abnormal level of the other 7 miRNAs, the altered level of which was reported in the peripheral blood of patients with CAD. These results suggesting that exosomes may provide more specific biomarkers for atherosclerosis clinical diagnosis.

Exosomes are full of common constituents which are fusion of multivesicular bodies attachment to target cells. In this study, we reported that miR-30e and miR-92a were up-regulated in the serum exosomes of patients with coronary atherosclerosis and ABCA1 is a direct target of miR-30e and miR-92a. These two exosomal miRNAs may have the potential to be a biomarker for atherosclerosis diagnosis. Meanwhile, miRNAs can be delivered from the donor cells to the recipient cells by exosomes (18). So whether overexpressed miR-92a and miR-30e can be functionally delivered to target cells or not need to be further examined. Meanwhile, our study partially explained the function of disturbed miR-30e and miR-92a in the cell proliferation, apoptosis and cholesterol efflux, however, the role of miR-30e and miR-92a in the pathogenesis of atherosclerosis needs to be further unveiled.

The ABCA1 is a member of the ABC1 subfamily that moves phospholipids and cholesterol across the cell membrane to HDL-C and has an important role in the pathogenesis of atherosclerotic vascular diseases due to their involvement in cholesterol homeostasis, blood pressure regulation, endothelial function, vascular inflammation, as well as platelet production and aggregation (19). It is confirmed the more than XX miRNAs modulate ABCA1 expression directly including miR-33a, miR-122, miR-467b, miR-183, miR-28 and so on (20,21). In the present study, we confirm ABCA1 is a direct target of miR-92a and miR-30e for the first time, which is an import supplement of our knowledge of ABCA1 modulation system.

In conclusion, the level of plasma exosomal miR-30e and miR-92a was up-regulated in patients with atherosclerosis and negative correlate with the plasma cholesterol and ABCA1 level, which may provide a new biomarker for clinical diagnosis and treatment of coronary atherosclerosis.

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