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Characteristics of *CXCL2* expression in coronary atherosclerosis and negative regulation by microRNA-421

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Abstract

Objective: The study investigated expression of microRNA (miR)-421 in plaques, peripheral blood mononuclear cells (PBMCs), and serum from patients with coronary atherosclerosis.

Methods: Thirty-three patients with coronary atherosclerosis and 29 healthy individuals were included. Plaque tissue and adjacent intimal tissue were collected from patients. Peripheral blood was collected from patients and healthy individuals. Quantitative real-time PCR was used to determine expression of C-X-C motif chemokine ligand 2 (*CXCL2*) mRNA and miR-421. Western blotting was used to measure expression of CXCL2 protein in plaques and PBMCs, and ELISA was used to detect serum levels of CXCL2. A dual luciferase reporter assay was carried out to test whether *CXCL2* mRNA directly interacts with miR-421.

Results: Patients with coronary atherosclerosis had elevated expression of CXCL2 mRNA and protein in plaques, PBMCs, and serum compared with healthy controls but reduced expression of miR-421. The dual luciferase reporter assay showed that miR-421 could bind with the 3′-untranslated seed region of *CXCL2* mRNA to regulate its expression.

Conclusion: We demonstrated that elevated expression of CXCL2 in plaques, PBMCs, and serum of patients with coronary atherosclerosis was related to downregulation of miR-421 expression. miR-421 plays a role in the occurrence of coronary atherosclerosis, probably through CXCL2.

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Introduction

Coronary atherosclerosis, a serious vascular disease, results in coronary artery stenosis, which is a common cause of angina pectoris and myocardial infarction.[1] Coronary atherosclerotic plaques, especially thrombotic "vulnerable plaques," are an important cause of coronary stenosis.[2] Coronary atherosclerosis usually has a long clinical treatment cycle, frequent relapse, poor prognosis, and a great impact on the quality of life. Therefore, exploring the mechanism of coronary atherosclerosis at the molecular level is important for improving its clinical treatment.

Atherosclerosis is a type of inflammation of the artery intima, and it is accompanied by basic characteristics of inflammation.[3,4] C-X-C motif chemokine ligand 2 (CXCL2) is a key inflammatory factor that has a strong chemotactic effect,[5] playing important physiological roles in atherosclerosis.[6] MicroRNAs (miRNA or miR) are small non-coding RNA molecules (18 to 22 nucleotides) that are ubiquitous in eukaryotes and that can regulate protein expression at the mRNA level.[7–9] Expression of many miRNAs and proteins is altered in coronary atherosclerosis, suggesting that miRNAs may play important roles in the regulation of atherosclerosis-related proteins.[10,11] Abnormal expression of miR-421 is observed in various diseases, such as epilepsy, gastric cancer, glioma, and breast cancer,[12–15] suggesting its special roles in the pathological environment. However, the regulatory mechanism of CXCL2 in coronary atherosclerotic plaques and the upstream miRNA regulation of CXCL2 are not yet fully understood.

In the present study, we determined the expression of CXCL2 mRNA and protein in plaque tissues, blood monocytes, and serum from patients with coronary atherosclerotic plaques to better understand the relationship between CXCL2 and miR-421 in this disease.

Materials and methods

Ethical approval

All procedures performed in the current study were approved by the Ethics Committee of Zaozhuang Municipal Hospital. Written informed consent for participation and for publication of associated data and accompanying images was obtained from all patients or their parents, guardians, or next of kin.

Patients

Thirty-three patients with coronary atherosclerosis undergoing routine coronary artery endarterectomy at our hospital between January 2014 and December 2017 were included in the experimental group (18 men and 15 women; age range, 39 to 69 years; median age, 56 years). In addition, 29 healthy individuals who underwent physical examinations at our hospital in the same period were included as a control group for blood testing (16 men and 13 women; age range, 36 to 72 years; median

Table 1. Demographic data for healthy controls and patients with coronary atherosclerosis

	Controls	Patients
No. of subjects	29	33
No. of men	16	18
No. of women	13	15
Age range (years)	36–72	39–69
Median age (years)	58	56
Atherosclerosis	No	Yes
Complications and infections of liver and kidney	No	No
Diabetes	No	No
Tumors	No	No

age, 58 years). None of the patients had complications or infections of liver or kidney, diabetes, or tumors (Table 1). Age, sex, diabetes history, and hypertension history of the patients were recorded, and the degree of coronary atherosclerosis was evaluated using the Gensini scoring system.[16]

Samples

Plaque tissues and adjacent intimal tissues (control tissue) were collected from all patients by coronary artery endarterectomy. Peripheral blood was collected from all patients and healthy controls and centrifuged at $400 \times g$ for 10 minutes to separate the serum. To obtain peripheral blood mononuclear cells (PBMCs), a mixture of heparin-anticoagulated venous blood and an equal amount of serum-free Iscove's modified Dulbecco's medium (v/v, 1:1) was added gently onto lymphocyte separation medium before centrifugation at $400 \times g$ for 30 minutes. After centrifugation, the middle layer was aspirated and mixed with five volumes of Hanks' balanced salt solution before centrifugation at $300 \times g$ for 10 minutes. The cells were washed twice, counted, and diluted to a density of 1×10^6/mL. Finally, 3×10^6 cells were seeded onto a round culture plate with a bottom area of 9 cm^2, followed by incubation at 37°C and 5% CO_2 for 1 to 2 hours. The cells that attached on the bottom were PBMCs.

Quantitative real-time PCR

Samples (1,000 μL of serum or plaque or 3×10^7 PBMCs) were lysed using 10 mL of TRIzol reagent following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted using the phenol/chloroform method.[17] The concentration (15–30 ng/μL from serum; 50–100 ng/μL from plaques) and quality of RNA were measured using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Fisher Scientific). Then, cDNA was obtained by reverse transcription from 1 μg of RNA and stored at −20°C. Reverse transcription of mRNA was performed using TIANScript II cDNA First Strand Synthesis Kit (Tiangen, Beijing, China), and reverse transcription of miRNA was carried out using miRcute miRNA cDNA First Strand Synthesis Kit (Tiangen).

The SuperReal PreMix (SYBR Green) quantitative real-time (qRT)-PCR kit (Tiangen) was used to detect mRNA expression of *CXCL2*, using *ACTB* (*β*-actin) as internal reference. The sequences of *CXCL2* were 5′-CTCAAGAACATCC AAAGTGTG-3′ (forward) and 5′-ATTC TTGAGTGTGGCTATGAC-3′ (reverse). The sequences of *ACTB* were 5′-CACCA GGGCGTGATGGT-3′ (forward) and 5′-CTCAAACATGATCTGGGTCAT-3′ (reverse). The reaction system (25 μL) was composed of 12.5 μL of SYBR Premix EXTaq, 0.5 μL of upstream primer, 0.5 μL of downstream primer, 1 μL of cDNA, and 10.5 μL of distilled, deionized (dd)H_2O. The PCR conditions were initial denaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 40 s,

and elongation at 72°C for 15 s; and final elongation at 72°C for 5 minutes (iQ5; Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression of *CXCL2* mRNA against *ACTB*. Each sample was tested in triplicate.

The expression of miR-421 was determined by miRcute miRNA RT-PCR Kit (Tiangen), using U6 as internal reference. The sequences of miR-421 primers were 5′-TTCACAGTGCCTAATCCGG-3′ (forward) and 5′-GGCGCCCAATTAAT GTCTG-3′ (reverse). The sequences of U6 primers were 5′-CTCGCTTCGGCAG CACA-3′ (forward) and 5′-AACGCT TCACGAATTTGCGT-3′ (reverse). The reaction system (20 μL) contained 10 μL of qRT-PCR mix, 0.5 μL of upstream primer, 0.5 μL of downstream universal primer, 2 μL of cDNA, and 7 μL of ddH_2O. The reaction protocol was initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s (iQ5; Bio-Rad). The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression of miR-421 against U6. Each sample was tested in triplicate.

Western blotting

Before lysis, tissues (100 mg) were ground into powder, and cells (1×10^6) were trypsinized and collected. Then, tissue samples or cells were lysed with cooled radioimmunoprecipitation assay (RIPA) lysis buffer [600 μL; 50 mM Tris base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China] for 30 minutes on ice. The mixture was centrifuged at $10{,}000 \times g$ and 4°C for 10 minutes. The supernatant was used to determine protein concentration using a bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co. Ltd., Beijing, China). The samples were then mixed with 5× SDS loading buffer before denaturation in a boiling water bath for 10 minutes. Afterward, the samples (20 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skim milk at room temperature for 1 hour. Then, the membranes were incubated with rabbit anti-human CXCL2 (1:1000; ab91511; Abcam, Cambridge, UK) or ACTB (1:5000; ab129348; Abcam) polyclonal primary antibodies at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 three times (15 minutes each), the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000; ab6721; Abcam) for 1 hour at room temperature before washing with phosphate-buffered saline with Tween 20 three times (15 minutes each). Then, the membrane was developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image Lab v3.0 software (Bio-Rad) was used to acquire and analyze imaging signals. The relative content of target protein was expressed against ACTB.

ELISA

A CXCL2 ELISA kit (ab184862; Abcam) was used to determine the concentration of CXCL2 in serum. In microplates, standards (50 μL) and samples (10 μL of serum and 40 μL of diluent) were added into predefined wells, and blank wells were left empty. In the wells for standards and samples, horseradish peroxidase-labeled conjugates (100 μL) were added before sealing the plates for incubation at 37°C for 1 hour. After washing the plates 5 times, substrates A (50 μL) and B (50 μL) were added into

each well. After incubation at 37°C for 15 minutes, stop solution (50 µL) was added into each well, and the absorbance of each well was measured at 450 nm within 15 minutes.

Bioinformatics

Bioinformatics prediction is a powerful tool for the study of miRNA functions. We used miRanda (August 2010 release; http://www.microrna.org/microrna/home.do), Target Scan (v6.2; http://www.targetscan.org), PiTa (v6 on 31 August 2008; http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (18 September 2017; http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), and PICTA (26 March 2007; http://pictar.mdc-berlin.de/) to predict target genes that might be regulated by miR-421 and found that *CXCL2* was a potential target gene of miR-421 (Figure 1).

Dual luciferase reporter assay

According to the bioinformatics results, wild-type (WT) and mutant seed regions of miR-421 in the 3′-untranslated region (UTR) of *CXCL2* were chemically synthesized in vitro. Then, their two ends were attached with *Spe*I and *Hin*dIII restriction enzymes and cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.8 µg) with WT or mutant 3′-UTR sequences were co-transfected with agomiR-421 (100 nM; Sangon Biotech, Shanghai, China) into 293T cells. For control, 293T cells were transfected with agomiR-negative control (NC). After cultivation for 24 hours, the cells were lysed using the dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions, and luminescence intensity was measured using GloMax 20/20 luminometer (Promega). Using Renilla luminescence activity as the internal reference, the luminescence values of each group of cells were measured.

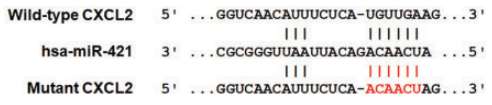

Figure 1. Direct interaction between miR-421 and CXCL2. Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. We used miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and PICTA (http://pictar.mdc-berlin.de/) to predict target genes that might be regulated by miR-421 and found that CXCL2 was a potential target gene of miR-421. miR, microRNA; CXCL2, C-X-C motif chemokine ligand 2.

Statistical analysis

The results were analyzed using IBM SPSS 20.0 statistical software (IBM Corp., Armonk, NY, USA) and data expressed as means ± standard deviations. Data were tested for normality, and multigroup measurement data were analyzed using one-way ANOVA. In case of homogeneity of variance, least significant difference and Student–Newman–Keuls methods were used; in case of heterogeneity of variance, Tamhane's T2 or Dunnett's T3 method was used. Comparison between two groups was carried out using Student's *t*-test. $P < 0.05$ indicated statistically significant differences.

Results

Expression of CXCL2 mRNA

We measured the expression of *CXCL2* mRNA using qRT-PCR. The levels of *CXCL2* mRNA in plaques, PBMCs, and serum from patients with coronary atherosclerosis were significantly higher than those in the control group ($P < 0.05$ for

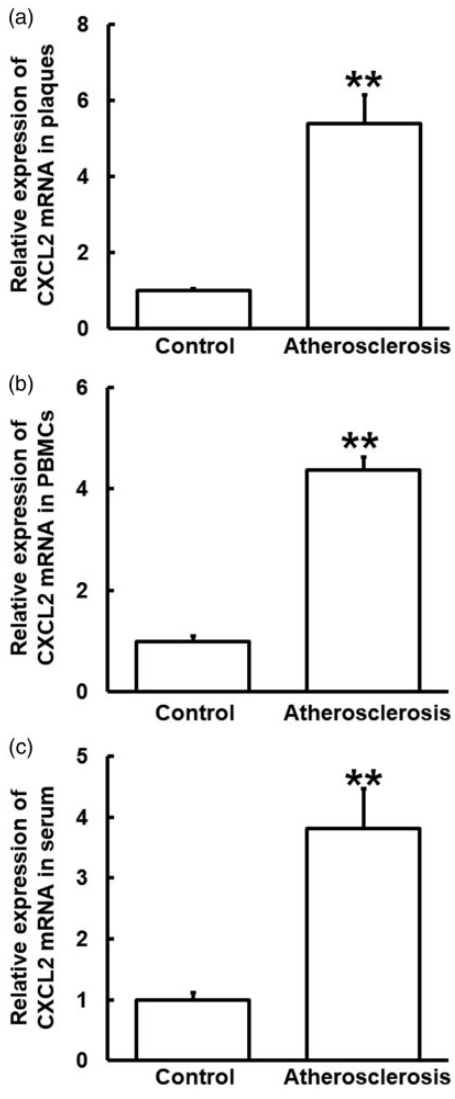

Figure 2. Expression of *CXCL2* mRNA in (a) plaques, (b) PBMCs, and (c) serum from healthy individuals (control group) and patients with coronary atherosclerosis. Quantitative real-time PCR was used to determine the expression of mRNA. $^{**}P < 0.01$ compared with control group. CXCL2, C-X-C motif chemokine ligand 2; PBMCs, peripheral blood mononuclear cells.

all; Figure 2). Thus, patients with coronary atherosclerosis had elevated expression of *CXCL2* mRNA compared with healthy individuals.

Expression of CXCL2 protein in plaques and PBMCs

To determine the expression of CXCL2 protein in plaques and PBMCs, western blotting was carried out. The levels of CXCL2 protein in plaques and PBMCs from patients with coronary atherosclerosis were significantly higher than those in the control group ($P < 0.05$ for all; Figure 3), indicating that expression of CXCL2 was elevated in plaques and PBMCs from patients with coronary atherosclerosis compared with healthy individuals.

CXCL2 protein in serum

We used ELISA to determine the content of CXCL2 protein in serum. The data showed that the content of CXCL2 protein in serum from patients with coronary atherosclerosis was significantly higher than that in the control group ($P < 0.05$; Figure 4), probably due to the increased release of CXCL2 protein from PBMCs.

Expression of miR-421

We used qRT-PCR to examine the expression of miR-421. The level of miR-421 in plaques, PBMCs, and serum was significantly lower in patients with coronary atherosclerosis than in the control group ($P < 0.05$ for all; Figure 5).

Interaction of miR-421 and 3′-UTR of CXCL2 mRNA

We used the dual luciferase reporter assay to evaluate the interaction between miR-421 and the 3′-UTR of *CXCL2* mRNA. The luminescence value of cells co-transfected with agomiR-421 and pMIR-REPORT-WT luciferase reporter plasmids was significantly lower than that in the negative control group ($P < 0.05$). In contrast, the luminescence value of cells co-transfected with agomiR-421 and pMIR-

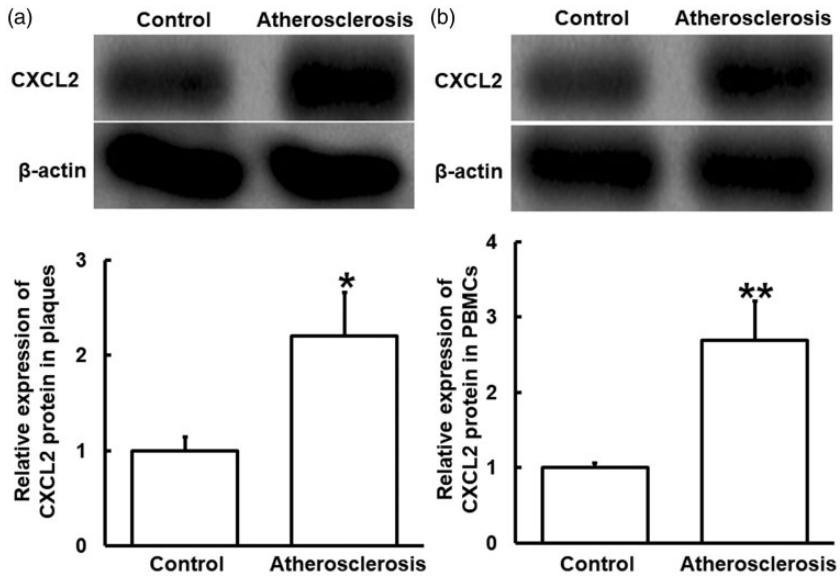

Figure 3. Expression of CXCL2 protein in (a) plaques and (b) PBMCs from healthy individuals (control group) and patients with coronary atherosclerosis. Western blotting was used to determine the expression of protein. *$P < 0.05$ and **$P < 0.01$ compared with control group. CXCL2, C-X-C motif chemokine ligand 2; PBMCs, peripheral blood mononuclear cells.

Figure 4. Expression of CXCL2 protein in serum from healthy individuals (control group) and patients with coronary atherosclerosis. ELISA was used to determine the content of protein. **$P < 0.01$ compared with control group. CXCL2, C-X-C motif chemokine ligand 2.

REPORT-mutant luciferase reporter plasmids was not significantly different from that in negative control group (Figure 6). These results indicate that miR-421 can bind with the 3′-UTR seed region of *CXCL2* mRNA to regulate its expression.

Discussion

CXCL2 plays important roles in the occurrence and development of chronic inflammation. Chemokines are important mediators involved in recruitment, maturation, and activation of immune cells, and their chemical constituents are low-molecular-weight secretory proteins.[18] Studies show that CXCL2 has potent neutrophil chemotactic activity, mediates inflammatory damages, and promotes tumor cell growth and angiogenesis.[19–21] Abnormal expression of *CXCL2* has been found in a variety of immune cells and tumors, including colon cancer, non-small-cell lung cancer, pancreatic cancer, esophageal squamous cell carcinoma, and primary chronic lymphoblastic leukemia.[22–27] Atherosclerosis is inflammation in the

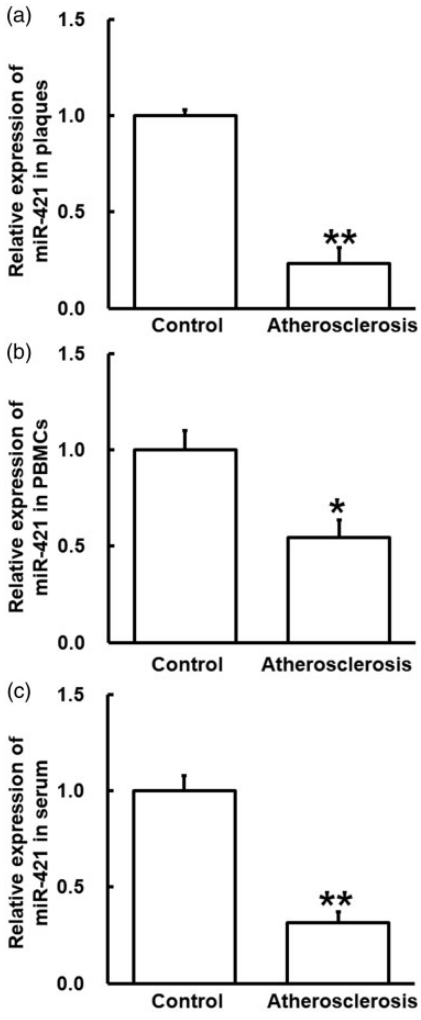

Figure 5. Expression of miR-421 in (a) plaques, (b) PBMCs, and (c) serum from healthy individuals (control group) and patients with coronary atherosclerosis. Quantitative real-time PCR was used to determine the expression of miR-421. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group. CXCL2, C-X-C motif chemokine ligand 2; PBMCs, peripheral blood mononuclear cells.

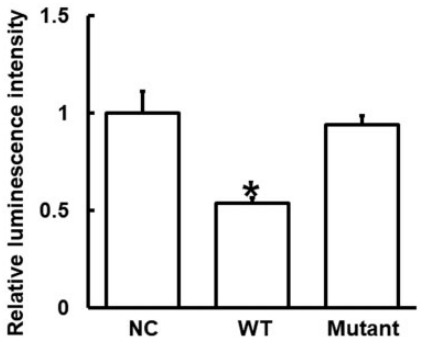

Figure 6. Identification of interaction between miR-421 and *CXCL2* mRNA using dual luciferase reporter assay. Plasmids (0.8 μg) with wild-type (WT) or mutant 3′-UTR sequences were co-transfected with agomiR-negative control (NC) or agomiR-421 (100 nM; Sangon Biotech, Shanghai, China) into 293T cells. After cultivation for 24 hours, the cells were lysed using dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer's manual, and luminescence intensity was measured using GloMax 20/20 luminometer (Promega). Using Renilla luminescence activity as internal reference, the luminescence values of each group of cells were measured. $^{*}P < 0.05$ compared with NC group. miR, microRNA; CXCL2, C-X-C motif chemokine ligand 2; UTR, untranslated region.

artery intima.[3,4] In the present study, the expression of CXCL2 mRNA and protein was upregulated in plaques, PBMCs, and serum of patients with coronary atherosclerosis, suggesting that the pathogenesis of coronary atherosclerosis is similar to that of intimal inflammation, in which monocytes and lymphocytes are activated and secrete a large amount of CXCL2 to produce chemotaxis.

Bioinformatics prediction showed that miR-421 is closely related to CXCL2 and may be an upstream miRNA that regulates *CXCL2*, perhaps by cleaving *CXCL2* mRNA and inhibiting its translation.[28] Regulation by miRNAs in this way up- or downregulates the expression of mRNA and thus plays an important role in the occurrence and development of diseases.[29,30] For example, abnormal expression of miR-421 has been detected in gastric cancer,[31] and expression of miR-421 is upregulated in neuroblastoma, pancreatic cancer, and prostate cancer.[32–34]

Moreover, miR-421 is closely related to cell growth. For example, the expression of miR-421 in gastric cancer tissues is higher than that in normal tissues, indicating that miR-421 may play an important role in the early growth of gastric cancer.[35] Moreover, transfection with miR-421 inhibitor in animals inhibits the growth of gastric cancer.[36] These studies suggest that miR-421 is closely related to the occurrence and development of human diseases. Our results demonstrated that miR-421 expression was reduced and *CXCL2* expression increased in plaques and PBMCs of patients with coronary atherosclerosis, suggesting that the body negatively regulates the cleavage of *CXCL2* by miR-421 by downregulating miR-421. Subsequently, increased expression of *CXCL2* promotes immune responses. Our observation on the expression of miR-421 and *CXCL2* in serum showed similar results, indicating that increased CXCL2 in PBMCs is released into the blood. Therefore, levels of miR-421 and CXCL2 in serum can partially reflect the inflammatory reaction and tissue injury in coronary atherosclerosis. Results of the dual luciferase reporter assay showed that miR-421 can directly bind with the 3′-UTR of *CXCL2* mRNA to regulate its expression.

In conclusion, we demonstrated that reduced expression of miR-421 in coronary artery intima tissue and blood decreases the ability of miR-421 to regulate CXCL2 expression, thereby altering the expression of related proteins and playing a biological role in the occurrence and development of coronary atherosclerosis.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work. JY collaborated in design of the study; JY, HL, QC, and WZ were responsible for performing experiments; JY analyzed the data; and JY and HL collaborated to interpret results and develop the manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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