Roles of miR-16 in vascular endothelial injury in patients with coronary heart disease.

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Abstract

Objective: This study is to detect miR-16 expression in peripheral blood of patients with Coronary Heart Disease (CHD), and explore the role of miR-16 in vascular endothelial injury.

Methods: Peripheral blood was collected from coronary patients. QRT-PCR was applied to detect miR-16 expression and autophagy related 14 (AGT14) mRNA expressions. After transfection with miR-16 inhibitor in vitro, the biological functions of miR-16 were detected in endothelial cells by CCK-8, apoptosis assay, and cell cycle analysis. Bioinformatics methods predicted that ATG14 was one of target genes regulated by miR-16. Dual luciferase assay was used to validate whether miR-16 directly regulated ATG14. Expression changes of ATG14, Beclin1 and microtubule-associated protein 1 light chain 3 beta (LC3B) were detected by Western blot.

Results: miR-16 expression was significantly increased in blood in CHD patients, and miR-16 expression was higher in patients with unstable angina than patients with stable angina (P<0.05), which indicated that miR-16 was related to the development of CHD. In vitro experimental results showed that miR-16 inhibited the proliferation and promoted apoptosis of endothelial cells significantly (P<0.05). Compared with control group, the percentage of cells in G1 phase in miR-16 inhibitor group significantly decreased (58.8 ± 3.51 vs. 40.7 ± 3.32) whereas the percentage of cells in S phase significantly increased (21.8 ± 1.62 vs. 32.7 ± 2.47) (P<0.05), indicating that miR-16 inhibited G1/S conversion of endothelial cells. The dual luciferase assay validated that miR-16 directly targeted ATG14. Western blot and qRT-PCR results demonstrated that expressions of Beclin1 and LC3B were repressed when miR-16 was over-expressed while were significantly increased after down-regulated miR-16. And, electronic microscopy showed that miR-16 inhibited the formation of autophagy bodies in endothelial cells.

Conclusion: MiR-16 expression was up-regulated in peripheral blood of patients with CHD. MiR-16 regulated ATG14 expression in endothelial cells. Thus, miR-16 may play a role in the development of vascular endothelial injury and CHD.

Keywords: MiR-16, Vascular endothelial injury, Autophagy related 14 (AGT14), Coronary heart disease (CHD).

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Introduction

Coronary Heart Disease (CHD) is a type of serious disease, which frequently occurs in cardiovascular system and is closely related to the environment, autoimmune, and genetic factors [1-3]. The pathogenic processes of CHD are very long, and the chronic vascular endothelial injury is a major cause of this disease [4,5]. It is demonstrated that endothelial cells have endocrine, paracrine and autocrine ability and can secrete biological active substances to regulate vascular function, such as nitric oxide, prostacyclin I2, thromboxane A2, which play important roles in occurrence and development of CHD [6,7]. In addition, the basic pathological change of atherosclerosis is the injury of vascular endothelial cells, which is a risk factor for CHD [8,9]. However, the exact pathogenesis mechanisms are still unclear [10].

MiRNA is a class of small non-coding RNA with 18-22 nt in length, which can inhibit protein translation through binding to 3’ UTR of target miRNA and then regulate gene expression as an important post-transcriptional regulator [11]. Bioinformatics algorithm predicts that about 1/3 genes can be regulated by miRNA [12]. It is demonstrated that miRNA expression profile changes significantly in peripheral blood in patients with cardiovascular diseases, and some of them could be used as diagnosis markers, such as miR-1, miR-135, and miR-126 [13,14]. Mature miR-16 is processed from precursor miR-16-1 and miR-16-2. MiR-16-1 is located in one fragile site on 11q24, and miR-16-2 is located in 21q211 near Let-7c [10]. MiR-16
could inhibit tumor proliferation, metastasis and promote apoptosis and its expression is down-regulated in lung cancer, liver cancer, ovarian cancer, and pancreatic cancer, etc. [15-17]. miR-16 is also related to cardiovascular diseases. For example, miR-16 inhibits angiogenesis through down-regulating expressions of VEGFR2 and FGFR1 [18]. The expression change of miR-16 can induce myocardial cell hypertrophy [19]. The role and mechanism of miR-16 in CHD are not clear.

In this study, we aim to detect the expression of miR-16 in peripheral blood in patients with CHD and explore its role in vascular endothelial cells.

Materials and Methods

Clinical data and sample collection

This study included 67 cases of CHD patients, which was consisted by 37 cases of Unstable Angina Pectoris (UAP) and 30 cases of Stable Angina Pectoris (SAP) from February 2013 to August 2014 in Department of Cardiology in Shanghai Ninth People's Hospital. Among all the patients, 31 cases were male and 36 cases were female, and the mean age was (63.2 ± 5.36) years old. For control, 30 healthy individuals (21 males and 9 females) were included from center of health examination with the mean age of (60.80 ± 4.5) years old. All the patients were diagnosed based on 2011 “ACC/AHA Unstable Angina (UA)/non-ST Segment Elevation Myocardial Infarction (NSTEMI) Guide" and standard treatment guidelines of chronic stable angina by the Chinese Medical Association. Totally 5 ml fasting blood was collected from each patient and each healthy individual within 24 h of admission. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Shanghai Ninth People's Hospital.

Human umbilical vein endothelial cell (HUVEC) culture

DMEM medium with 10% FBS was used to culture HUVEC cells under 37°C in 5% CO₂. When cell confluence reached about 70%-90%, trypsin digestion method was used for cell passage, and the first 3-6th generation cells were used for experiments.

Quantitative real time PCR (qRT-PCR)

Total RNAs of peripheral blood and cultured HUVEC cells were extracted by Trizol (Invitrogen, California, USA) method. Then, RNA was reverse transcribed into cDNA. For the detection of miR-16b, the primers for miR-16b were 5’-AGCAAGCAGTGAATATTGG-3’ and universal primer. The 20 μl reaction system included 10 μl qRT-PCR-Mix, 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl cDNA template and 7 μl ddH2O. The cycle conditions were the following: 95°C for 10 min, and followed by 40 cycles of 95°C for 1 min, 60°C for 30 s. The relative expression level was calculated by the 2-ΔΔCT method.

For detection of autophagy related 14 (ATG14) mRNA, the primers for ATG14 were as follows: Forward: 5’-TTCATGCCGCTCGTGCATG-3’ and Reverse: 5’-GGATCTGCGCTGCAATTTCG-3’. The reaction system included 10 μl qRT-PCR-Mix, 0.5 μl forward primer, 0.5 μl reverse primer, 5 μl cDNA template and 14 μl ddH2O. The PCR procedures were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 2 min. The relative expression level was calculated by the 2-ΔΔCT method.

Transfection of miR-16b inhibitor to HUVEC cells

The HUVEC cells were divided into Control group, Negative Control (NC) group, and miR-16b inhibitor group. The cells were cultured in antibiotics free DMEM medium containing 10% FBS. When cell confluence reached about 70%-90%, the cells were used for transfection. Totally 1.25 ul of 25 pmol/ul miR-16 inhibitor and 1 ul of Lipo 2000 (Invitrogen Co., Carlsbad, CA, USA) were added into EP tubes containing 50 ul DMEM medium, respectively. The 2 tubes were mixed together after 5 min incubation at room temperature. The mixture was added to each well after incubation for 20 min at room temperature. After 6 h, fresh DMEM with 10% FBS was used to replace medium, then cultured for 72 h.

CCK-8 assay

After transfection with miR-16b, each group of cells were washed by PBS twice and digested by trypsin. The cells were then seeded in 96-well plate. Each well contained about 2 × 10³ cells and each well had 3 replicates. At 24 h, 48 h and 72 h, CCK-8 solution was added into each well and incubated for 1 h at 37°C. The absorbance of cells was measured at 450 nm wavelength to estimate the proliferation of cells.

Flow cytometry analysis of apoptosis

Each group of HUVEC cells was collected by centrifugation after trypsin digestion, and then washed by pre-cooled PBS twice. The apoptosis was detected with the ANXN V FITC APOPTOSIS DTEC KIT I (BD Biosciences, Franklin Lakes, NJ, USA) based on the standard protocol of manufactures. Briefly, cells were collected by trypsin digestion. After centrifugation at 500 g for 5 min, the cell pellet was collected. After washing with pre-cooled PBS for 3 times, cells were incubated with Annexin V and PI in the dark. Cell apoptosis was detected by flow cytometry.

Flow cytometry analysis of cell cycle

After transfection for 48 h, cells of each group were digested by trypsin and washed by pre-cooled PBS twice. Then, 1 × 10⁶ cells were used for cell cycle analysis based on standard protocol of BD cell Cycle/DNA Kits (BD Biosciences, Franklin Lakes, NJ, USA) and Templates kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were collected and washed with pre-cooled PBS. Then, cells were incubated with 200 μl of Solution A at room temperature. After incubation for
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10 min, 120 µl of Solution B was added and incubated at room temperature for 15 min. After washing, cells were incubated with 10 µl of Solution C in the dark at room temperature for 10 min. Finally, cell cycle was analysed by flow cytometry. Modfit software (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyse cell cycle changes.

**Bioinformatics prediction and dual luciferase validation**

Target Scan software was used to analyse target genes of miR-16. For validating whether miR-16 can bind to ATG14, dual luciferase assay was applied. Based on the bioinformatics prediction, the wild-type 3’ UTR and the mutant 3’ UTR of ATG14 were synthesized in vitro and were cloned into the downstream of pMIR-REPORT luciferase vector by Spe-1 and Hind III enzyme. HEK293T cells were co-transfected with agomiR-16 (100 nM) mimics and wild-type ATG14 3’ UTR or the mutant 3’ UTR. After transfection for 24 h, cells were lysed and luciferase intensity was measured by GloMax 20/20 luminometer (Promega, Madison, WI, USA) based on the standard protocol of the luciferase kit (Promega, Madison, WI, USA). The intensity of Renilla was used as control, and the fluorescence intensity in different groups was analysed.

**Western blot**

After transfection for 48 h, the HUVEC cells were washed by pre-cooled PBS twice, and then RIPA lysis buffer containing 1% PMSF was added for lysis for 5 min on ice. Then, protein was loaded into 10% SDS-PAGE (constant voltage 100 V) and transferred to PVDF membrane. After blocking by 5% non-fat milk, the primary antibodies of AGT14 (1:800; rabbit anti-human polyclonal antibody; Cat# ab139727), Beclin1 (1:800; rabbit anti-human polyclonal antibody; Cat# ab55878), microtubule-associated protein 1 Light Chain 3 Beta (LC3B) (1:800; rabbit anti-human polyclonal antibody; Cat# ab48394) and GAPDH (1:5000; mouse anti-human monoclonal antibody; Cat# ab8245) were added and incubated at 4°C overnight. The secondary antibodies of HRP-conjugated goat anti-mouse (1:8000; Cat# ab6789) and goat anti-rabbit IgG (1:8000; Cat# ab6721) were added incubated at room temperature for 1 h. All the antibodies were purchased from Abcam Company (Boston, USA). Finally, the membrane was developed by enhanced chemiluminescence reagent. The developed film was scanned using the Alpha Imager gel imaging systems (Alpha Imager, Santa Clara, California, USA). And the Western blot images were analysed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal control.

**Analysis of autophagy by electron microscope**

After transfection with miR-16 inhibitor for 48 h, cells were collected and were fixed by 2.5% glutaraldehyde at 4°C overnight. After washing once with PBS, the 1% osmium tetroxide was added and incubated for 1 h. Then electron microscopy was used to observe autophagy bodies.

**Statistical analysis**

The SPSS 16.0 software was used to do statistical analysis. All the data were shown as the mean ± SD. Standard t-test was used to compare difference between groups. P<0.05 was considered as statistically significant.

**Results**

**MiR-16 is up-regulated in peripheral blood of patients with CHD**

To investigate the expression of miR-16 in peripheral blood of CHD patients, qRT-PCR was performed. As shown in Figure 1A, miR-16 expression was significantly up-regulated in peripheral blood of CHD patients when compared with normal control (P<0.05). And, miR-16 expression was significantly higher in peripheral blood of UAP patients than in SAP patients (P<0.05) (Figure 1B). These results indicate that miR-16 may play roles in the development of CHD.

**Effects of miR-16 on proliferation of HUVEC**

To check the influence of miR-16 on HUVEC proliferation, miR-16 expression was inhibited in HUVEC after transfection with miR-16 inhibitor and then CCK-8 assay was used to detect the changes of cell proliferation. As shown in Figure 2A, compared with NC and control, cell proliferation of HUVEC at 48 h and 72 h was significantly higher in cells transfected with miR-16 inhibitor (P<0.05). Thus, miR-16 may slow down the proliferation of HUVEC.

**Effects of miR-16 on apoptosis of HUVEC**

To check the influence of miR-16 on HUVEC apoptosis, we inhibited miR-16 expression in HUVEC after transfection with miR-16 inhibitor. Then PI/ANNEXIN double staining method was used to detect the changes of cell apoptosis. As shown in Figure 2B, the apoptosis rate in miR-16 inhibitor group was significantly lower than NC group and control group (P<0.05), which indicate that miR-16 may promote the apoptosis of HUVEC.

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**Effects of miR-16 on cell cycle of HUVEC**

To determine the effect of miR-16 on HUVEC cell cycle, flow cytometry analysis was performed. As shown in Figure 2C, the percentage of cells in G1 phase was significantly lower and that in S phase was significantly higher in miR-16 inhibitor group (P<0.05). The results indicate that miR-16 may inhibit proliferation of HUVEC through inhibiting G1/S transition.

![Figure 2](image)

**Figure 2.** Effects of miR-16 on proliferation, apoptosis and cell cycle of HUVEC. A) Cell proliferation was measured by CCK8 assay. MiR-16 inhibits endothelial cell proliferation (*P<0.05). B) Cell apoptosis was measured by flow cytometry. MiR-16 promotes endothelial cell proliferation (*P<0.05). C) Cell cycle was detected with flow cytometry. Quantitative flow cytometry results were shown. MiR-16 inhibits endothelial G1/S conversion of HUVEC (*P<0.05).

**ATG14 mRNA level is regulated by miR-16**

To further detect the correlation of miR-16 with ATG14, qRT-PCR was applied to detect ATG14 mRNA expression after miR-16 inhibition. As shown in Figure 4, ATG14 mRNA expression in HUVEC was gradually increased at 24 h, 48 h, and 72 h after transfection with miR-16 inhibitor. Statistically, there was significant difference between NC group and miR-16 inhibitor group at all-time points (P<0.05). The results further validated that miR-16 negatively regulated ATG14 expression in HUVEC.

![Figure 4](image)

**Figure 4.** Analysis of ATG14 mRNA expression. ATG14 mRNA expression was detected with qRT-PCR. Compared with NC, *P<0.05.

**Expression of ATG14 and autophagy associated proteins by Western blot**

To explore the function of miR-16 in HUVEC, Western blot was used to detect the expressions of autophagy related proteins after abnormally expressing miR-16. As shown in Figure 5, ATG14 protein expression was significantly increased when down-regulating miR-16 (P<0.05). The autophagy associated proteins Beclin1 was up-regulated, and the ratio of LC3B I/II was significantly increased (P<0.05). These results indicate that miR-16 may suppress cell autophagy of HUVEC.

**Effects of miR-16 on the formation of autophagosome by electron microscope**

We also applied electron microscope to observe the autophagosome in HUVEC. As shown in Figure 6, we found the number of autophagosomes was increased after down-regulating miR-16, which indicated that autophagy level was
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increased. The results indicate that miR-16 may affect autophagy of HUVEC.

**Figure 5.** Analysis of ATG14, Beclin1, and LC3B. Expression levels of ATG14, Beclin1, and LC3B were detected with Western blot. A) Representative Western blot results. B) Quantitative Western blot results. Compared with NC, *P<0.05.

**Figure 6.** Analysis of autophagy. Autophagy was observed under microscope. Down-regulated miR-16 increases capability of autophagy. Red arrows show the autophagosome. A) miR-16 inhibitor group. B) Negative control group.

**Discussion**

Vascular endothelial cell damage is an initiation process of atherosclerosis, which further leads to CHD, stroke and other complications [20]. It is reported that a variety of miRNA are changed during processes of cardiovascular diseases. For example, Wang et al. found that the expression levels of miR-1, miR-133a, miR-499, and miR-208a were lower in plasma of healthy human, while these expression levels were significantly increased in peripheral blood of acute myocardial infarction patients [21]. Among these miRNAs, miR-208a has better sensitivity and specificity in the diagnosis of myocardial infarction [21]. Other study demonstrated that miR-122 can be used as an early diagnosis marker for liver injury as it was significantly increased in plasma in patients with liver tissue damage [22]. In this study, the expression and the role of miR-16 expression in patients with CHD were analysed.

The progression of CHD is accompanied with aggravation of vascular endothelial cell damage [23]. Studies show that miRNA plays an important role in vascular endothelial cell damage. For example, Hu et al. reported that miR-21 was closely related with the proliferation of vascular endothelial cells [24]. Xu et al. found that miR-103 affected the viability of HUVEC cells through regulating Bcl-2 [25]. Importantly, it is shown that miR-16 could inhibit the cell proliferation of nasopharyngeal carcinoma cells and human cervical cancer cells [26,27]. However, the role of miR-16 in CHD was unknown. Our results showed that miR-16 expression was significantly increased in peripheral blood of patients with CHD than healthy people. And, miR-16 expression was significantly higher in peripheral blood of UAP patients than SAP patients, which indicated that miR-16 might be involved in the progression of CHD. We further studied the biological functions of miR-16 in endothelial cells. After down-regulating miR-16, HUVEC proliferation was promoted and G1/S conversion was also accelerated. Consistent with previous studies [26,27], the results indicated that the proliferation capability of endothelial cells was inhibited by miR-16, which might slow down the damage repair of endothelial cell injury.

Zhang et al. found that miR-16 inhibited the apoptosis of PC12 cells [28]. MiR-16 can regulate chemo resistance of human glioma cells by regulating Bcl-2 [29]. In this study, apoptosis analysis demonstrated that miR-16 promoted the apoptosis of endothelial cells. Together, we speculated that the highly expressed miR-16 strengthened endothelial cell damage.

Furthermore, we found that ATG14 was one of target genes of miR-16 by bioinformatics analysis. ATG14 is one of the autophagy related genes, which plays important roles in the development of autophagy [30]. We hypothesized that miR-16 might inhibit cell proliferation, promote apoptosis and inhibit cell autophagy. After transfection with miR-16 inhibitor, we found that autophagy associated genes (Beclin1, LC3B, and ATG14) were up-regulated, which indicated that cell autophagy was increased. The electron microscopy observation showed that the number of autophagosomes was significantly increased after down-regulating miR-16, which further validated that miR-16 can inhibit the development and progression of cell autophagy.

It is of great importance to identify molecular markers for diagnosis of CHD. For example, soluble ST2, a receptor of IL-33 may be used as a novel cardiac bio-marker [31,32]. Whether miR-16 could be used as a diagnostic marker for CHD is unknown and needs further investigation.

In summary, up-regulated miR-16 in peripheral blood was related to the development and progression of CHD.

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**Disclosures**

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