MiR-365 participates in coronary atherosclerosis through regulating IL-6

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Abstract. – OBJECTIVE: This study aims to investigate the role and mechanism of miR-365 in the coronary atherosclerosis (AS).

PATIENTS AND METHODS: Blood were collected from AS patients and healthy people, coronary plaque and adjacent arterial tissue were collected from AS patients. QRT-PCR method was used to detect expressions of miR-365 and IL-6 in coronary plaque, blood monocytes, and serum. Western blot was applied to detect IL-6 protein expression in coronary plaque and blood monocytes, and ELISA was used to detect IL-6 protein expression in serum.

RESULTS: Compared with the normal group, IL-6 expression was significantly increased in coronary plaque, blood monocytes, and serum both in mRNA level and in protein level, while miR-365 expression was significantly decreased ($p < 0.05$).

CONCLUSIONS: IL-6 expression was significantly up-regulated in coronary plaque, blood monocytes, and serum, whereas miR-365 expression was down-regulated. MiR-365 may regulate the pathogenesis and immune response in AS through targeting IL-6.

Key Words:
    MiR-365, Coronary Atherosclerosis, Plaque, IL-6.

Introduction

Coronary atherosclerosis (AS) is a serious vascular disease and can induce coronary artery stenosis, which is one common reason to cause angina and myocardial infarction. The long-term myocardial ischemia and hypoxia can lead to enlargement of the heart, reduction of compliance, and dysfunction of myocardial contraction[1], which further induces chronic heart failure[2]. The coronary atherosclerotic plaque, especially the "vulnerable plaque" of coronary atherosclerosis with a tendency of thrombosis, is an important cause of coronary artery stenosis[3]. AS greatly affects the life quality of patients.

MiRNA is a class of small non-coding RNA in eukaryotes, with a length of 18-22 nt, which can regulate target gene expression in mRNA level and protein level[4-6]. In pathological processes of AS, expression levels of many miRNAs and proteins were changed, which indicated that miRNA could play important roles in regulating associated proteins of AS[7,8]. There are suggestions that atherosclerosis is an inflammation of the arterial intima and has the basic characteristics of inflammation[9,10]. IL-6 is a key inflammatory cytokine. However, the regulatory mechanisms of IL-6 in AS and the upstream regulation for IL-6 are still unclear.

In this study, we applied qRT-PCR, Western blot, bioinformatics prediction, and ELISA assay to detect IL-6 expression both in mRNA level and in protein level in coronary plaque, blood monocytes, and serum. We also predicted and validated the relationship of IL-6 expression with miR-365 expression to further analyze the mechanisms of miR-365 in AS.

Patients and Methods

Patients

This study included 38 cases of AS patients with coronary endarterectomy and 26 healthy control people from June 2012 to August 2015 in Henan Provincial People's Hospital. Among AS patients, there were 21 males and 17 females, and their ages were 40-75 years old, with a median of 65 years old. Among healthy control people, 18 males and 8 females were included, with age of 38-75 years old, and the median age was 59 years old. All AS patients were confirmed by electrocardiogram, echocardiography, and coronary angiography. Patients with complications and infection in liver and kidney, diabetes mellitus, cancer or other immune-related diseases were excluded. All patients were diagnosed by pathological professors. The healthy control group was also confirmed by above examination to exclude

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AS. The basic information (age, gender, diabetes, and hypertension history) of patients was collected. The Gensini scoring system was applied to evaluate the degree of AS. Prior written and informed consent was obtained from every patient and the study was approved by the Ethics Review Board of Henan Provincial People's Hospital.

Arterial plaque tissue and adjacent endometrial tissue were taken by coronary endarterectomy and then stored in liquid nitrogen. Peripheral blood was collected and serum was separated by centrifugation at 400 g for 10 min. Monocytes were isolated from peripheral blood as previously described[11]. Briefly, the solutions of 5 ml lymphocyte-separating medium, venous blood without heparin and IMDM without FBS were mixed equally (1:1). Then, the mixture was slowly added on hierarchical layers. After that, 8 ml Hanks solution proportionally diluted blood was added and centrifuged at 400 g for 30 min. Three layers were obtained after centrifugation. Monocytes were isolated from the middle layer. The isolated monocytes were washed with D-Hank's solution for 3 times. After centrifugation at 300 g for 10 min, the monocytes were re-suspended at $1*10^6$/ml. Each $3*10^6$ cells were seeded into 9 cm^2 dish and cultured at 37 °C in 5% CO_2 incubator for 1-2 h. The adherent cells were monocytes.

Methods

RNA isolation and Reverse Transcription

Totally 100 mg tissues were grinded into powder in liquid nitrogen and then 1 ml Trizol (Invitrogen, California, CA, USA) was added. RNA was extracted by phenol-chloroform methods. The RNA quality was checked by gel electrophoresis and the ratio of 260/280 by Spectrophotometer. The cDNA of mRNA were reversed transcribed by TIANScript II cDNA kit (TIANGEN, Beijing, China) based on the standard protocol. The cDNA was stored at -20 °C.

QRT-PCR Analysis

MiR-365 expression was detected in coronary plaque, blood monocytes, and serum by SYBR GREEN qRT-PCR (Nuoweizan Biocompany, Nanjing, China). The U6 was used as internal reference. The primers for miR-365 were 5'- GCGTA-ATGCCCCTAAAAATCC -3' and Uni-miR qPCR Primer provided in kit (Angran Biocompany, Shenzhen, China). The primers for U6 were 5'-AAC-GCTTCACGAATTTGCGT-3' and 5'-CTCGCT-TCGGCAGCACA-3'. The system (total 25 μl) included 2 μl cDNA, 12.5 μl SYBR PremixETaq, 1 μl PCR Forward Primer, 1 μl Uni-miR qPCR Primer, and 8.5 μl ddH$_2$O. The cycle conditions were the following: 95 °C for 30 s, and followed by 40 cycles of 95 °C for 5s, 60 °C for 34s. Each sample had 3 replicates. The relative expression of miR-365 was calculated by the $2^{-\Delta\Delta T}$ method.

For detection of IL-6 mRNA in coronary plaque, blood monocytes, GAPDH was used as internal reference. The primers for IL-6 were 5'- GGCACTGGCAGAAAACAACC -3' and 5'-GCAAGTCTCCTCATTGAATCC -3'. And the primers for GAPDH were 5'-GGGAAACTGCG-GCGTGAT-3' and AAAGGTGGAGGAGTGG-GT. The 25 μl reaction system included 12.5 μl SYBR EXTaqTM, 0.5 μl forward primer, 0.5 μl reverse primer, 1 μl cDNA template and 10.5 μl ddH$_2$O. Each sample had 3 replicates. The cycle conditions were the following: 95 °C for 30 s, and followed by 45 cycles of 95 °C for 5s, 57 °C for 30s, then additional melting curve was analyzed by 95 °C for 15s, 60 °C for 23 s and 95 °C for 15s. The relative expression of IL-6 was calculated by the $2^{-\Delta\Delta T}$ method.

Western Blot

Total proteins were extracted by the standard protocol of lysis (Nuoweizan Biocompany, Nanjing, China). The protein concentration was detected by BCA assay kit (Beyotime Biotechnology, Jiangsu, China). The proteins were loaded into 10% SDS-PAGE and then transferred to PVDF membrane (100 V constant voltage in an ice bath). After blocking by 5% skim milk at room temperature for 1h, the primary antibodies (rabbit anti-human IL-6, 1:1000 and rabbit anti-human β-actin antibody, 1:5000) were added and incubated at 4 °C overnight. Then, the secondary antibody of HRP-conjugated goat anti-rabbit IgG (1:3000) was added and incubated at room temperature for 1 h. All the antibodies were purchased from Abcam Company (Boston, MA, USA). Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned and analyzed by image lab software (Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as an internal control to calculate the relative expression of IL-6.

ELISA

ELISA was performed according to the instructions provided by the ELISA Kit (Abcam, Burlingame, CA, USA). Each 50 μl standard sam-

ples at different concentrations were added into different wells on the ELISA plate, and 10 μl serum and 40 μl dilution buffer were added into different well too. Except for blank well, each well was added with 100 μl HRP labeled antibody, covered with microplate sealers and then incubated at 37 °C for 1 h. After washing for 5 times, 50 μl substrate A and 50 μl substrate B were added into each well. After incubation at 37 °C for 15 min, 50 μl stop solution was added into each well. The OD value was measured at a wavelength of 450 nm within 15 min.

Bioinformatics Prediction

The software of miRanda, TargetSean, PicTar, MiRanda and BibiServ were used to predict the potential miRNAs that can regulate IL-6 directly. The miR-365 was selected for further study. The complementary binding site of miR-365 with IL-6 was shown in Figure 1.

Statistical Analysis

The SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used. All the data were shown as mean ± SD and normality test was performed. One-way ANOVA was used to compare difference among multiple groups. When variance was homogenous, LSD and SNK method were used; otherwise, Tamhane's T2 or T3 methods were applied. The $p < 0.05$ was considered as statistically significant.
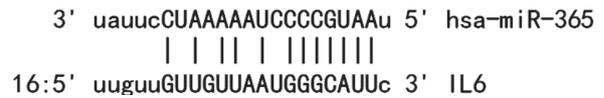
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3'  uauucCUAAAAAUCCCCGUAAu 5'  hsa-miR-365
          | | || | |||||||
16:5'  uuguuGUUGUUAAUGGGCAUUc 3'  IL6
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Figure 1. The "seed" region of miR-365 and complementary binding site in 3'UTR of IL-6 mRNA.

Results

Expression of IL-6 mRNA in Different specimens in AS Patients

To detect the expression changes between coronary plaque and adjacent tissues in AS patients, we applied qRT-PCR to detect IL-6 mRNA. We also compared IL-6 mRNA expression in serum and monocytes in AS patients with healthy control people. Compared with adjacent tissues, IL-6 mRNA expression was significantly increased in AS plaques ($p < 0.01$, as shown in Figure 2A). Similar to the results in plaque, IL-6 mRNA expression was also significantly increased in serum and blood monocytes of AS patients than healthy control ($p < 0.05$, as shown in Figure 2B and Figure 2C). The results indicate that IL-6 may play regulatory roles in the pathological processes of AS.

Expression of IL-6 Protein In Coronary Plaque and Blood Monocytes

To validate the expression of IL-6 protein in plaque and blood monocytes in AS patients, We-
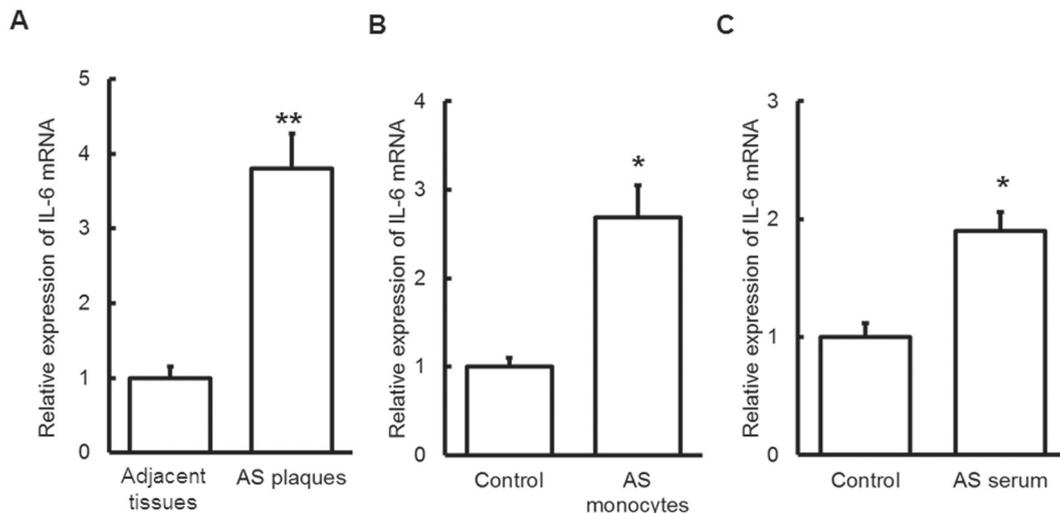
Figure 2. IL-6 mRNA expression in different types of specimens in AS patients. *A,* Compared with adjacent tissues, the expression of IL-6 mRNA was significantly increased in arteriosclerosis (AS) plaques ($p < 0.01$). *B,* Compared with control group, the expression of IL-6 mRNA was significantly increased in blood monocytes in AS patients ($p < 0.05$). *C,* Compared with control group, the expression of IL-6 mRNA was significantly increased in serum in AS patients ($p < 0.05$).

stern blot was used. Compared with adjacent tissues and control group, IL-6 protein was significantly increased in plaque and blood monocytes in AS patients ($p < 0.05$, as shown in Figure 3A and Figure 3B). We also applied ELISA to detect the expression of IL-6 protein in serum in AS patients. Compared with control group, IL-6 protein was significantly increased in serum of AS patients ($p < 0.05$, as shown in Figure 3C). The results demonstrated that IL-6 protein expression was
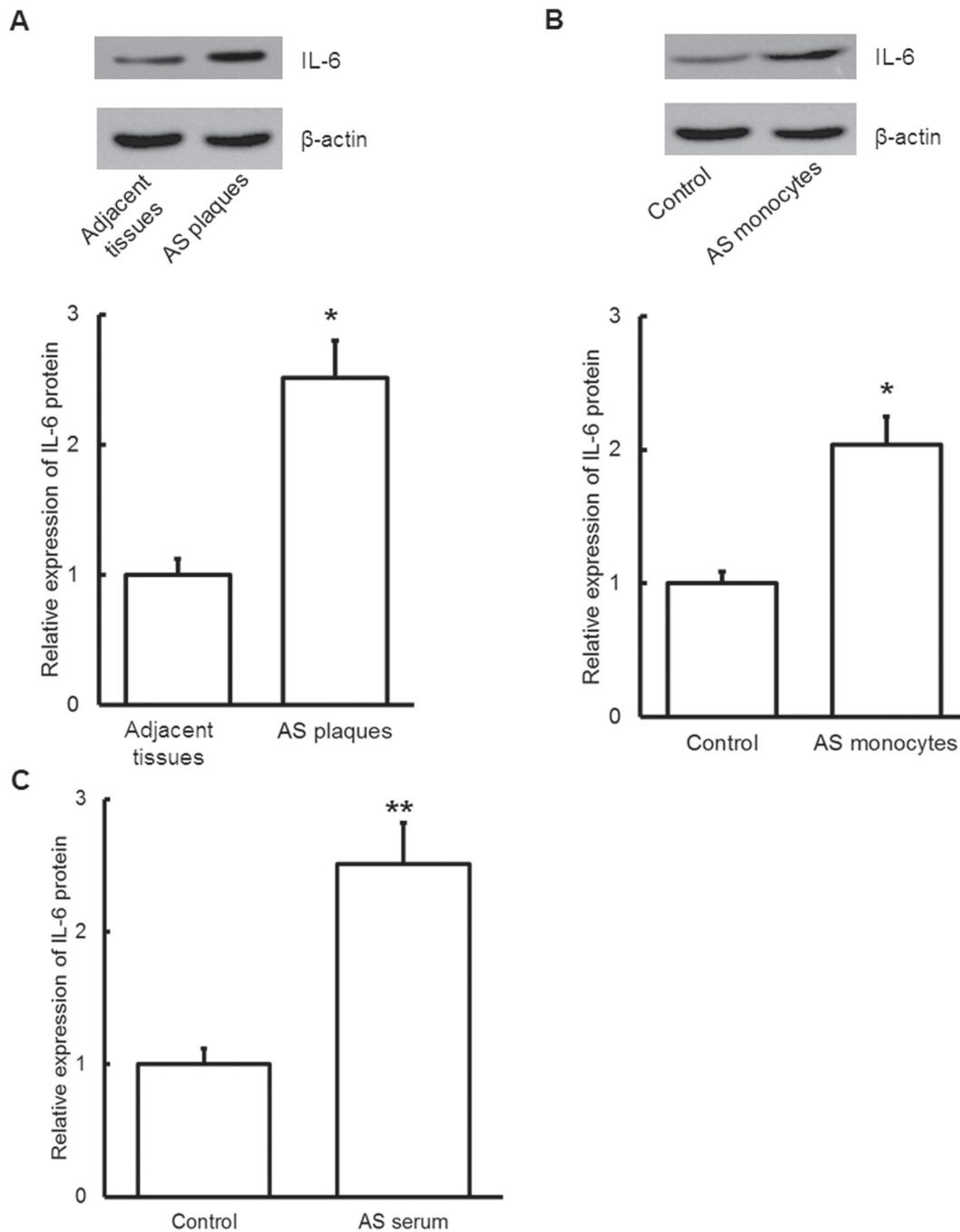

Figure 3. IL-6 protein expression in plaques and blood monocytes in AS patients. **A,** Compared with adjacent tissues, the expression of IL-6 protein was significantly increased in arteriosclerosis (AS) plaques by Western blot ($p < 0.05$). **B,** Compared with control group, the expression of IL-6 protein was significantly increased in blood monocytes in AS patients by Western blot ($p < 0.05$). **C,** Compared with control group, IL-6 protein expression was significantly increased in serum of AS patients ($p < 0.01$).
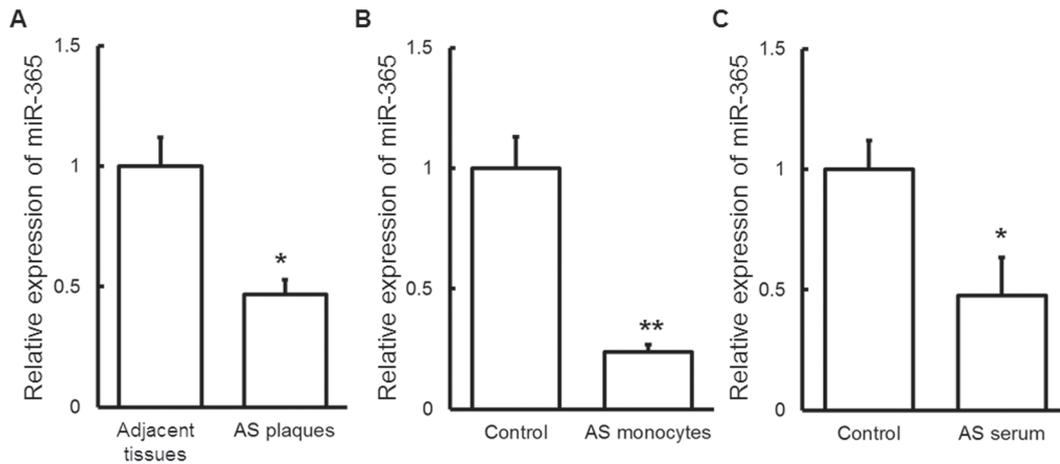
Figure 4. MiR-365 expression in different types of specimens in AS patients. *A,* Compared with adjacent tissues, the expression of miR-365 was significantly decreased in AS plaques ($p < 0.05$). *B,* Compared with control group, miR-365 expression was significantly increased in blood monocytes in AS patients ($p < 0.01$). *C,* Compared with control group, miR-365 expression was significantly increased in serum in AS patients ($p < 0.05$).

consistent with IL-6 mRNA expression, which further indicate that IL-6 might play roles in the development of AS.

Expression of miR-365 in Different Types of Specimens in AS Patients

To investigate the roles of miR-365 in AS patients, qRT-PCR was used to detect its expression in coronary plaque, serum, and blood monocytes. As shown in Figure 4, miR-365 expression was significantly down-regulated in coronary plaque, serum, and blood monocytes when compared with control samples ($p < 0.05$). Considering the above detection of IL-6 expression and bioinformatics prediction about miR-365 with IL-6, these results indicate that miR-365 may play roles in the pathological processes of AS through regulating IL-6 expression.

Discussion

In this study, we observed miR-365 expression in coronary atherosclerotic plaque, blood monocytes, and serum of AS patients, and we also detected down-stream target gene (IL-6) expression both in mRNA level and in protein level. We discussed the potential mechanisms of how miR-365 participated in the pathogenesis of AS through down-regulating IL-6.

IL-6 is a cytokine with multiple functions, which is produced by the stimulation of bacteria, endotoxin, dust and other foreign substances[12,13].

IL-6 plays important roles in immune response, inflammation, cell differentiation, blood clotting and tumor development, and its expression is significantly increased in the inflammation reaction induced by injury, trauma, stress and infections[14,15]. In inflammation, IL-6 may induce expressions of C-reactive protein and fibrinogen, and may promote thrombosis[16]. Through binding to IL-6 receptor, IL-6 can induce the occurrence of inflammatory diseases such as rheumatoid arthritis and Crohn's disease[17]. In rheumatoid arthritis, IL-6 can stimulate T cell and B cell to secrete inflammatory mediators and can promote maturation of B cells, which increases the effects of IL-1β and TNF-α[18]. In inflammation, IL-6 has a chemotactic effect to other inflammatory cells, such as neutral lymphocytes and monocytes macrophages[19,20]. All these studies indicate that IL-6 plays important roles in inflammation. In this work, we detected the increased expression of IL-6 in plaque, serum, and blood monocytes of AS patients both in mRNA level and in protein level, which indicates that the occurrence of AS may be similar to one kind of vascular intima inflammation. Then, monocytes and lymphocytes are activated, and these cells can secrete large amounts of IL-6 to enhance immune response, which is consistent at the reaction of cell damages in human[21,22].

MiRNA participates in different pathological and physiological processes, such as proliferation, invasion and metastasis of tumor cells, hypertension, diabetes, and atherosclerosis[23,24]. It is still unclear about the roles of miRNA in AS patho-

genesis. MiR-365 was reported to be abnormally expressed in breast cancer and uterine endometriosis[25], and in NIH3T3 cells, miR-365 expression was significantly increased after ultraviolet radiation, which showed some tumor suppressing effect[26]. Over-expressed tumor suppressor gene NGX6 may induce the decreased expression of miR-365, which indicates that miR-365 may play roles as oncogene[27]. In the resting or senescence of lung fibroblasts, miR-365 expression was down-regulated, which indicated that miR-365 could regulate cell proliferation[28]. Through bioinformatics prediction, we found that miR-365 was an upstream regulator of IL-6. After reviewing literature, we found that miR-365 negatively regulated IL-6 expression in HEK293 and HeLa cells[29]. In this research, the down-regulated miR-365 and up-regulated IL-6 in plaques and monocytes in AS patients indicate that the increased IL-6 may be induced by decreased miR-365. In serum, we also observed the similar expression pattern of miR-365 and IL-6.

Conclusions

We found that miR-365 expression was decreased in coronary endometrial tissue and blood, which may induce the up-regulation of IL-6 expression. Through regulating IL-6 expression, miR-365 plays important roles in the development and progression of AS.

Conflict of interest

The authors declare no conflicts of interest.

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