Original Article

Significant role and mechanism of microRNA-143-3p/KLLN axis in the development of coronary heart disease

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Abstract: Cardiovascular disease predominantly includes coronary heart disease (CHD) and stroke, results in high morbidity and mortality. MicroRNA-143-3p (miR-143-3p) is a tumor suppressor and is involved in many cancers. However, the role and mechanism of miR-143-3p in coronary heart disease is still unclear. In this study, we identified that miR-143-3p was up-regulated in rabbit CHD model. The results of TargetScan and the dual luciferase reporter assay indicated that KLLN (killin, p53 regulated DNA replication inhibitor) was a direct target of miR-143-3p. Besides, we revealed that KLLN was down-regulated in rabbit coronary heart disease model. In addition, we found that the related-markers of CHD such as TC (total cholesterol), TG (triglyceride), and LDLC (low-density lipoprotein cholesterol) in the model group were significantly increased than that in the control group. And compared with the model group, miR-143-3p inhibitor significantly reduced TC, TG, LDLC expression, while miR-143-3p mimic further increased the expression of TC, TG, and LDLC. We next found that miR-143-3p mimic promoted cell viability and migration of vascular smooth muscle cells, inhibited apoptosis; and these changes were reversed by KLLN-plasmid. And miR-143-3p inhibitor had the counter effects. Our study provided a new target for the treatment of CHD and deserves further study.

Keywords: Coronary heart disease, vascular smooth muscle cells, miR-143-3p-3p, KLLN

Introduction

Coronary heart disease (CHD) is one of the most common types of heart disease and remains the primary cause of death in adults worldwide. In addition, there are more than 350,000 deaths of CHD patients each year. And CHD is a complex and multifaceted condition. The causes of CHD include myocardial ischemia, hypoxia, necrosis and coronary atherosclerosis [1, 2]. Atherosclerosis is the main cause of CHD and myocardial ischemia is the characteristic physiological and pathological change. At present, the treatment of CHD has caused people’s widespread concern, therefore it is very important to find new targets for the treatment of CHD.

MicroRNA (miRNA) is a highly conserved, single-stranded, non-coding and small RNA, which contains 22-24 nucleotides and exists on non-coding regions [3, 4]. MiRNA negatively regulates gene expression in the post-transcriptional level by specific binding to the 3’ untranslated region (3’UTR) of target genes [5]. Besides, miRNA plays a crucial role in physiological and pathological processes, including cell differentiation, organ development, gene expression, cell proliferation, apoptosis and tumorigenesis [6, 7]. Previous researches reported that miRNA is abnormal expression in many tumors, such as breast cancer [8], non-small cell lung cancer [9], gastric cancer [10], osteosarcoma [11], ovarian cancer [12]. MiR-143-3p is a special miRNA associated with a variety of diseases, including age-related defective muscle regeneration, gastric cancer and esophageal squamous cell carcinoma [13]. Furthermore, some studies have indicated that miR-143-3p is highly expressed in adipose tissue of obese people, and its expression is related to glucose metabolism, colorectal tumorigenesis, cancer glycolysis and damage to myogenogenesis [14-17].

Moreover, miR-143 activation regulates smooth muscle and endothelial cell crosstalk in pulmo-
Coronary arterial hypertension [18]. MiR-143-3p controls TGF-β1-induced cell proliferation and extracellular matrix production in airway smooth muscle via negative regulation of the nuclear factor of activated T cells 1 [19]. However, till now, the specific molecular mechanism through which miR-143-3p affects the progression of CHD remains unclear. Therefore, the main purpose of the present study was to reveal the role of miR143-3p in CHD and explore its action mechanism.

Materials and methods

CHD rabbit model establishment and treatment

Rabbits are another animal model widely used in cardiovascular diseases [20-24]. Rabbits are a better choice of human lipoproteins than rodents. In the present study, all rabbits were randomly divided into 2 groups (n=5): control group and CHD model group. The CHD rabbit model was established based on the Ishikawa method [25]. All rabbits were housed separately in cages and given a fat diet formulation (80.5% basal diet + 5% egg yolk powder + 0.5% cholesterol + 4% lard, fed for 4 weeks). We injected the rabbits with 2.5 nmol/kg of miRNA-143-3p mimic, miR-143-3p-3p inhibitor, or the negatively control (NC) intravenously for 48 h. Then these rabbits were divided into 5 groups: control group, model group, model + NC, model + miR-143-3p mimic, model + miR-143-3p inhibitor. Then, we collected the blood of rabbits from each group to detect the expression of biochemical indicators.

The in vivo animal experiments were carried out according to the Recommended Guideline for the Care and Use of Laboratory Animals issued by Chinese Council on Animal Research. This study was approved by the Animal Ethics Committee of The Second Clinical Medical College (Shenzhen People’s Hospital).

Peripheral blood mononuclear cells (PBMCs) separation

Blood was collected from the ear vein of the rabbits and injected into the anticoagulant tube of heparin. Equal volume of phosphate buffer saline (PBS) was added to the blood, and the mixture was gently blowed. Ficoll was added into a clean centrifuge tube and tilt the tube, and diluted blood was slowly added to Ficoll. After centrifugation at 2000 rpm for 20 min, we inserted the pipette into the cloud layer, gently aspirated PBMC and placed it into a new centrifuge tube. Then, PBMC was centrifuged with PBS at 2000 rpm for 10 min for two times. Added fresh medium to PBMC, and the mixture was mixed by pipetting to prepare a PBMC cell suspension.

Cell culture and transfection

Rabbit vascular smooth muscle cells (rVSMCs) were isolated from rabbit tissues as previously described. rVSMCs were cultured in dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics and incubated in a 5% CO2 humidified atmosphere at 37°C. rVSMCs were transfected with miR-143-3p mimic, miR-143-3p inhibitor, KLLN-plasmid, control-plasmid, miR-143-3p mimic + KLLN-plasmid, KLLN-shRNA, control-shRNA or miR-143-3p inhibitor + KLLN-shRNA for 48 h using lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Quantitative real-time PCR (QRT-PCR) analysis

Total RNA was acquired from cells with Trizol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized by using cDNA synthesis kit (Takara). qRT-PCR was performed by using SYBR® Green (TaKaRa). GAPDH was used as an internal control for mRNA, and U6 was used for miRNA. Relative expression of genes was calculated using the 2(-∆∆Ct) method. All experiments were repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was performed to examine the expression of TC, TG, and LDLc in the blood of rabbits from each group. Specific ELISA kits were used to detect related markers expression according to the manufacturer’s instructions.

Western blot analysis

Total protein was extracted by using RIPA Lysis (RIPA) buffer with phenylmethanesulfonyl fluoride (PMSF). And the protein samples were boiled, centrifuged and then separated by 12%
sodium dodecyl sulfate (SDS)-Polyacrylamide-Gel Electrophoresis (PAGE) for 40 min. Then proteins were then transferred to Polyvinylidene difluoride (PVDF) membranes (Millipore), followed by blocked with 5% nonfat milk for 1.5 h at room temperature. The membranes were then incubated with primary antibodies at 4°C overnight. The next day, the membranes were incubated with a secondary antibody at room temperature for 2 h. Protein blots were visualized and analyzed using a chemiluminescence system. β-actin was used as an internal control.

Dual-luciferase reporter assay

TargetScan was used to predict the potential targets of miR-143-3p, and the binding sites between miR-143-3p and KLLN were observed. To confirm the relationship between miR-143-3p and KLLN, dual-luciferase reporter assay was conducted in this study. pmiR-RB-Reporter dual luciferase reporter gene plasmid vectors (Guangzhou RiboBio Co., Ltd., Guangzhou, China) which contain the wild-type 3'Untranslated Regions (UTRs) of KLLN (WT-KLLN), or mutant 3'UTRs of KLLN (MUT-KLLN) were conducted. rVSMCs were seeded into 24-well plates and then co-transfected with WT-KLLN or MUT-KLLN and miR-143-3p mimic or mimic control using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. After the cells were transfected for 48 h, the luciferase activity was detected by using dual luciferase reporter assay system (Promega, USA). Relative luciferase activity was calculated as the ratio of the raw firefly luciferase activity and Renilla luciferase activity.

Cell viability assay

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromid (MTT) assay was performed to examine the cell viability of rVSMCs. Cells were plated into 96-well plates at 5,000 cells per well, then the cells were transfected with NC, miR-143-3p mimic, miR-143-3p inhibitor, miR-143-3p mimic + KLLN-plasmid, or miR-143-3p inhibitor + KLLN-shRNA for 48 h. Subsequently, each well was added with 20 μl MTT reagent. After incubation for 4 h at 37°C, dimethyl sulfoxide (DMSO) was added to each well and the plate was shaken for 15 min to dissolve formazan. The absorbance of each well was measured at 490 nm using a micro-plate reader.

Flow cytometric analysis of apoptosis

Annexin V-FITC/PI kit was performed to detect cell apoptosis. After cells were transfected with NC, miR-143-3p mimic, miR-143-3p inhibitor, miR-143-3p mimic + KLLN-plasmid, or miR-143-3p inhibitor + KLLN-shRNA for 48 h, the cells were harvested and washed with PBS for three times. Then, the cells were centrifuged, and re-suspended in 100 μl of FITC-binding buffer. Approximately 5 μl of ready-to-use Annexin V-FITC (BD Bioscience) and 5 μl of PI were added to the mixture. Cells were incubated for 30 min in the dark at room temperature. Annexin V-FITC and PI fluorescence were assessed by BD FACSCalibur flow cytometer (BD Technologies).

Transwell assay

Cell migration assay was performed by using the Transwell inserts (Corning Incorporated, Corning, NY, USA). The experiment was performed according to the manufacturer’s protocol. The cells were re-suspended in serum-free medium and added to upper chamber at 30000 cells per well, and the lower chamber was added with 600 μl cell culture medium with 10% FBS. Then, after incubation for 48 h, the cells that migrated into the lower chamber were stained and quantified by counting under microscopy. The cell number was calculated by Image J software.

Statistical analysis

All data were presented as the mean ± standard deviation (SD) from at least three independent experiments. SPSS software version 17.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and Student’s t-test or one-way analysis of variance with Tukey’s post hoc test was performed to examine whether differences between groups were significant. P<0.05 was known as statistically significant difference.

Results

miR-143-3p was significantly up-regulated in rabbit coronary heart disease model

To explore the role of miR-143-3p in CHD, we established rabbit CHD model and then collected the rabbit PBMC cells. qRT-PCR assay showed that compared with the control group,
miR-143-3p was significantly up-regulated in the PBMC cells of rabbits in the model group (Figure 1).

**KLLN was a direct target of miR-143-3p**

We performed TargetScan to analyze the potential targets of miR-143-3p, and the predicted sequence analysis data showed the binding sites between KLLN and miR-143-3p (Figure 2A). To further verify the result, dual-luciferase reporter assay was performed, and the results indicated that following co-transfection with miR-143-3p mimic, the luciferase reporter activity of WT-KLLN was significantly decreased, but not the luciferase reporter activity of MUT-KLLN (Figure 2B). The results suggested that KLLN was a direct target of miR-143-3p. Besides, we found that KLLN was significantly down-regulated in the PBMC cells of rabbits in the model group (Figure 2C and 2D).

**Effect of miR-143-3p on the rabbit model of coronary heart disease**

In order to examine the function role of miR-143-3p in CHD, we detected related indicators expression in the occurrence of CHD after rabbit CHD model were intraperitoneally injected with miR-143-3p mimic or miR-143-3p inhibitor for 48 h. ELISA assay results demonstrated that compared with the control group, total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDLC) were significantly increased in the blood of rabbits from the model group (Figure 3A-C); compared to the model group, miR-143-3p inhibitor obviously reduced TC, TG and LDLC expression in the blood of CHD rabbits (Figure 3A-C), while miR-143-3p mimic further improved TC, TG and LDLC expression in the blood of CHD rabbits (Figure 3A-C).

**Effect of miR-143-3p mimic on rabbit vascular smooth muscle cells**

To explore the effects of miR-143-3p on vascular smooth muscle cells, rVSMCs were transfected with NC, miR-143-3p mimic, KLLN-plasmid, control-plasmid or miR-143-3p mimic + KLLN-plasmid for 48 hours, qRT-PCR was used to detect transfection efficiency. Compared with the control group, miR-143-3p mimic significantly increased the expression of miR-143-3p in rVSMCs (Figure 4A). KLLN-plasmid could obviously improve the expression of KLLN in rVSMCs (Figure 4B and 4C). The results showed that compared with the control group, miR-143-3p mimic markedly decreased KLLN expression in rVSMCs, which was reversed by KLLN-plasmid (Figure 4D and 4E). To investigate the biological functions of miR-143-3p mimic in rVSMCs, MTT assay, transwell assay and flow cytometry assay were performed to detect cell proliferation (Figure 4F), migration (Figure 4G), and apoptosis (Figure 4H). The results indicated that miR-143-3p mimic promoted cell viability and cell migration, and inhibited apoptosis of rVSMCs compared with the control group. However, these changes were reversed by KLLN-plasmid.

**Effect of miR-143-3p inhibitor on rabbit vascular smooth muscle cells**

Next, we transfected rVSMCs with NC, miR-143-3p inhibitor, KLLN-siRNA, control-siRNA or miR-143-3p inhibitor + KLLN-siRNA for 48 h. qRT-PCR assay results showed that miR-143-3p inhibitor significantly reduced the expression of miR-143-3p in rVSMCs (Figure 5A). KLLN-siRNA significantly decreased KLLN expression in rVSMCs (Figure 5B and 5C). Besides, miR-143-3p inhibitor markedly improved the expression of KLLN, which was reversed by KLLN-siRNA (Figure 5D and 5E). MTT assay, transwell assay and flow cytometry assay results showed that miR-143-3p inhibitor inhibited cell viability (Figure 5F), cell migration (Figure 5G), and induced cell apoptosis (Figure 5H). All these effects of miR-143-3p inhibitor
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Figure 2. KLLN was the target gene of miR-143-3p. A. miR-143-3p potential targets were predicted by the miRNA target-prediction programme TargetScan. B. The relative luciferase activity of luciferase reports with wild type or mutant KLLN-3’UTR were determined in rabbit PBMC cells, which were co-transfected with the miR-143-3p mimic or mimic control for 48 h. C and D. The mRNA and protein level of KLLN in the PBMC cells of rabbit coronary heart disease model using qRT-PCR and western blotting respectively. Data were displayed as mean ± SD. **P<0.01 vs. mimic control; ##P<0.01 vs. Control.

Figure 3. The expression level of related indicators in coronary heart disease. ELISA assay was used to measure the level of (A) total cholesterol (TC), (B) triglyceride (TG), and (C) low-density lipoprotein cholesterol (LDLC) in the serum of the rabbit coronary heart disease model. Data were displayed as mean ± SD. **P<0.01 vs. Control; #P<0.05 vs. Model.
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A

Relative miR-143-3p level

Control | NC | mimic

B

Relative KLLN mRNA level

Control | control-plasmid | KLLN-plasmid

C

D

Relative KLLN mRNA level

Control | NC | mimic | mimic-plasmid

E

KLLN | β-actin

F

Cell viability (%)

Control | NC | mimic | mimic-plasmid

G

Number of migratory cells

Control | NC | mimic | mimic-plasmid

H

Cell apoptosis (%)

Control | NC | mimic | mimic-plasmid
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Discussion

It has been reported that miRNAs can serve as either oncogenes or tumour suppressors by directly or indirectly modulating gene expression. In our study, we found that miR-143-3p was up-regulated in rabbit CHD model. It was confirmed that KLLN was a direct target of miR-143-3p. The study also demonstrated that CHD related markers such as TC, TG and LDLC increased significantly in the model group and miR-143-3p mimic could obviously improve these molecules' expression. In addition, miR-143-3p mimic could promote cell viability and migration of rVSMCs and inhibit rVSMC apoptosis; these changes were reversed by KLLN-plasmid. In contrast, miR-143-3p inhibitor could inhibit cell viability and migration of rVSMCs and induce rVSMC apoptosis; these changes were reversed by KLLN-plasmid.

Cardiovascular disease (CVD), mainly including CHD and stroke, has been considered as a major health killer in developed countries for more than half a century and has soared in many underdeveloped regions in recent decades. In addition, miRNA is involved in many cancers and was abnormally expressed in tumors, such as gastric cancer, liver cancer, pancreatic cancer and colorectal cancer [26-29]. It has been reported that miR-143-3p serves as a tumour suppressor in various cancers, including breast cancer and ovarian cancer [30, 31]. Yang et al. revealed that miR-143-3p was up-regulated in rheumatoid arthritis tissues [32]. miR-143-3p inhibition could inhibit proliferation and induce apoptosis of MH7A cells by targeting insulin like growth factor 1 receptor (IGF1R) and insulin like growth factor binding protein 5 (IGFBP5). Zhang and Li [33] reported that miR-143-3p mediates the proliferation of cytokine-induced killer cells. However, the effect and its mechanism of miR-143-3p in CHD remains unclear. In our present study, we found that miR-143-3p over-expression promoted rVSMC cell proliferation and migration and inhibited cell apoptosis. However, miR-143-3p inhibitor suppressed rVSMC cell proliferation and migration and induced cell apoptosis.

To further explore more specific mechanism in which how miR-143-3p regulated rVSMCs, a bioinformatics prediction system (TargetScan) was used to predict the targets of miR-143-3p. The results showed that KLLN was a direct target of miR-143-3p. Previous research demonstrated that integrin α6 (ITGA6) is a direct target gene of miR-143-3p, which was related to angiogenesis [34]. KLLN is a tumor suppressor protein that contributes to p53-mediated apoptosis in colon cancer cell lines [35], KLLN is associated with regulation of cell growth, KLLN over-expression leads to increased cell death, whereas KLLN knockdown leads to cell proliferation, colony formation and migration in breast and prostate cancer cell lines [36, 37]. It has previously been reported that KLLN is also a high-affinity DNA-binding protein that binds to the promoters of genes such as tumor protein p53 (TP53) and serine/threonine protein kinase (CHK1) and regulates their expression [38].

In addition, previous research have indicated that miR-143-3p regulates nfkb nuclear factor kappa B (NF-kB) and p38 MAPK pathways [39]. In the later stage, we will conduct a related pathway study of miR-143-3p in CHD. These results indicated that miR-143-3p is known as a potential target for CHD therapy.

In conclusion, miR-143-3p was obviously up-regulated in CHD, however, target gene KLLN expression was down-regulated in rabbit CHD model. MiR-143-3p down-regulation suppressed cell proliferation and migration and induced cell apoptosis in rVSMCs by targeting KLLN.
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Figure 5. miR-143-3p promoted cell proliferation and migration ability. A. Rabbit vascular smooth muscle cells were transfected with miR-143-3p inhibitor for 48 h, and transfection efficiency was determined with qRT-PCR assay. B and C. The cells were transfected with KLLN-siRNA, and qRT-PCR and western blot assay were performed to the mRNA and protein expression of KLLN. D and E. The cells were transfected with miR-143-3p inhibitor or miR-143-3p inhibitor + KLLN-siRNA for 48 h, and then qRT-PCR and western blot assay were performed to the mRNA and protein expression of KLLN. F. Cell viability was detected by MTT assay. G. Transwell assay was performed to detect cell migration ability. H. Flow cytometry assay was performed to examine cell apoptosis. Data were displayed as mean ± SD. **P<0.01 vs. Control; #, ##P<0.05, 0.01 vs. mimic.

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Disclosure of conflict of interest

None.

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