Original Paper

Effects of MicroRNA-499 On the Inflammatory Damage of Endothelial Cells During Coronary Artery Disease Via the Targeting of PDCD4 Through the NF- κ B/TNF- α Signaling Pathway

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Key Words

Microrna-499 • Programmed cell death 4 • Coronary artery disease • NF- κ B/TNF- α signaling pathway • Endothelial cells • Inflammatory damage

Abstract

Background/Aims: This study aimed to analyze the impact of microRNA-499 (miR-499) on the inflammatory damage of endothelial cells during coronary artery disease (CAD) via the targeting of PDCD4 through the NF- κ B/TNF- α signaling pathway. **Methods:** A total of 216 CAD patients (CAD group) and 90 healthy people (normal group) were enrolled in our study. Endothelial cells were collected and assigned into normal, OX-LDL, negative control (NC), miR-499 inhibitor, miR-499 mimic, PDCD4 siRNA, and miR-499 inhibitor + PDCD4 siRNA groups. The quantitative PCR and western blotting were performed to detect the mRNA and protein expression levels of PDCD4 and miR-499. The MTT assay was performed to determine cell viability, and flow cytometry assay to evaluate cell apoptosis. **Results:** Increased miR-499 expression and decreased PDCD4 expression in the plasma were observed in the CAD group compared with the normal group, demonstrating a negative correlation between miR-499 and PDCD4. Compared to the normal and miR-499 inhibitor groups, the survival rate of cells and PDCD4 expression were decreased; and the expressions of miR-499, IL-6, IL-8, IL-1 β , TNF- α , NF- κ B, VCAM-1, ICAM-1 and MCP-1 and the apoptosis rate were all elevated in the OX-LDL, NC, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups. Compared to the OX-LDL, NC and miR-499 inhibitor + PDCD4 siRNA groups, PDCD4 expression and the survival rate of cells were increased; and the IL-6, IL-8, IL-1 β , TNF- α , NF- κ B, VCAM-1, ICAM-1 and MCP-1 expression levels and the apoptosis rate were all reduced in the miR-499 inhibitor group. In the PDCD4 siRNA group, PDCD4 expression and the survival rate of cells were lower, and the expression levels of IL-6, IL-8, IL-1 β , TNF- α , NF- κ B, VCAM-1, ICAM-1 and MCP-1 and the apoptosis rate were all higher compared with the miR-499 mimic group. In the miR-499

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inhibitor + PDCD4 siRNA group, PDCD4 expression and the survival rate of cells were higher, and the expression levels of IL-6, IL-8, IL-1 β , TNF- α , NF- κ B, VCAM-1, ICAM-1, and MCP-1 and the apoptosis rate were all lower than those in the PDCD4 siRNA group. **Conclusion:** Down-regulated miR-499 expression increased PDCD4 expression and protected endothelial cells from inflammatory damage during CAD by inhibiting the NF- κ B/TNF- α signaling pathway.

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Introduction

Coronary artery disease (CAD), as the most common etiology of heart attack and death in the world, is a multifactorial disease caused by the combined impact of genes [1]. The main etiology of CAD is characterized by defective endothelial function, however, the underlying mechanism has not been fully elucidated despite the high incidence of CAD [2]. Although there are many recent advancements in treatment, the mortality caused by CAD is still increasing. Genetic features are crucial and constant risk factors for CAD pathogenesis. Recently, one study investigated the relationship between CAD and genetic predisposition and showed that 40% to 60% of the risk for CAD is related to genetic predisposition [3].

MicroRNAs (miRNAs) are a group of single-strand non-coding RNAs of approximately 22 nucleotides that regulate gene expression either translational repression or mRNA degradation [4]. MicroRNA-499 (miR-499) is a recently discovered member of the miRNAs encoded by myosin [5]. Under normal physiological circumstances, there is a low expression level of miR-499 regulating the expression of the beta myosin heavy chain, leading to the enhancement of myocardial oxygen metabolism and tolerance [6]. It has recently been demonstrated that miR-499 expression is significantly changed in certain heart diseases such as valvular heart disease, ischemic heart disease and heart failure [7]. MiR-499 has been proven to target genes such as FOXO4 and programmed cell death 4 (PDCD4) [8].

PDCD4 is a highly conserved RNA binding protein that inhibits the translation of specific mRNAs [9]. As a significant tumor suppressor, PDCD4 affects translation and transcription of multiple genes, and regulates various signal transduction pathways [10]. It may play a pivotal role in arresting cell cycle progress, inhibit cell proliferation and tumor metastasis [11]. One study showed that PDCD4 can modulate various signal transduction pathways; specifically, the study results demonstrated that up-regulated miR-182 and down-regulated PDCD4 were observed in ovarian cancer cell lines, while blocking miR-182 in ovarian cancer cell lines resulted in opposite alteration in endogenous PDCD4 protein expression, which was beneficial for cancer treatment [10]. Nuclear factor kappa B (NF- κ B), one of the most critical signal pathways, regulates genes correlated with proliferation, apoptosis, immune response, inflammation and tumorigenesis [12]. It can react to harmful stimuli immediately modulating molecules that involved in the early immunological reaction as well as inflammation in all stages [13]. A study revealed that NF- κ B caused apoptosis resistance in cancer and played a role in carcinogenesis [14]. Inflammation is of great significance in cardiovascular disease like atherosclerosis and especially, during the early phase of atherosclerosis, inflammation occurs in vascular endothelium and the inflamed endothelial cells can release the pro-inflammatory mediators, including chemokines, pro-inflammatory cytokines, and adhesion molecules [15]. Tumor necrosis factor-alpha (TNF- α) is regarded as a pleiotropic cytokine that is involved in the pathophysiology of inflammatory diseases [16]. CAD, as a chronic inflammatory disease that results from atherosclerosis, is mediated to some extent by proinflammatory cytokines, especially TNF- α , which is often expressed in atherosclerotic plaques [17]. Because the effects of miRNAs have not been systematically explained, we intended to determine the potentially regulatory mechanism of miRNA on certain target genes. Here, we investigated the effects of miR-499 on inflammatory damage in endothelial cells during CAD via the inhibition of the PDCD4 gene through the NF- κ B/TNF- α signaling pathway.

Materials and Methods

Ethics statement

All patients were informed and provided signed informed consent. The experiment was approved by the Experiment Ethics Committee of Jiangxi Pingxiang People's Hospital.

Study subjects

A total of 216 CAD patients were recruited in the Department of Cardiovascular Diseases of Jiangxi Pingxiang People's Hospital from January to December of 2015; they formed the CAD group with 132 males and 84 females aged 40-88 years old with a mean age of 59.82 years old. The inclusion criteria were based on the CAD diagnostic criteria [18]: patients with chest discomfort and/or ischemic ST-T changes as determined by an electrocardiograph (ECG), and coronary angiography (CAG) showing the four main coronary arteries of the left main (LM), left anterior descending (LAD), left circumflex (LCX) and right coronary artery (RCA), with at least one artery containing pathological changes of stenosis $\geq 50\%$. The exclusion criteria [19] were as follows: patients with other severe systemic diseases such as cancer, lung disease, chronic kidney disease, severe infection, acute infectious disease, severe liver and kidney dysfunction, hemorrhagic disease, autoimmune disease and tuberculosis or those who recently underwent major surgeries. Ninety healthy people who underwent physical examination in the medical examination center of Jiangxi Pingxiang People's Hospital were recruited for the normal group, including 54 males and 36 females. They were 37-81 years old with a mean age of 56.20 ± 9.82 years old. No significant difference was found in age or gender between the two groups ($P > 0.05$).

Collection and preservation of plasma

In the morning, 3 mL of peripheral venous blood was extracted from the patients in the CAD and normal groups. All blood samples were collected using ethylenediamine tetraacetic acid (EDTA) anticoagulant tubes, and they were centrifuged (3,000 g) at 4°C for 10 min for plasma separation. The supernatant (plasma) was placed in RNase-free eppendorf (EP) tubes and stored at -80°C.

Primary culture of human umbilical vein endothelial cells (HUVECs)

Healthy maternal-fetal umbilical cords were extracted under sterile conditions. The surface and the stump of the umbilical cord were cleaned with biological safety cabinet with 75% ethyl alcohol. After the part with the clamp mark was cut off, the remaining residual blood was rinsed with phosphate-buffered solution (PBS), and the umbilical cord was sequentially perfused with preheated 0.25% trypsin and 0.02% EDTA digestion liquid to fill the umbilical cord. Then, the umbilical cord was put into a water bath at 37°C for digestion for 8 h. The digestive enzyme was collected, and the vein was rinsed with PBS. The two solutions were collected and centrifuged for 10 min. The supernatant was removed. Cells were resuspended with complete Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, CAS: 1000044, Gibco, New York, USA), 100 μ /mL penicillin, 100 μ /mL streptomycin and 100 mg/mL endothelial cell growth supplement (ECGS), inoculated in a flask and placed in the incubator at 37°C with 5% CO₂ for 24 h. After the cells adhered to the wall of the flask, the medium was changed, and the cells as well as red blood cells were removed. Thereafter, the medium was changed every two days. Subculture was performed when the cells grew to 80%-90% confluence. The experiments were performed on the third-generation cells.

Morphological observation and identification of HUVECs

Acquired HUVECs were inoculated to a 6-well plate containing coverslips. After the HUVECs reached 60% confluence, the coverslip was removed, rinsed three times with sterile PBS, fixed with 3% paraformaldehyde at room temperature for 15 min and washed with sterile PBS three times. After that, the coverslip was incubated in 3% H₂O₂ for 8 min, washed with PBS three times, and incubated with goat serum for 15 min. Rabbit anti-human VIII factor-related antigen (No. SP0731, Ascend Biotechnology Co., Ltd, Guangzhou, China) was incubated with the coverslips overnight at 4°C. In the NC group, the primary antibody was replaced by PBS for incubation. On the second day, the sample was washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (No. ZDR-5118, ZSGB-BIO, Beijing, China) at 37°C for 30 min. After washing with PBS, the coverslip was put under a microscope for

cell morphology observation and imaging. VIII factor-related antigen positive expression in the endothelial cells was detected by immunofluorescence.

Establishment of an inflammatory injury model using endothelial cells

The HUVEC inflammatory injury model was established through the induction of oxidized low-density lipoprotein (OX-LDL) (No. UBC_oxldl, Peking Union-Biology Co. Ltd, Beijing, China) [20]. HUVECs in the logarithmic phase were digested with 0.25% trypsin. A cell suspension was made, and the cell concentration was adjusted to 1×10^6 cells/mL; then, 2 mL of DMEM medium containing 100 μ g/mL OX-LDL was added. Cells were put in the incubator with 5% CO₂ at 37°C for 24 h. When the cells reached 80%-90% confluence, subculture was performed for subsequent experiments.

Dual-luciferase reporter assay

The miR target gene prediction tool Targetscan (<http://www.targetscan.org/>) was used to predict the binding site of miR-499 in the PDCD4 3'UTR. The PDCD4 3'UTR promoter sequence containing the miR-499 binding site was synthesized and inserted at the 5' end of BglII of the control vector to construct the PDCD4 3'UTR wild-type (WT) plasmid (named as PDCD4 3'UTR-WT). Using this plasmid, the miR-499 binding site was mutated, and the resultant sequence was used to construct the PDCD4 3'UTR mutant (MUT) plasmid (named as PDCD4 3'UTR-MUT). According to the instructions of the plasmid extraction kit (12145, QIAGEN, Valencia, CA), NC + PDCD4-WT, NC + PDCD4-MUT, miR-499 mimic + PDCD4-WT, miR-499 mimic + PDCD4-MUT, miR-499 inhibitor + PDCD4-WT and miR-499 inhibitor + PDCD4-MUT were transfected into HUVECs. The reporter gene vector used in the experiment was pcDNA3.1-luc, which encodes firefly luciferase. To measure the transfection efficiency, pRL-TK was co-transfected and used as the internal control. The pRL-TK plasmid encoded renilla luciferase. The preheated fluorescent agent was used to set the parameters. After adjusting to zero, 40 μ L of RLU and 40 μ L of STOP&Glo reagent were added to the sample tube, which was put in the fluorimeter for reading. The fluorescence value was recorded.

Cell grouping and transfection

MiR-499, PDCD4 inhibitor and the analogs were used in the cell transfection were synthesized by Shanghai GenePharma. Based on the different treatment methods, HUVECs were divided into seven groups: the normal group (normal HUVECs); the OX-LDL group (endothelial cell inflammatory injury model induced by OX-LDL without transfection); the NC group (OX-LDL group with transfection of irrelevant siRNA); the miR-499 inhibitor group (OX-LDL group with transfection of miR-499 inhibitor); the miR-499 mimic group (OX-LDL group with transfection of miR-499 mimic); the PDCD4 siRNA group (OX-LDL group with transfection of PDCD4 siRNA); and the miR-499 inhibitor + PDCD4 siRNA group (OX-LDL group with transfection of miR-499 inhibitor and PDCD4 inhibitor). Using liposomal Lipofectamine 3000 (CAS: L3000-015, Invitrogen, Carlsbad, USA), transient transfection was performed. HUVECs were subcultured one day before the transfection. The cells were inoculated into 6-well plates. After cell confluence reached 90 ~ 95%, 500 μ L of serum-free medium was used to dilute 1.0 μ g of DNA, and then, another 50 μ L of serum-free medium was used to dilute 1 μ L of Lipofectamine 3000 transfection reagent. After evenly mixing, the mixture was added with the diluted DNA and put at room temperature for 20 min. After the medium was removed, each well was supplemented with the compound, and the culture plate was shaken to mix the solution evenly. Then, the cells were cultured in the incubator with 5% CO₂ at 37°C. Four hours later, the medium was replaced. After a 48-hour transfection, the subsequent experiment was carried out.

Quantitative real-time polymerase chain reaction (qRT-PCR)

According to the instruction of the kit (CAS: 74104, QIAGEN, Valencia, CA), total RNA of the plasma and cells was extracted. The OD260/280 value of the extracted RNA was detected with an ultraviolet spectrophotometer, and the RNA concentration was calculated. Then, the samples were stored properly at -80°C. Reverse transcription of cDNA was performed following the instructions of the kit (CAS: 205311, QIAGEN, Valencia, CA). Gene sequences published by the GenBank database and the Primer5.0 primer design software were used to design specific primers. The designed primers are displayed in Table 1. The designed primers were synthesized by Shanghai Sangon Biotech (Shanghai, China). The PCR reaction system was generated in a 20- μ L volume: 10 μ L of SYBR PremixExTaq, 0.8 μ L of forward primer, 0.8 μ L of reverse primer, 0.4 μ L of ROX Reference Dye II, 2 μ L of DNA template and 6.0 μ L of dH₂O. The reaction conditions

Table 1. The primer sequences for qRT-PCR, Note: miR-499, microRNA-499; PDCD4, programmed cell death 4; ICAM-1, intercellular cell adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; VCAM-1, vascular cell adhesion molecule-1; NF- κ B, nuclear factor-kappa B; TNF- α , tumor necrosis factor-alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, F, forward; R, reverse, qRT-PCR, quantitative real-time polymerase chain reaction

Primers	Sequences
miR-499	F: CAAAGTCTTCACTTCCCTGCCA R: GATGTTAACTCCTCTCCAGGTGATC
PDCD4	F: TGGATGTCCACATTCATACTCTG R: TCTGGTTAAAGACGACCTCCATCT
ICAM-1	F: GGAACCCATTGCCCGAGC R: GGTGAGGATTGCATTAGGTC
MCP-1	F: ATCCTCCAGCATGAAGGTCT R: ACTTGCTGCTGGTGACTCTT
VCAM-1	F: CCCTTGACCGGCTCTGATT R: CTGGGGGCAACATTGACCTAAAT
NF- κ B	F: TGCTGTGCGGCTCTGCT R: AGGCTGGGCTCTGCTCTGCT
TNF- α	F: CAGAGGGAACCTGCTCCAG R: CCTTGGTCTGCTCTGCTCTGCT
U6	F: CTCGCTTCCTCAGCA R: AACGCTTCGGAATTTGCGT
GAPDH	F: GAAGGTCTGCTGCGGAGT R: CATGGTTCGCGGATTTC

were pre-denaturation at 95°C for 30 s and underwent 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. U6 was the internal control for the detection of miR-499, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was as the internal control for the detection of PDCD4, intercellular cell adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), nuclear factor-kappa B (NF- κ B) and tumor necrosis factor-alpha (TNF- α) expression. The solubility curve was used to evaluate the reliability of the results. The CT value (the inflection point of the power amplification curve) and the formula of 2^{-CT} were used to calculate the expression levels of the target genes, and each experiment was repeated three times [21].

Western blotting

Proteins were extracted from each group, and the concentration was determined in accordance with the instructions of the BCA kit (Bio-Rad, Beijing, China). In each well, 30 μ g of protein was added and separated by 10% polyacrylamide gel electrophoresis. The electrophoresis voltage was changed from 80 V to 120 V. Wet transfer was carried out, and the transmembrane voltage was 100 mv for 45-70 min. After transferring the protein to polyvinylidene fluoride (PVDF) membrane at room temperature, the samples were sealed with 5% bovine serum albumin (BSA) for 1 h. Then, primary anti-mouse monoclonal antibodies against ICAM-1 (CAS: sc-8439, SANTA CRUZ, California, USA), VCAM-1 (CAS: sc-13160, SANTA CRUZ, California, USA), NF- κ B p65 (CAS: sc-8008, SANTA CRUZ, California, USA), TNF- α (CAS: sc-133192, SANTA CRUZ, California, USA) and GAPDH (CAS: sc-47724, SANTA CRUZ, California, USA) were added. All of the antibodies were diluted to 1:1000 and incubated overnight at 4°C. The membrane was washed with Tris-buffered saline Tween (TBST) three times for 5 min each. A goat anti-mouse IgG secondary antibody (1:1000) was incubated with the membrane for 1 h at room temperature. Then, the membrane was washed three times for 5 min each. The Gel Doc EZ imager (Bio-Rad, California, USA) was used to visualize the protein bands. GAPDH was used as the internal reference. The Image J software was used to analyze the gray values of the target bands. Each experiment was repeated three times.

Methyl-thiazolyl-tetrazolium (MTT) assay

The diluted cell suspension was inoculated onto a 96-well plate with 5×10^4 cells per well. When cells adhered to the wall and the cell confluence reached 80%, the cells were treated as indicated by the experimental grouping. In each group, 6 parallel wells were used. The medium was discarded after 48 h in culture. Serum-free medium was supplemented to every well, and then, the cells were washed 3 times. Then, 20 μ L of MTT solution (CAS: M2128-1G, Sigma, CA, USA) was added, and the plates were put in the incubator at 37°C for 4 h; then, the MTT solution was discarded. Then, 150 μ L of dimethyl sulfoxide (DMSO) was added into each well. After shaking for 10 min, the optical density (OD) value of each well was recorded at the wavelength of 490 nm in the microplate reader. The experiment was carried out in triplicate, and the

average OD value was calculated. The cell survival rate = (experiment group OD value - control group OD value) / control group OD value. Each experiment was repeated three times.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (No. EK0997, Boster, Wuhan, China) were utilized to determine the levels of tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 8 (IL-8). First, the standard solutions, the biotin-labeled antibody and the avidin-biotin-peroxidase complex (ABC) were prepared. Diluted ABC and tetramethylbenzidine (TMB) developing solutions were preheated. The number of ELISA plates was determined. Cell supernatants were taken from each group, and the samples to be detected were acquired following centrifugation; then, 100 μ L of the standard samples and the diluted samples were added to the ELISA plate. After reacting with the wells of the plate, the liquid in the plate was discarded. In each well, 100 μ L of biotin antibody was added to react for 60 min. The sample was washed three times with PBS, and 100 μ L of ABC liquid was added to react for 30 min. Then, the samples were washed three times with PBS, and 90 μ L of the TMB developing liquid was added to react without light for 30 min. Finally, 90 μ L of TMB stopping buffer was added, and the OD value was measured at a wavelength of 450 nm in a microplate reader. Based on the OD value, the corresponding concentration on the standard curve was found. The final concentration of the sample was detected by multiplying the dilution factor. Each experiment was repeated three times.

Flow cytometry assay

Cell apoptosis was detected using the Annexin V-FITC (product No. 1002, Bogoo, Shanghai, China). The medium in each group was collected and placed in a centrifuge tube. The sample was washed with PBS, followed by the addition of 0.25% trypsin for cell digestion. After cell percussion, the digestion liquid was collected and added into the originally collected medium. Samples were centrifuged at 1000 g for 5 min at 4°C, and the cell sediment was collected. The samples were adjusted to achieve a 5 ~ 10 million cell suspension and were centrifuged at 1000 g for 5 min. The supernatant was removed, and 195 μ L of Annexin V-FITC binding buffer was added; then, the cells were resuspended. After an addition of 5 μ L of Annexin V-FITC, the solution was mixed evenly and incubated for 10 min at room temperature without light and centrifuged at 1000 g for 5 min. The supernatant was removed, and 190 μ L of Annexin V-FITC binding buffer was added. Then, 10 μ L of propidium iodide (PI) single staining solution was added. After evenly mixing, the sample was restored in an ice bath and prepared for flow cytometry assay (No. FACSCanto II, BD, Jersey, USA). Early apoptosis was set as the lower right quadrant, late apoptosis was set as the upper right quadrant, and the live cells were in the lower left quadrant. The apoptosis rate = the early apoptosis rate + the late apoptosis rate. Each experiment was repeated three times.

Statistical analysis

The SPSS 21.0 statistical software (SPSS, Inc, Chicago, IL, USA) was used. The measurement data are presented as the mean $\pm$ standard deviation. Comparisons of measurement data with normal distribution between two groups were made using *t* tests, while comparisons among multiple groups were made using one-way analysis of variance (ANOVA). Correlation analyses were performed using Pearson correlation coefficient. *P* < 0.05 was considered statistically significant.

Results

Relative expression of plasma miR-499 and PDCD4 in the normal and CAD groups

The qRT-PCR was used to detect the mRNA expressions of miR-499 and PDCD4 in the normal and CAD groups. The results showed that compared with the normal group, the expression of miR-499 in the CAD group was significantly increased while that of PDCD4 was significantly decreased (*P* < 0.05) (Fig. 1A). The results of the Pearson correlation analysis showed that mRNA expressions of miRNA-499 and PDCD4 were negatively correlated (*r* = -0.512, *P* < 0.05) (Fig. 1B).

Morphological observation and identification of HUVECs

The morphology of HUVECs was observed under an inverted microscope. It was noted that HUVECs were round, polygonal or spindle shaped. The cells showed monolayer growth without overlapping. A few cells extended and presented in arrangements similar to cobblestones or paving stones after confluence (Fig. 2A and B). VIII factor-related antigen was specific to endothelial cells. The rabbit anti-human VIII factor-related antigen primary antibodies were incubated with a secondary antibody. Green fluorescence was emitted in HUVECs. In the control group, the primary antibody was replaced by PBS, and thus, green fluorescence was not observed (Fig. 2C and D). The results suggested that the cells isolated from human umbilical vein were endothelial cells.

PDCD4 was verified as target gene of miR-499

The online miRNA target gene prediction tool TargetScan (http://www.targetscan.org/vert_71/) predicted the target genes of miR-499. The results showed that the 3'UTR of PDCD4 was a potential miR-499 binding site (Fig. 3A). Luciferase reporter gene activity assay results showed that in the group co-transfected with wild-type PDCD4 3'UTR-WT compared with the NC group, the relative activity of luciferase in the miR-499 mimic group

was significantly decreased while that in the miR-499 inhibitor group was significantly increased ($P < 0.05$). A change in luciferase relative activity was not observed following the co-transfection of the NC, miR-499 mimic and miR-499 inhibitor with mutant PDCD4 3'UTR-MUT ($P > 0.05$) (Fig. 3B). The results were consistent with the bioinformatics prediction and further confirmed that miR-499 could bind with the 3'-UTR of PDCD4 mRNA, suggesting the targeted inhibition of PDCD4 by miR-499.

Fig. 1. Relative mRNA expressions of plasma miR-499 and PDCD4 in the normal and CAD groups as detected by qRT-PCR. A: relative mRNA expressions of miR-499 and PDCD4 in plasma as detected by qRT-PCR; and B: correlation of the mRNA expressions of miR-499 and PDCD4 as analyzed by Pearson correlation analysis; *, $P < 0.05$ compared with the normal group. qRT-PCR, quantitative real-time polymerase chain reaction; miR-499, microRNA-499; PDCD4, programmed cell death 4; and CAD, coronary artery disease.

Fig. 2. The morphology and identification of HUVECs as observed under an inverted microscope. A, primary cultured HUVECs under an inverted microscope (100 ×); B, primary cultured HUVEC under an inverted microscope (200 ×); C, endothelial cells of the control group of the VIII factor-related antigen labeling experiment; and D, endothelial cells with positive VIII factor-related antigen expression (× 200); HUVECs, human umbilical vein endothelial cells.

Fig. 3. Analysis of the relative luciferase activity from the PDCD4 3'UTR and miR-499. A: prediction of the relationship between miR-499 and PDCD4 3'UTR; and B: luciferase activity analysis; *, $P < 0.05$, compared with the NC group; NC, negative control; miR-499, microRNA-499; and PDCD4, programmed cell death 4; Each experiment was repeated three times.

Fig. 4. Comparison of the mRNA expressions of miR-499 and PDCD4 in each group as detected by qRT-PCR. A, miR-499 mRNA expression in plasma as detected by qRT-PCR; B, PDCD4 mRNA expression in plasma as detected by qRT-PCR; $P < 0.05$, statistically significant differences ($P < 0.05$) were presented using different letters. qRT-PCR, quantitative real-time polymerase chain reaction; miR-499, microRNA-499; and PDCD4, programmed cell death 4. Each experiment was repeated three times.

Comparison of the mRNA expressions of miR-499 and PDCD4 in each group

Compared with the normal group, miR-499 expression was up-regulated in the OX-LDL, miR-499 mimic and PDCD4 siRNA groups ($P < 0.05$). MiR-499 expression was significantly down-regulated in the miR-499 inhibitor group (Fig. 4A). Compared with the normal group, PDCD4 expressions in the OX-LDL, NC, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups were significantly decreased ($P < 0.05$). There was no significant difference between the miR-499 inhibitor and normal groups ($P > 0.05$). Compared with the OX-LDL and NC groups, PDCD4 expression in the miR-499 inhibitor group was increased while those in the miR-499 mimic and PDCD4 siRNA groups were decreased ($P < 0.05$). No significant differences were found in the miR-499 inhibitor + PDCD4 siRNA group ($P > 0.05$) (Fig. 4B).

Effect of miR-499 targeting PDCD4 on cell proliferation in each group

The MTT assay was performed to measure the cell proliferation rate in each group. The results showed that compared with the normal group, the cell survival rate was significantly

Fig. 5. Effect of miR-499 targeting PDCD4 on cell viability in each group as detected by the MTT assay. miR-499, microRNA-499; PDCD4, programmed cell death 4; and MTT, methyl-thiazolyl-tetrazolium. Each experiment was repeated three times. Statistically significant differences ($P < 0.05$) were presented using different letters.

Table 2. Comparisons of expressions of inflammatory cytokines in each supernatant group (pg/mL). Note: miR-499, microRNA-499; PDCD4, programmed cell death 4; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-1, Interleukin-1; TNF- α , Tumor necrosis factor alpha; NC, negative control. Different letters showed significant difference, miR-499, microRNA-499; PDCD4, programmed cell death 4; qRT-PCR, quantitative real-time polymerase chain reaction; $P < 0.05$, each experiment was repeated for three times, NC, negative control

Groups	IL-6	IL-8	IL-1 β	TNF- α
The normal group	73.29 $\pm$ 13.53 ^a	35.08 $\pm$ 5.53 ^a	101.14 $\pm$ 16.64 ^a	253.39 $\pm$ 35.01 ^a
The OX-LDL group	327.18 $\pm$ 15.42 ^b	144.4 $\pm$ 6.87 ^b	293.45 $\pm$ 18.05 ^b	1023.90 $\pm$ 94.90 ^b
The NC group	312.31 $\pm$ 10.08 $\pm$ 7.17 ^b	301.71 $\pm$ 18.95 ^b	998.92 $\pm$ 86.48 ^b	
The miR-499 inhibitor group	95.08 $\pm$ 8.87 ^a	47.05 $\pm$ 8.44 ^a	132.33 $\pm$ 15.44 ^a	327.31 $\pm$ 42.41 ^a
The miR-499 mimic group	327.18 $\pm$ 15.42 ^b	144.4 $\pm$ 6.87 ^b	293.45 $\pm$ 18.05 ^b	1023.90 $\pm$ 94.90 ^b
The PDCD4 siRNA group	59.08 $\pm$ 3.37 ^c	144.10 $\pm$ 7.31 ^c	376.24 $\pm$ 19.82 ^c	1307.99 $\pm$ 43.52 ^c
The miR-499 inhibitor + PDCD4 siRNA	339.24 $\pm$ 17.74 ^b	101.46 $\pm$ 6.45 ^b	297.57 $\pm$ 17.37 ^b	1000.58 $\pm$ 75.19 ^b

decreased in the OX-LDL, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups ($P < 0.05$). Compared to the normal group, there was no significant difference in the miR-499 inhibitor group ($P > 0.05$). Compared with the OX-LDL and NC groups, the cell survival rate significantly increased in the miR-499 inhibitor group while those in the miR-499 mimic and PDCD4 siRNA groups were significantly decreased ($P < 0.05$). There was no significant difference in the miR-499 inhibitor + PDCD4 siRNA group ($P > 0.05$). Compared with the PDCD4 siRNA group, the cell survival rate was significantly increased in the miR-499 inhibitor + PDCD4 siRNA group ($P < 0.05$). No significant differences were observed between the miR-499 mimic and PDCD4 siRNA groups ($P > 0.05$) (Fig. 5).

Effect of miR-499 targeting PDCD4 on inflammatory cytokines in each group

ELISA was utilized to detect the levels of IL-6, IL-8, IL-1 β and TNF- α in the supernatant. The results showed that compared with the normal group, the levels of IL-6, IL-8, IL-1 β and TNF- α were significantly increased in the OX-LDL, NC, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups ($P < 0.05$). Compared with the normal group, there was no significant difference in the miR-499 inhibitor group ($P > 0.05$). Compared with the OX-LDL and NC groups, the levels of IL-6, IL-8, IL-1 β and TNF- α were significantly decreased in the miR-499 inhibitor group while those in the miR-499 mimic and PDCD4 siRNA groups were significantly increased ($P < 0.05$). There was no significant difference in the miR-499 inhibitor + PDCD4 siRNA group ($P > 0.05$). Compared with the PDCD4 siRNA group, the levels of IL-6, IL-8, IL-1 β and TNF- α were significantly decreased in the miR-499 inhibitor + PDCD4 siRNA group ($P < 0.05$). There was no significant difference between the miR-499 mimic and PDCD4 siRNA groups ($P > 0.05$) (Table 2).

Fig. 6. Comparison of cell apoptosis as detected by flow cytometry in each group. Each experiment was repeated three times. Statistically significant differences ($P < 0.05$) were presented using different letters.

Table 3. Comparisons of mRNA expressions of NF- κ B/TNF- α signaling pathway-related proteins detected by qRT-PCR in each group Note: miR-499, microRNA-499; PDCD4, programmed cell death 4; ICAM-1, intercellular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1; NF- κ B, nuclear factor-kappa B; TNF- α , tumor necrosis factor- α ; different letters showed significant difference, $P < 0.05$; qRT-PCR, quantitative real-time polymerase chain reaction; each experiment was repeated for three times, NC, negative control

Groups	NF- κ B	TNF- α	VCAM-1	ICAM-1	MCP-1
The normal group	0.67 ± 0.04^a	0.57 ± 0.03^a	0.73 ± 0.06^a	0.84 ± 0.07^a	0.44 ± 0.02
The OX-LDL group	3.08 ± 0.11^b	2.03 ± 0.08^b	2.24 ± 0.10^b	3.52 ± 0.13^b	1.74 ± 0.06
The NC group	3.13 ± 0.09^b	2.06 ± 0.06^b	2.29 ± 0.14^b	3.58 ± 0.11^b	1.80 ± 0.04
The miR-499 inhibitor group	0.67 ± 0.04^a	0.57 ± 0.03^a	0.80 ± 0.04^a	0.89 ± 0.08^a	0.48 ± 0.03
The miR-499 mimic group	6.7 ± 0.15^c	6.35 ± 0.19^c	4.34 ± 0.17^c	5.81 ± 0.18^c	3.32 ± 0.15
The PDCD4 siRNA group	6.01 ± 0.12^c	6.51 ± 0.13^c	4.37 ± 0.19^c	5.93 ± 0.22^c	3.36 ± 0.03
The miR-499 inhibitor + PDCD4 siRNA group	3.08 ± 0.11^b	4.06 ± 0.10^b	2.23 ± 0.12^b	3.61 ± 0.15^b	1.73 ± 0.04

Effect of miR-499 targeting PDCD4 on cell apoptosis in each group

Flow cytometry was used to detect cell apoptosis. The results showed that compared with the normal group, the cell apoptosis rate was significantly increased in the OX-LDL, NC, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups ($P < 0.05$). Compared with the normal group, there was no significant difference in the miR-499 inhibitor group ($P > 0.05$). Compared with the OX-LDL and NC groups, the cell apoptosis rate was significantly decreased in the miR-499 inhibitor group while those in the miR-499 mimic and PDCD4 siRNA groups were significantly increased (both $P < 0.05$). There was no significant difference in the miR-499 inhibitor + PDCD4 siRNA group ($P > 0.05$). Compared with the PDCD4 siRNA group, the cell apoptosis rate was significantly decreased in the miR-499 inhibitor + PDCD4 siRNA group ($P < 0.05$). There was no significant difference between the miR-499 mimic and PDCD4 siRNA groups ($P > 0.05$) (Fig. 6).

Effect of miR-499 targeting PDCD4 on the NF- κ B/TNF- α signaling pathway-related proteins in each group

The qRT-PCR and western blotting were used to determine the mRNA and protein expressions of NF- κ B/TNF- α signaling pathway-related proteins (NF- κ B, TNF- α , VCAM-1, ICAM-1 and MCP-1) in each group. The results showed that compared with the normal group, the mRNA and protein expressions of NF- κ B, TNF- α , VCAM-1, ICAM-1 and MCP-1 were significantly increased in the OX-LDL, NC, miR-499 mimic, PDCD4 siRNA and miR-499 inhibitor + PDCD4 siRNA groups ($P < 0.05$). Compared with the normal group, there was no significant difference in the miR-499 inhibitor group ($P > 0.05$). Compared with the OX-LDL

Fig. 7. Comparison of the expressions of NF- κ B/TNF- α signaling pathway-related proteins as detected by Western blotting in each group. A: Western blotting bands of NF- κ B, TNF- α , VCAM-1 and ICAM-1 proteins; and B, C, D, E and F: histograms for the NF- κ B, TNF- α , VCAM-1, ICAM-1 and MCP-1 proteins; NF- κ B, nuclear factor-kappa B; TNF- α , tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; and MCP-1, monocyte chemoattractant protein-1. Each experiment was repeated three times. Statistically significant differences ($P < 0.05$) were presented using different letters.

and NC groups, the mRNA and protein expression of NF- κ B, TNF- α , VCAM-1, ICAM-1 and MCP-1 was significantly increased in the miR-499 inhibitor group while those in the miR-499 mimic and PDCD4 siRNA groups were significantly increased ($P < 0.05$). There was no significant difference in the miR-499 inhibitor + PDCD4 siRNA group ($P > 0.05$). Compared with the PDCD4 siRNA group, the mRNA and protein expressions of NF- κ B, TNF- α , VCAM-1, ICAM-1 and MCP-1 were significantly decreased in the miR-499 inhibitor + PDCD4 siRNA group ($P < 0.05$). There was no significant difference between the miR-499 mimic and PDCD4 siRNA groups ($P > 0.05$) (Table 3, Fig. 7).

Discussion

Currently, CAD incidence is still a main cause of mortality in the world [22]. Endothelial cells are the essential components of various inflammatory diseases, such as psoriasis, diabetes, cancer and rheumatoid arthritis, and chronic inflammation is related to increased atherosclerosis and accelerated cardiovascular mortality and morbidity [23]. Endothelial dysfunction is regarded as one of the significant reason for cardiovascular disease [24]. Increasing evidence has elucidated that mRNAs play a key role in regulating myocardial apoptosis [25, 26]. In our study, we verified the roles of miR-499 and PDCD4 in endothelial cells during CAD.

Our results provided evidence that miR-499 expression was elevated and PDCD4 was down-regulated in the plasma of CAD patients compared to that in the normal group, indicating that miR-499 was negatively correlated with PDCD4. A change in miR-499 expression might be involved in the modulation of the expression of multiple genes and signaling transduction

pathways. Recent studies have identified several target genes of miR-499, such as Drp1 and Ets1 [27, 28]. The tumor suppressor PDCD4 is often down-regulated in several malignancies, and its protein expression could be modulated by miR-21, indicating that PDCD4 down-regulation is a reliable biomarker for early-stage squamous cell esophageal neoplasia [29]. In addition, compared to the normal group, NF- κ B and TNF- α expression and the apoptosis rates were all increased. The NF- κ B/TNF- α signaling pathway has been reported to promote cancer cell growth, angiogenesis, invasion, and eventually metastasis in many human tumors [30, 31]. As an important transcription factor, NF- κ B is responsible for a variety of biological processes that participate in the transcription of proinflammatory genes, thus mediating cellular inflammatory responses, including the elevated expression of chemokines and cytokines [32]. Activation of constitutive NF- κ B has been reported to modulate different biological functions, such as cell proliferation, angiogenesis, and inhibition of NF- κ B which protect against atherosclerosis [33]. Silencing of miR-499 could lead to reduced migration and invasion of colorectal cancer (CRC) cells in vitro by modulating PDCD4 [34]. Thus, we concluded that down-regulated miR-499 expression inhibited apoptosis and the activation of inflammatory factors as well as improved CAD by modulating its functional target, PDCD4, by inhibiting the NF- κ B/TNF- α signaling pathways.

In addition, we also found that the levels of IL-6, IL-8, and MCP-1 were decreased in the miR-499 inhibitor and miR-499 inhibitor + PDCD4 siRNA groups while increased in the PDCD4 siRNA group. The endothelial cells can express 59 inflammatory factors, including chemokines, cytokines, chemokine and cytokine receptors, and inflammatory transcription factors, and thus, inflammatory cytokines will be consequently secreted by the inflammatory activation of endothelial cells [34]. As small and active proteins, cytokines regulate cellular growth, function, and differentiation as well as control the immune response and inflammation, including some proinflammatory cytokines such as IL-6 and IL-8 [35].

Additionally, the results showed that the levels of TNF- α , NF- κ B, VCAM-1, ICAM-1 and MCP-1 were reduced in the miR-499 inhibitor and miR-499 inhibitor + PDCD4 siRNA groups while enhanced in the PDCD4 siRNA group. NF- κ B is a host of proinflammatory master switch and also induces transcription factors which are capable of controlling the production of some inflammatory factors [36]. In the vascular endothelium, NF- κ B activation induces the expression of proinflammatory genes, including those encoding adhesion molecules, cytokines, and chemoattractant proteins, which collectively contribute to the initiation and development of atherosclerosis [37]. As a proinflammatory, pleiotropic, and homotrimeric soluble molecule, TNF- α is involved in various metabolic disorders, such as diabetes mellitus and rheumatoid arthritis [39], and disorders with an inflammatory background, including but not limited to, atherosclerosis [40]. TNF- α results in the initiation of transcriptional responses via the rapid activation of NF- κ B [41]. Chronic inflammation is also a cardinal feature of atherosclerotic vascular disease, in which TNF- α causes endothelial dysfunction that leads to reduced vasodilatory responses [42]. It has been shown that inhibition of inflammatory pathways may be beneficial for the prevention of vascular disease. TNF- α has also been shown to stimulate endothelial microparticles (EMPs) that are released from endothelial cells and participate in the apoptosis of endothelial cells. Therefore, TNF- α inhibitor have an emerging role in the prevention of vascular dysfunction [43]. A study demonstrated that elevated expression of adhesion cytokines, including VCAM-1 and ICAM-1 in arterial endothelium, might enhance recruitment and adhesion of inflammatory cells, contributing to the progression of plaque instability and atherosclerosis [44]. Elevated levels of ICAM-1 have been found in patients with coronary heart disease, and circulating VCAM-1 levels could be employed to determine the stage of atherosclerosis; additionally, a role for MCP-1 in atherogenesis has also been recently proven [45]. The expressions of VCAM-1, ICAM-1 as well as MCP-1 can be mediated by NF- κ B [46]. Thus we can reach a conclusion that down-regulated miR-499 expression protected endothelial cells from inflammatory damage during CAD through the NF- κ B/TNF- α signaling pathway.

Conclusion

Our findings have illustrated that low miR-499 expression contributes to high PDCD4 expression and protects the endothelial cells against inflammatory damage through the NF- κ B/TNF- α signaling pathway during CAD. Therefore, miR-499 could be a potential target for the treatment of CAD.

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Disclosure Statement

The authors declare that they have no competing interests.

References

- Jung JH, Song GG, Kim JH, Seo YH, Choi SJ: Association between Endothelin Receptor XIII Val363Leu polymorphism and coronary artery disease: A meta-analysis. *Cardiol J* 2016;10.5603/CJ.16.0070.
- Mahmoodi K, Nasehi L, Karami E, Soltanpour MS: Association between Nitric Oxide Levels and Endothelial Nitric Oxide Synthase G894T Polymorphism with Coronary Artery Disease in the Iranian Population. *Vasc Specialist Int* 2016;32:105-112.
- Yayla C, Okyay K, Yilmaz A, Sahinarslan A, Sahin AS, Eyiyl A, Bolayir HA, Sezenoz B, Menevse S, Cengel A: Association of rs10757274 and rs2381111 polymorphisms on 9p21 locus with Coronary Artery Disease in Turkish Population. *Korean J Prev Med* 2016;20:1-11.
- Ning FL, Wang F, Li ML, Yu ZS, Chen SS: MicroRNA-182 modulates chemosensitivity of human non-small cell lung cancer to cisplatin by targeting PDCD4. *Diagn Pathol* 2014;9:143.
- Xin Y, Yang C, Han Z: Circulating miR-499 is a potential biomarker for acute myocardial infarction. *Ann Transl Med* 2016;4:135.
- Zhao CH, Cheng GC, He R, Wang QL, Wang ZZ, Pan ZY: Analysis and clinical significance of microRNA-499 expression levels in serum of patients with acute myocardial infarction. *Genet Mol Res* 2015;14:401-406.
- Chua SH, Wang J, Li J, Lo HM, Chiu CZ, Shyu KG: Mechanical Stretch Inhibits MicroRNA499 via p53 to Regulate Calcineurin Expression in Rat Cardiomyocytes. *PLoS One* 2016;11:e0148683.
- Liu Z, Wang Z, Sun L, Chai N, Tang S, Jin J, Hu H, Nie Y, Wang X, Wu K, Jin H, Fan D: MicroRNA-499-5p promotes cellular invasion and tumor metastasis in colorectal cancer by targeting FOXO4 and PDCD4. *Oncogenesis* 2011;32:1798-1805.
- Wang J, Ferlemann E, Haas A, Muller JP, Werwein E, Fehler O, Biyanee A, Klempnauer KH: Programmed cell death 4 protein (Pdcd4) and homeodomain-interacting protein kinase 2 (Hipk2) antagonistically control translation of Hipk2 mRNA. *Biochim Biophys Acta* 2015;1853:1564-1573.
- Wang YQ, Guo RD, Guo RM, Sheng W, Yin LR: MicroRNA-182 promotes cell growth, invasion, and chemoresistance by targeting programmed cell death 4 (PDCD4) in human ovarian carcinomas. *J Cell Biochem* 2013;114:1464-1473.
- Wei N, Liu SS, Chan KK, Ngan HY: Tumour suppressive function and modulation of programmed cell death 4 (PDCD4) in ovarian cancer. *PLoS One* 2012;7:e30311.
- Cao W, Hu C, Wu L, Xu L, Jiang W: Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-kappaB signaling in H22 tumor-bearing mice. *J Pharmacol Sci* 2016;132:131-137.
- Li L, Hu M, Zheng L, Zhang C, Li J, Rong R, Zhu T, Jia Y: Endothelin Receptor Down-Regulation Mediated Ligand Regulation Mechanisms Protect Against Cellular Hypoxia Injury in Rat Vascular Endothelial Cells.

- Cell Physiol Biochem 2016;40:1443-1454.
- 14 Xiang N, He M, Ishaq M, Gao Y, Song F, Guo L, Ma L, Sun G, Liu D, Guo D, Chen Y: The DEAD-Box RNA Helicase DDX3 Interacts with NF-kappaB Subunit p65 and Suppresses p65-Mediated Transcription. PLoS One 2016;11:e0164471.
- 15 Song L, Kang C, Sun Y, Huang W, Liu W, Qian Z: Crocetin Inhibits Lipopolysaccharide-Induced Inflammatory Response in Human Umbilical Vein Endothelial Cells. Cell Physiol Biochem 2016;40:443-452.
- 16 Gane JM, Stockley RA, Sapey E: TNF-alpha Autocrine Feedback Loops in Human Monocytes: The Pro- and Anti-Inflammatory Roles of the TNF-alpha Receptors Support the Concept of Selective TNFR1 Blockade *Vivo*. J Immunol Res 2016;2016:1079851.
- 17 Cho HC, Yu G, Lee MY, Kim HS, Shin DH, Kim YN: TNF-alpha polymorphisms and coronary artery disease: a population study in the Korean population. Cytokine 2013;62:104-109.
- 18 O'Rourke RA, Brundage BH, Froelicher VF, Greenland P, Grundy SM, Hachamovitch R, Heuser GM, Lerman JB, Lij, Weintraub WS, Winters WL, Jr.: American College of Cardiology/American Heart Association Expert Consensus Document on electron-beam computed tomography for the diagnosis and prognosis of coronary artery disease. J Am Coll Cardiol 2000;36:326-340.
- 19 Kadi H, Ceyhan K, Sogut E, Koc F, Celik A, Onalan O, Sahin S: Mildly decreased glomerular filtration rate is associated with poor coronary collateral circulation in patients with coronary artery disease. Clin Cardiol 2011;34:617-621.
- 20 Jiang YR, Miao Y, Yang L, Xue M, Guo CY, Ma XJ, Yin HJ, Shi DZ, Chen Y: Effect of Chinese herbal drug-containing serum for activating-blood and dispelling-toxin on ox-LDL-induced inflammatory factors' expression in endothelial cells. Chin J Integr Med 2014;18:30-33.
- 21 Chen X, Zhang L, Su T, Li H, Huang Q, Wu D, Yang C, Han Y: Kinetics of plasma microRNA-499 expression in acute myocardial infarction. J Thorac Dis 2015;7:890-895.
- 22 Hanifehpour R, Motevalli M, Ghanaati H, Shahriari M, Aghajani M, Ghaheri M: Diagnostic Accuracy of Coronary Calcium Score Less than 100 in Excluding Coronary Artery Disease. Iran J Radiol 2016;13:e16705.
- 23 Gerhardt S, Konig V, Doll M, Hailemariam-Jahromi H, Hrgovcic M, Kasper N, Kaufmann R, Kippenberger S, Meissner M: Dimethylfumarate protects against TNF-alpha-induced secretion of inflammatory cytokines in human endothelial cells. J Inflamm (Lond) 2015;2015:147.
- 24 Han F, Hui Z, Zhang S, Hou N, Yang Y, Sun Y: Overexpression of haemeoxygenase-1 improves FFA-induced endothelial dysfunction in rat aorta. Cell Physiol Biochem 2015;35:1230-1240.
- 25 Skommer J, Rana I, Marques FZ, Zhang Z, Charchar FJ: Small molecules, big effects: the role of microRNAs in regulation of cardiomyocyte death. Cell Death Dis 2014;5:e1325.
- 26 Lai KB, Sanderson JE, Izumiya MB, Yu SM: Micro-RNA and mRNA myocardial tissue expression in biopsy specimen from patients with heart failure. Int J Cardiol 2015;199:79-83.
- 27 Wang JX, Jiao M, Wang B, Wang K, Liu JP, Li YR, Li PF: miR-499 regulates mitochondrial dynamics by targeting cardiolipin and dynamin-related protein-1. Nat Med 2011;17:71-78.
- 28 Wei W, Li Z, Fu Y, Chen H, Wu Y, Zheng X: MicroRNA-1 and microRNA-499 downregulate the expression of the proto-oncogene in HepG2 cells. Oncol Rep 2012;28:701-706.
- 29 Fassanelli A, Realdon S, Pizzi M, Balistreri M, Battaglia G, Zaninotto G, Ancona E, Rugge M: Programmed cell death, nuclear loss and miR-21 or activated Akt overexpression in esophageal squamous cell carcinogenesis. Dis Esophagus 2012;25:263-268.
- 30 Li SY, Wu B: TRIM29 Overexpression Promotes Proliferation and Survival of Bladder Cancer Cells through NF-kappaB Signaling. Cancer Res Treat 2016;48:1302-1312.
- 31 Mulye RA, Bian Y, Wang L, Alemu PN, Cui H, Peng X, Li S: Placenta Peptide Can Protect Mitochondrial Dysfunction through Inhibiting ROS and TNF-alpha Generation, by Maintaining Mitochondrial Dynamic Network and by Increasing IL-6 Level during Chronic Fatigue. Front Pharmacol 2016;7:328.
- 32 Xie Y, Li Y, Cai X, Wang X, Li J: Interleukin-37 suppresses ICAM-1 expression in parallel with NF-kappaB down-regulation following TLR2 activation of human coronary artery endothelial cells. Int Immunopharmacol 2016;38:26-30.
- 33 Qin W, Xie W, Xia N, He Q, Sun T: Silencing of Transient Receptor Potential Channel 4 Alleviates oxLDL-induced Angiogenesis in Human Coronary Artery Endothelial Cells by Inhibition of VEGF and NF-kappaB. Med Sci Monit 2016;22:930-936.
- 34 Krogmann A, Staiger K, Haas C, Gommer N, Peter A, Heni M, Machicao F, Haring HU, Staiger H: Inflammatory response of human coronary artery endothelial cells to saturated long-chain fatty acids. Microvasc Res

2011;81:52-59.

- 35 Arican O, Aral M, Sasmaz S, Ciragil P: Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm* 2005;2005:273-279.
- 36 Han F, Guo Y, Xu L, Hou N, Han F, Sun X: Induction of Haemeoxygenase-1 Directly Improves Endothelial Function in Isolated Aortas from Obese Rats through the Ampk-Pi3k/Akt-Enos Pathway. *Cell Physiol Biochem* 2015;36:1480-1490.
- 37 Zhong Y, Cheng CF, Luo YZ, Tian CW, Yang H, Liu BR, Chen MS, Chen YF, Liu SM: C-reactive protein stimulates RAGE expression in human coronary artery endothelial cells in vitro via ROS generation and ERK/NF-kappaB activation. *Acta Pharmacol Sin* 2015;36:440-447.
- 38 Nystrom T, Nygren A, Sjöholm A: Increased levels of tumour necrosis factor-alpha (TNF-alpha) in patients with Type II diabetes mellitus after myocardial infarction are related to endothelial dysfunction. *Thromb Haemostas* (Lond) 2006;110:673-681.
- 39 Ohta S, Harigai M, Tanaka M, Kawaguchi Y, Sugiura T, Takagi K, Fukasawa C, Hara M, Kawanishi M: Tumour necrosis factor-alpha (TNF-alpha) converting enzyme contributes to production of TNF-alpha in synovial tissues from patients with rheumatoid arthritis. *J Rheumatol* 2001;28:1756-1761.
- 40 Bruunsgaard H, Skinhoj P, Pedersen AN, Schroll M, Pedersen BK: Age-related tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. *Clin Exp Immunol* 2000;121:23-29.
- 41 Szukiewicz D, Wojciechowska M, Bilska A, Stangret A, Szewczyk G, Goral T, Kozłowska M, Kochanowski J: Aspirin Action in Endothelial Cells: Different Patterns of Response Between Chemokine CX3CL1/CX3CR1 and TNF-alpha/TNFR1 Signaling Pathways. *Cardiovasc Drugs Ther* 2010;24:219-229.
- 42 Yang WS, Moon SY, Lee MJ, Park SK: Epigallocatechin gallate Attenuates the Effects of TNF-alpha in Vascular Endothelial Cells by Causing Ectodomain Shedding of TNF Receptor 1. *Cell Physiol Biochem* 2016;38:1963-1974.
- 43 Barbaro NR, de Araujo TM, Tanus-Santos JE, Anhe C, Fontana J, Moreno H: Vascular Damage in Resistant Hypertension: TNF-Alpha Inhibition Effects on Endothelial Cells. *Biomed Res Int* 2015;2015:631594.
- 44 Yang JX, Pan YY, Ge JH, Chen B, Mao W, Qiu J, Wang XX: Farnesyltransferase Inhibitor Farnesyltransferase Inhibitor II A Attenuates TNF-alpha-Induced Expression of VCAM-1 and ICAM-1 in Endothelial Progenitor Cells by Blocking Activation of NF-kappaB. *Cell Physiol Biochem* 2016;40:155-166.
- 45 Papayianni A, Alexopoulos E, Giamalis P, Giannakis L, Belechri AM, Koukoudis P, Memmos D: Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events. *Nephrol Dial Transplant* 2002;17:435-441.
- 46 Liang B, Wang X, Zhang H, Wang H, Bai R, Liu M, Bian Y, Xiao C, Yang Z: Angiotensin-(1-7) Attenuates Angiotensin II-Induced ICAM-1, and MCP-1 Expression via the MAS Receptor Through Suppression of NF-kappaB Pathways in HUVECs. *Cell Physiol Biochem* 2015;35:2472-2482.