



MiR-339 is a potential biomarker of coronary heart disease to aggravate oxidative stress through Nrf2/FOXO3 targeting Sirt2

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Background: MicroRNA (miRNA) is a class of small-molecule RNA that can regulate gene expression at post-transcription level. It is involved in the genesis and development of multiple diseases. The aim of this paper was to explore the mechanisms of miR-339 in coronary heart disease (CHD).

Methods: In this study, we enrolled patients with CHD from Beijing Chaoyang Hospital and performed animal experiments on CHD rats. *In vitro* experiments, such as histopathologic assay, quantitative real-time PCR assay, luciferase reporter assay, western blotting assay, and immunofluorescence assay were carried out to characterize the contents and associations of miR-339, Nrf2, FOXO3, and Sirt2 in CHD samples and cells. *In vivo* model was also established on rats.

Results: In CHD rat, miR-339 was up-regulated compared with control group. The expression of miR-339 up-regulation increased oxidative stress *in vitro* model via suppression of Sirt2/Nrf2/FOXO3. However, down-regulation of miR-339 expression inhibited oxidative stress *in vitro* model via activation of Sirt2/Nrf2/FOXO3. The Sirt2 or Nrf2 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model.

Conclusions: Down-regulation miR-339 may be the new targets to treat CHD through Nrf2/FOXO3 targeting Sirt2, and miR-339 may be a potential biomarker of CHD.

Keywords: miR-339; Nrf2; FOXO3; Sirt2; coronary heart disease (CHD)

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Introduction

Coronary heart disease (CHD) is a cardiovascular system disease with high morbidity. It is an organic lesion resulting from luminal occlusion and stenosis due to coronary atherosclerosis and thereby induces myocardial ischemia (1). CHD has severely threatened the health and life quality of the middle-aged and elderly patients (2). The stenosis of coronary atherosclerosis can reflect the extent of CHD (2). The pathogenic factors of CHD include diabetes, hypertension, obesity and unhealthy lifestyle (smoking, drinking, uptake of high-calorie food, lack of exercise,

and psychological factors) (3). CHD has been extensively studied in clinic recently (4). It is discovered that it is susceptible to multiple factors, including family history, unhealthy lifestyle and smoking. Research indicates that oxidative stress, inflammatory response, and vascular endothelial dysfunction play vital roles in the genesis and development of CHD (5,6).

Many studies suggested that CHD is closely related to the Sirtuin family during the genesis and development of its pathogenesis (7). Moreover, the Sirtuin family is also closely correlated with the risk factors of cardiovascular diseases, such as glucolipid metabolism. Sirtuin family is

a class of silence information regulators, which include 7 family members, from SIRT1-7 (7). However, recent studies find that SIRT2 has similar or contrary function in cardiovascular disease and its pathogenesis (7).

Recent findings have indicated that miRNA is related to the genesis and development of atherosclerosis (8,9). miRNA is extensively involved in the whole process of atherosclerosis (10). They include LDL deposition under the endarterium that leads to endothelial disturbance, cytokine release, monocyte adhesion and migration, monocyte transformation into macrophage, protective phagocytosis of macrophage, foam cell formation, inflammation amplification of macrophage and foam cell, smooth muscle cell proliferation and migration, as well as synthesis and degradation of fibrous cap matrix (8). Thus, miRNA plays a key role in the pathophysiological processes such as vascular endothelial dysfunction, inflammation, cell apoptosis, angiogenesis, atherosclerosis, neointima hyperplasia or restenosis (11). The aim of this paper was to explore the mechanisms of miR-339 in CHD. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-603>).

Methods

Patient with CHD

Patients with CHD and normal healthy volunteers were collected from Beijing Chaoyang Hospital, Capital Medical University and electrocardiogram of patient with CHD proceeded. The patients were diagnosed as CHD with angiographic evidence of at least one segment of a major coronary artery, including the left anterior descending, left circumflex, or right coronary artery, with >50% organic stenosis. The patients were included in this study if they have no family history of CHD and no history of significant concomitant diseases, including hepatic failure, renal failure, hepatitis, cardiomyopathy, congenital heart disease, bleeding disorders, previous thoracic irradiation therapy, and malignant diseases. Serum samples of patient with CHD and normal healthy volunteers were collected and saved at -80 °C. The experiments in the present study were approved by Beijing Chaoyang Hospital, Capital Medical University.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Beijing Chaoyang Hospital,

Capital Medical University (No.: 2016-6-4-1). Informed consent was obtained from every participant.

Animals and ethical approval

Wistar rats divided into two groups, normal control (n=6) and CHD model (n=6). CHD model rat was achieved by feeding with high-fat diet for 12 weeks and normal control rats were achieved by feeding with normal diet for 12 weeks. Experiments were performed under a project license granted by ethics board of Beijing Chaoyang Hospital, Capital Medical University (approval No.: F2016-025). Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Histopathologic assay

The heart tissues were fixed with 4% paraformaldehyde for 24 h. Tissue samples were embedded in paraffin, and the 5- μ m—thick sections were stained with hematoxylin-eosin (H&E). Tissue samples were obtained using fluorescence microscopy (Nikon Eclipse TE2000-U, Japan).

Quantitative real-time PCR assay

The total RNA samples from mice were obtained using TransZol according to the manufacturer's protocol (Beijing TransGen Biotech Co., Ltd. Beijing, China). Each RNA sample was reverse transcribed into cDNA using the kit of TransStart Top Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd. Beijing, China). Real-time PCR amplification was performed using the STEP ONE Real-time PCR Detection System (Foster City, CA, USA) with the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s.

Measurement of intracellular ROS. H9C2 cells were stained with DCFH-DA (10.0 μ M) for 30 min at 37 °C, and ROS levels were analyzed using fluorescence microscopy (Nikon Eclipse TE2000-U, Japan).

Luciferase reporter assay

Sirt2 plasmids were synthesized into luciferase reporter plasmid (pMIR-REPORT) (Promega Corporation, Fitchburg, WI, USA). Sirt2 plasmids and miR-339 mimics

were co-transfected into H9C2 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. 48 h after transfection, luciferase activities were measured using the Dual-Luciferase Assay (Promega Corporation) according to the manufacturer's instruction.

Enzyme-linked immunosorbent assay (ELISA)

The cell was collected and used to measure MDA, SOD, GSH, GSH-PX and ROS levels using ELISA kits according to the manufacturer's protocol. Absorbance values were measured using a microplate reader (Multiskan MK33; ThermoFisher systems, Helsinki, Finland) at 450 nm.

Western blotting assay

The total protein samples from the cells were homogenized using RIPA lysis buffer and the protein concentrations of the samples were determined using a BCA Protein Assay Kit. The proteins were separated by SDS-PAGE (10%) and then transferred to PVDF membranes (Millipore, Massachusetts, USA). Membranes were blocked with 5% non-fat in milk for 1 h at 37 °C and incubated with Sirt2, Nrf2, HO-1, FOXO3 and GAPDH (Cell Signaling Technology Inc.) at 4 °C overnight. Membranes were washed with TBST for 15 min and incubated with the anti-rabbit or anti-mouse secondary antibody (1:5,000, Cell Signaling Technology Inc.) for 1 h at 37 °C.

Immunofluorescence assay

The cell was washed with PBS and fixed with 4% paraformaldehyde for 15 min. The cell was incubated with 5% BSA+ 0.2% TrisX100 in TBST for 1 h at room temperature and incubated with Sirt2 (1:100, Cell Signaling Technology Inc.) at 4 °C overnight. The cell was stained with 555- anti-rabbit secondary antibody for 1 h at 37 °C and stained with DAPI assay for 15 min at darkness. Cell samples were imaged by fluorescence microscopy (Nikon Eclipse TE2000-U, Japan) at 200× magnification.

Vitro model of CHD

The H9C2 cells were purchased from the Chinese Academy of Medical Sciences, Shanghai, China. H9C2 cells were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ and 95% O₂ at

37 °C. MiR-339, anti-miR-339, and negative mimics were transfected into cell using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. They were subjected to hypoxia using DMEM/F12 without FBS and glucose in a hypoxia chamber (Forma 370; Thermo Fisher Scientific, Waltham, MA, USA) saturated with a gas mixture (95% N₂ and 5% CO₂) at 37 °C.

Statistical analysis

Data are presented as the mean ± SD using SPSS 17.0. Student's *t*-test or one-way analysis of variance (ANOVA) and Tukey's post-test was used to analyze the statistical significance. Statistical significance was considered to be *P*<0.05.

Results

The expression of miR-339 in patients or rats with CHD

Firstly, we analyzed the changes in microRNAs in patients or rats with CHD. As showed in *Figure 1A,B,C*, electrocardiogram showed sinus arrhythmia, and we found that the serum of miR-339 expression was increased in patients with CHD, compared with normal group. Then, in rats with CHD, SOD, GSH, and GSH-PX levels were inhibited, and MDA level was increased, compared with normal rat group (*Figure 1D,E,F,G*). HE staining showed that a large number of blood clots were formatted in rats with CHD, compared with normal group (*Figure 1H*). The serum of miR-339 expression was increased in rats with CHD, compared with normal group (*Figure 1I*). These results showed that miR-339 may be keeping in the formation of CHD.

Over-expression of miR-339 increased oxidative stress and not affected on ROS levels in vitro model

This study tested the function of miR-339 on the oxidative stress *in vitro* model. There was increase the expression of miR-339 in miR-339 mimics, compared with negative group (*Figure 2A*). Over-expression of miR-339 increased MDA levels, and inhibited SOD, GSH and GSH-PX levels *in vitro* model, compared with negative group (*Figure 2B,C,D,E*). However, over-expression of miR-339 not affected ROS level *in vitro*, compared with negative group (*Figure 2F,G*).

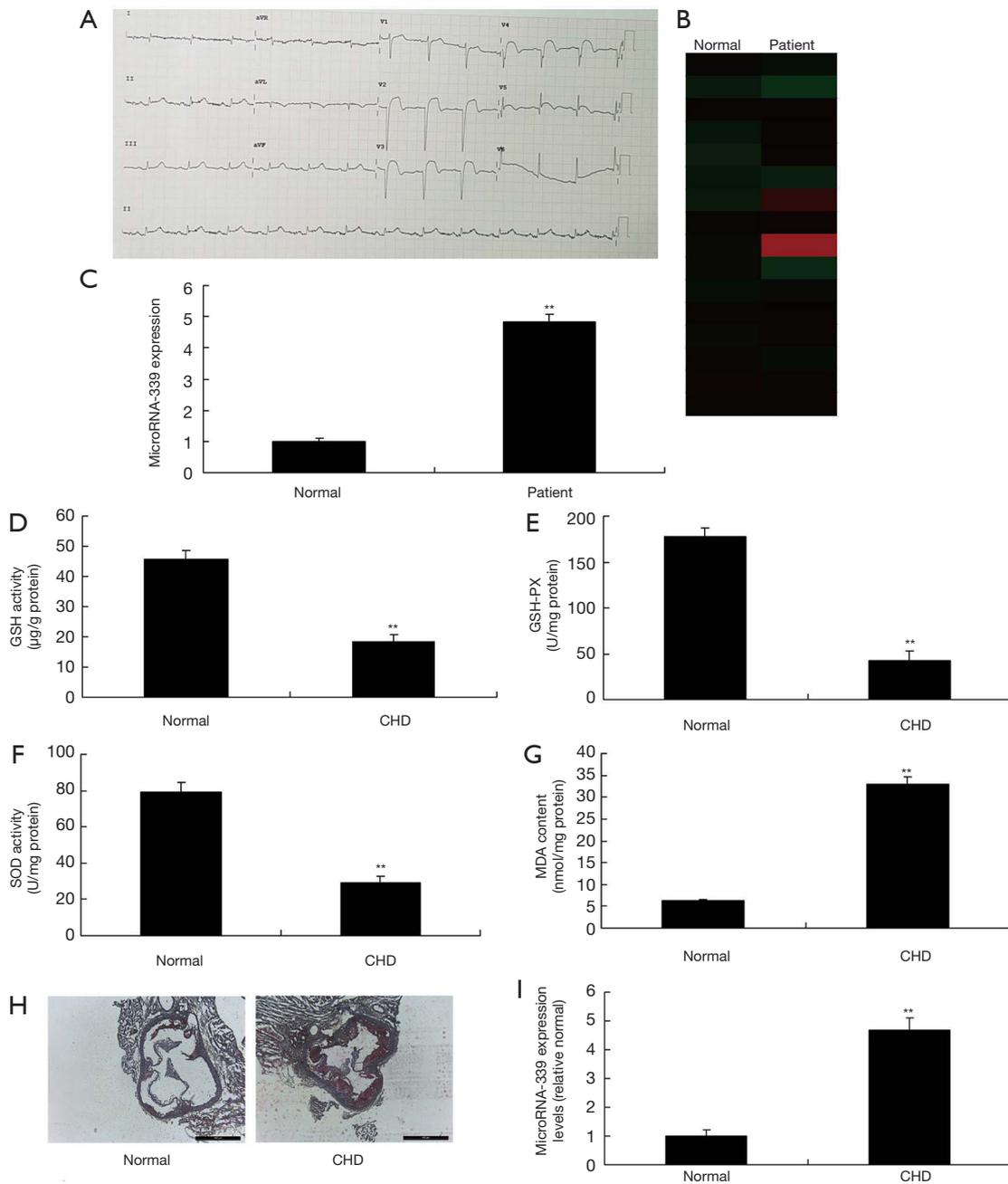


Figure 1 The expression of miR-339 in patients or rat with CHD. Electrocardiogram for patients with CHD (A), gene chip and QPCR for miR-339 expression (B,C) in patients with CHD; GSH (D), GSH-PX (E), SOD (F), MDA (G) level, HE staining (H) and QPCR for miR-339 expression (I) in rat with CHD. Normal, normal volunteer or normal rat group; patient, patients with CHD group; CHD, the rat with CHD group. **, $P < 0.01$ compared with normal volunteer or normal rat group. CHD, coronary heart disease; GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

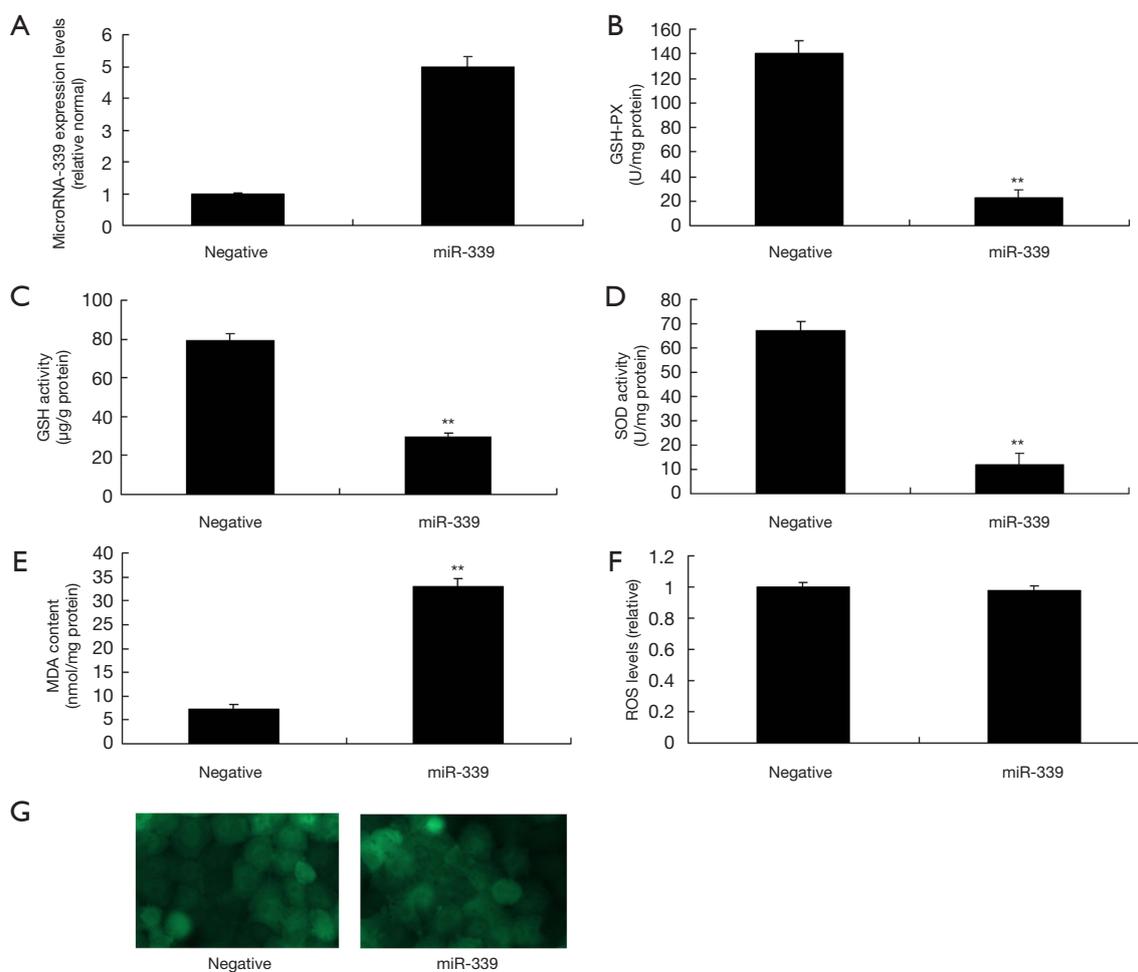


Figure 2 Over-expression of miR-339 increased oxidative stress and not affected on ROS levels *in vitro* model. QPCR for miR-339 expression (A), GSH (B), GSH-PX (C), SOD (D), MDA (E), ROS (F,G) levels. Negative, negative mimics group; miR-339, over-expression of the miR-339 group. ** $P < 0.01$ compared with negative mimics group. GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species.

Down-regulation of miR-339 expression inhibited oxidative stress and not affects ROS levels in vitro model

To validate the effect of miR-339 on oxidative stress *in vitro* model, we used anti-miR-339 to reduce the expression of miR-339 *in vitro* model. As showed in *Figure 3A*, there was inhibition of miR-339 expression *in vitro* model, compared with negative group. Meanwhile, we also found that down-regulation of miR-339 reduced MDA level, and increased SOD, GSH and GSH-PX levels *in vitro* model, compared with negative control group (*Figure 3B,C,D,E*). However, down-regulation of miR-339 not regulates ROS levels *in vitro* model, compared with negative group (*Figure 3F,G*).

So, these results showed that miR-339 only regulates oxidative stress and not affect ROS levels *in vitro* model of CHD.

MiR-339 regulates Sirt2/Nrf2/FOXO3 in vitro model of CHD.

We explored the mechanism of miR-339 on oxidative stress in CHD model. Over-expression of miR-339 suppressed Sirt2, Nrf2, HO-1 and FOXO3 mRNA expressions *in vitro* model, compared negative group (*Figure 4A,B,C,D,E*). Luciferase reporter assay showed that miR-339 targets Sirt2 mRNA and luciferase reporter activity levels were reduced

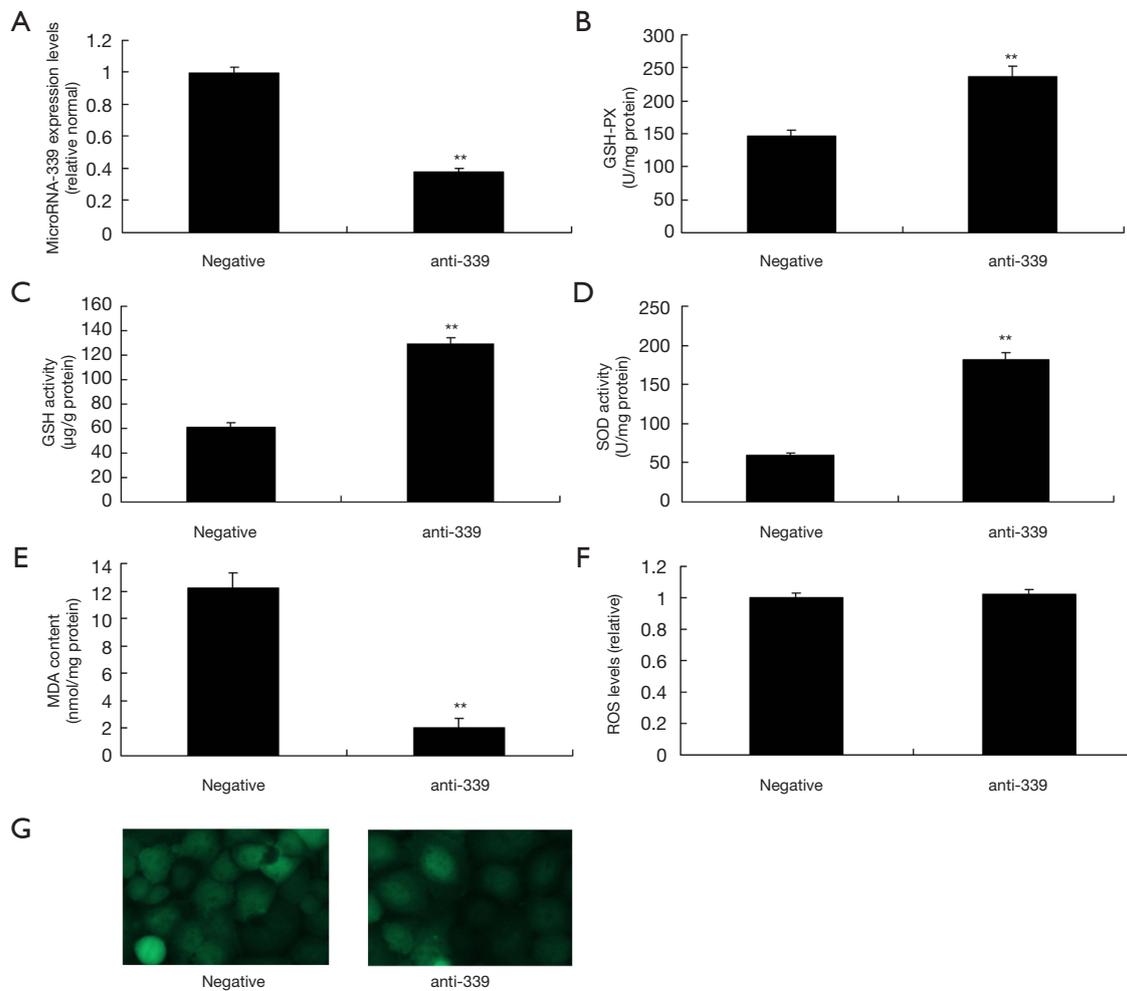


Figure 3 Down-regulation of miR-339 expression inhibited oxidative stress and not affects ROS levels *in vitro* model. QPCR for miR-339 expression (A), GSH (B), GSH-PX (C), SOD (D), MDA (E), ROS (F,G) levels. Negative, negative mimics group; anti-339, down-regulation of miR-339 group. **, $P < 0.01$ compared with negative mimics group. GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species.

in over-expression of miR-339 group, compared with negative group (Figure 4E,G). IF showed that miR-339 over-expression suppressed the protein expression of Sirt2 *in vitro* model group, compared with negative group (Figure 4H). Then, we also found that over-expression of miR-339 suppressed Sirt2, Nrf2, HO-1 and FOXO3 protein expressions *in vitro* model, compared negative group (Figure 5A,B,C,D,E). Down-regulation of miR-339 induced Sirt2, Nrf2, HO-1 and FOXO3 protein expressions *in vitro* model, compared negative group (Figure 5F,G,H,I,J). These results showed that miR-339 regulates Sirt2/Nrf2/FOXO3 to regulate oxidative stress *in vitro*.

The Sirt2 inhibitor reduced the protective effect of miR-339 down-regulation on the oxidative stress in vitro model

Next, this study analyzed the role of Sirt2 in the function of miR-339 on oxidative stress in CHD model. Sirt2 inhibitor could suppress the protein expression of Sirt2, Nrf2, HO-1 and FOXO3 protein expressions *in vitro* model by down-regulation of miR-339, compared with miR-339 down-regulation group (Figure 6A,B,C,D,E). The inactivation of Sirt2 reduced the protective effect of miR-339 on the inhibition of MDA levels, and induction of SOD, GSH and GSH-PX levels *in vitro* model of CHD, compared with down-regulation of miR-339 group (Figure 6F,G,H,I).

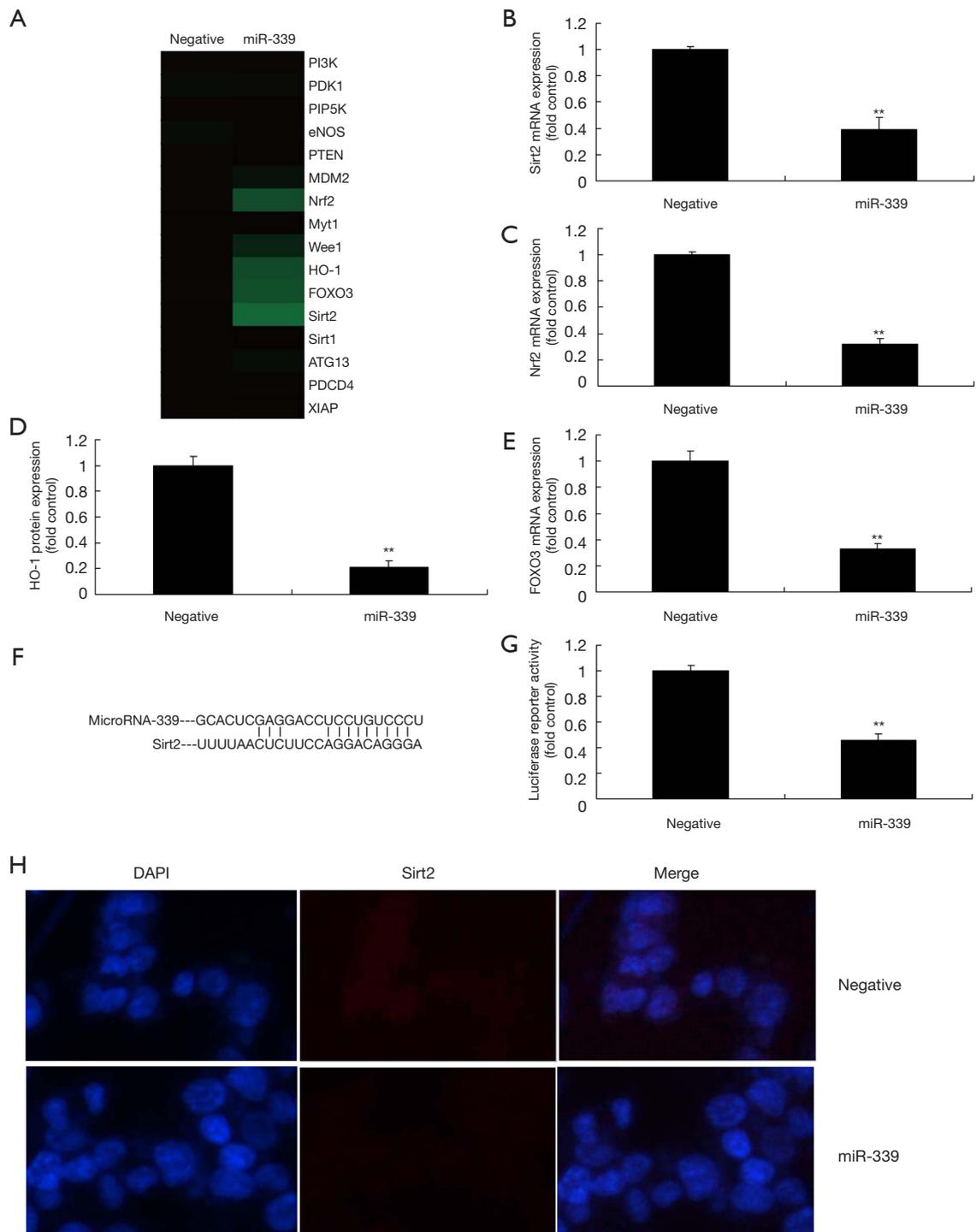


Figure 4 Over-expression of miR-339 regulates Sirt2/Nrf2 /FOXO3 in vitro model of CHD. Gene chip and QPCR for Sirt2, Nrf2, HO-1, FOXO3 expressions (A-E), Luciferase reporter assay showed that miR-339 targets Sirt2 mRNA (F) and luciferase reporter activity levels (G), IF for Sirt2 expression (H). Negative, negative mimics group; miR-339, over-expression of miR-339 group. **, $P < 0.01$ compared with negative mimics group. Sirt2, silent information regulator 2 protein; Nrf2, nuclear factor E2-related factor 2; HO, heme oxygenase; FOXO3, forkhead box protein O3.

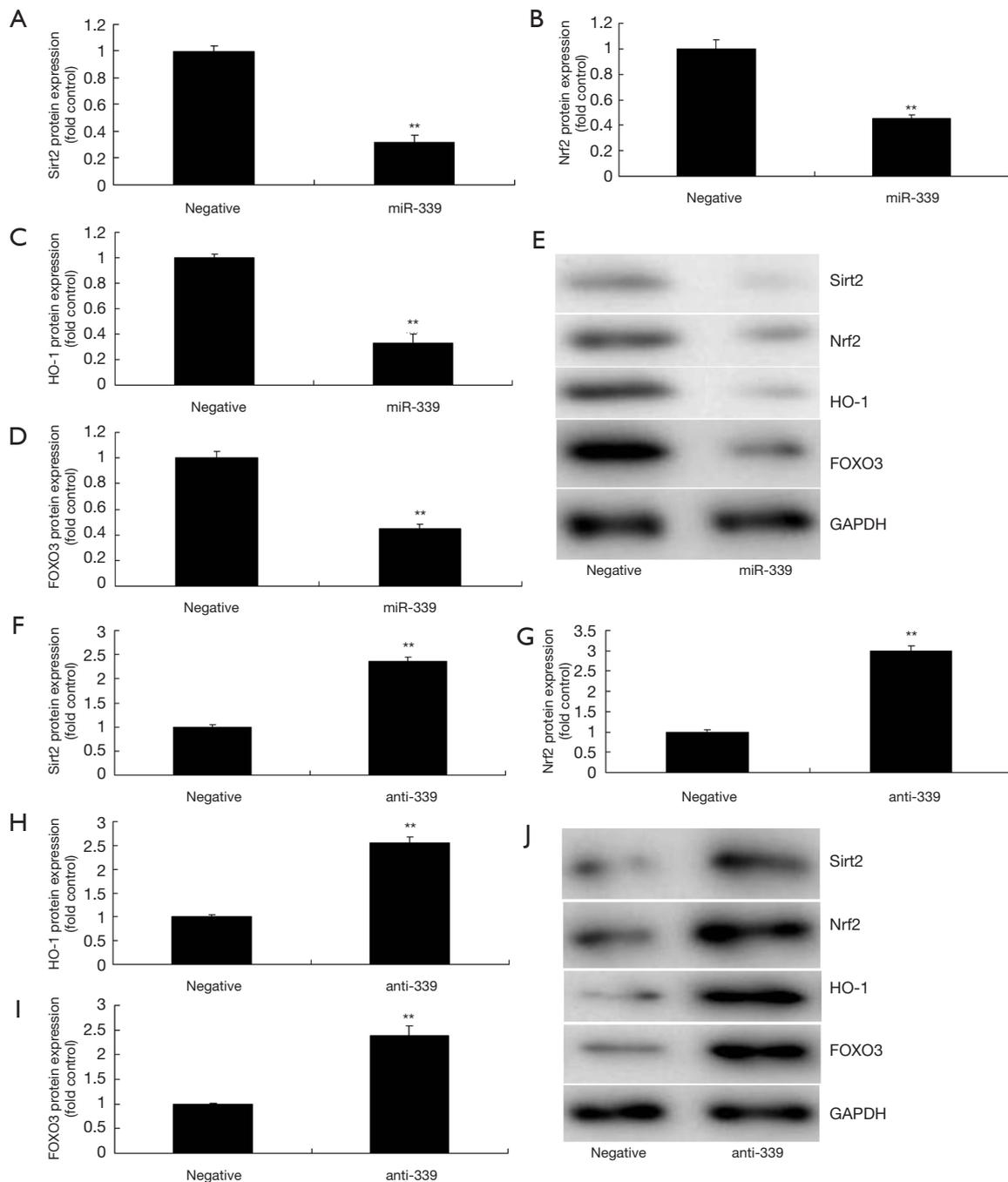


Figure 5 MiR-339 regulates Sirt2/Nrf2 /FOXO3 *in vitro* model of CHD. Sirt2, Nrf2, HO-1, FOXO3 protein expressions by statistical analysis (A,B,C,D) and western blot analysis (E) by over-expression of miR-339; Sirt2, Nrf2, HO-1, FOXO3 protein expressions by statistical analysis (F,G,H,I) and western blot analysis (J) by down-regulation of miR-339. Negative, negative mimics group; miR-339, over-expression of miR-339 group; anti-339, down-regulation of miR-339 group. **, $P < 0.01$ compared with negative mimics group. Sirt2, silent information regulator 2 protein; Nrf2, nuclear factor E2-related factor 2; HO, heme oxygenase; FOXO3, forkhead box protein O3.

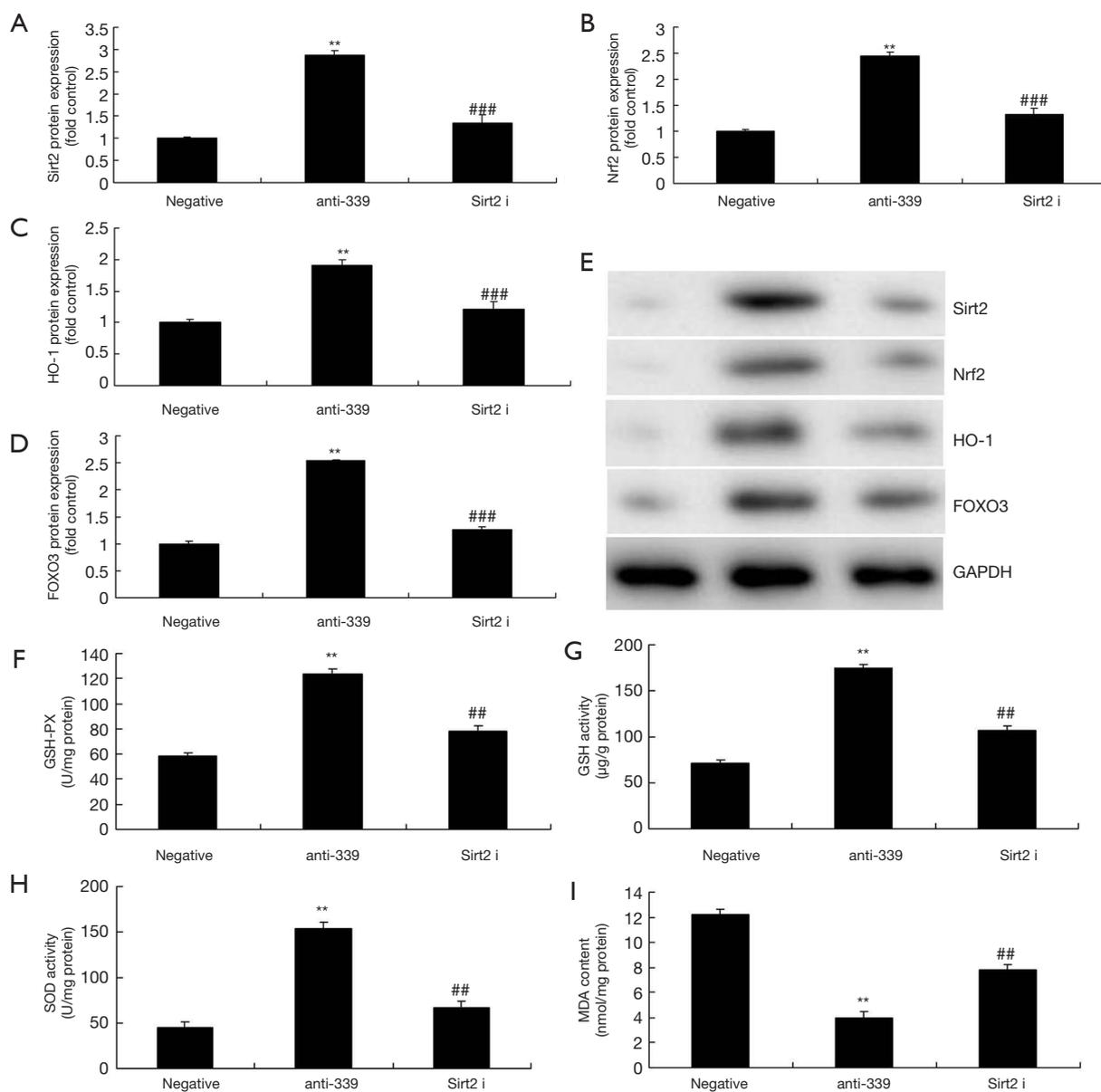


Figure 6 The Sirt2 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model. Sirt2, Nrf2, HO-1, FOXO3 protein expressions by statistical analysis (A,B,C,D) and western blot analysis (E) by over-expression of miR-339; GSH (F), GSH-PX (G), SOD (H), MDA (I). Negative, negative mimics group; anti-339, down-regulation of miR-339 group; Sirt2 i, down-regulation of miR-339 and Sirt2 inhibitor group. **, P<0.01 compared with negative mimics group; #, P<0.01 compared with anti-339 group; GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; Sirt2, silent information regulator 2 protein; Nrf2, nuclear factor E2-related factor 2; HO, heme oxygenase; FOXO3, forkhead box protein O3.

The Nrf2 inhibitor reduced the protective effect of miR-339 down-regulation on the oxidative stress in vitro model

Then, we used Nrf2 inhibitor to reduce the protein expression of Nrf2 and HO-1 *in vitro* model of CHD by

down-regulation of miR-339, compared with miR-339 down-regulation group (Figure 7A,B,C). Meanwhile, the inhibition of Nrf2 reduced GSH and GSH-PX levels, and increased MDA levels *in vitro* model of CHD by down-regulation of miR-339, compared with miR-339 down-

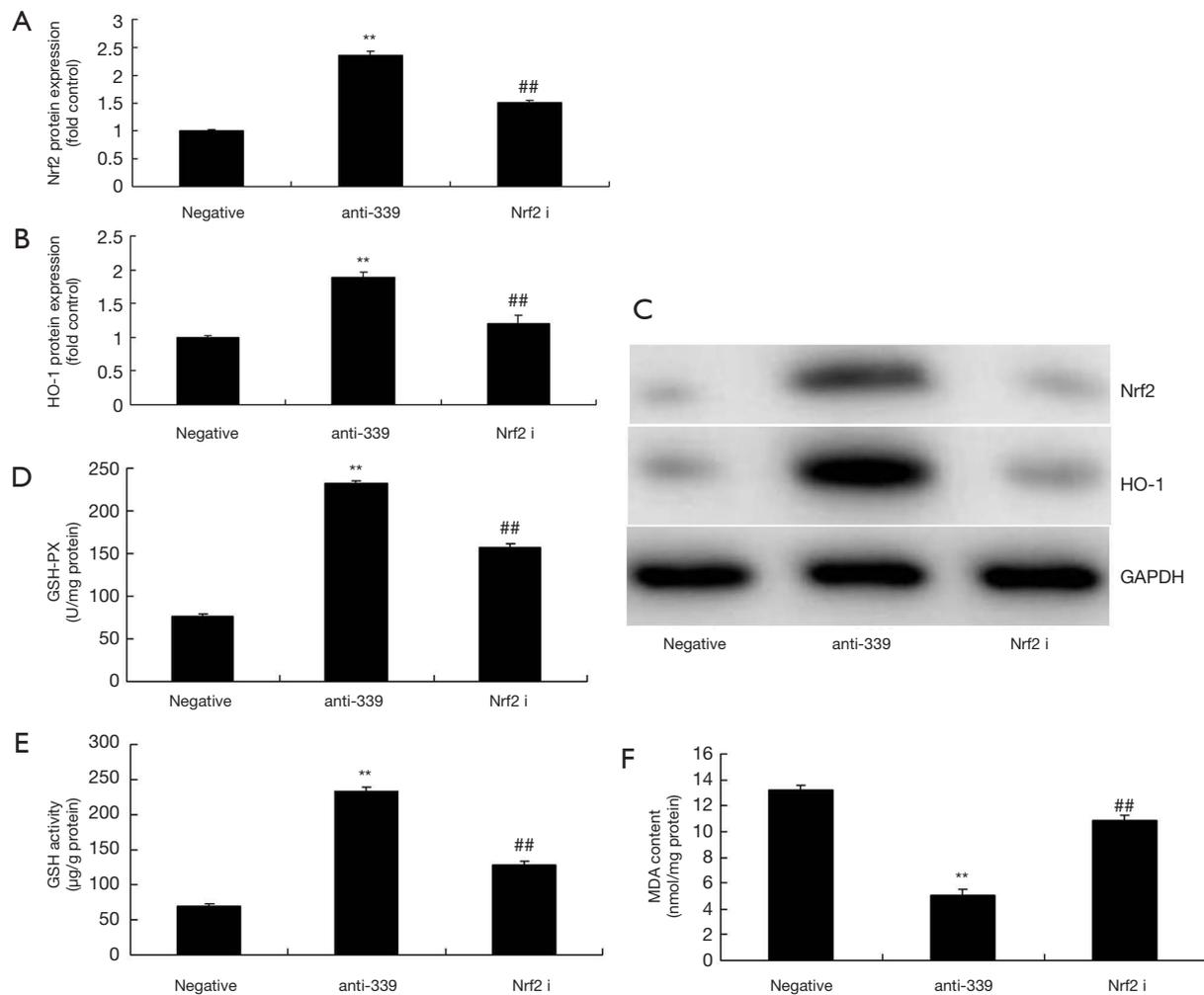


Figure 7 The Nrf2 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model. Nrf2 and HO-1 protein expressions by statistical analysis (A,B) and western blot analysis (C), GSH (D), GSH-PX (E), MDA (F). Negative, negative mimics group; anti-339, down-regulation of miR-339 group; Nrf2 i, down-regulation of miR-339 and Nrf2 inhibitor group. **, $P < 0.01$ compared with negative mimics group; ##, $P < 0.01$ compared with anti-339 group. GSH, glutathione; MDA, malondialdehyde; Nrf2, nuclear factor E2-related factor 2; HO, heme oxygenase.

regulation group (Figure 7D,E,F).

The FOXO3 inhibitor reduced the protective effect of miR-339 down-regulation on the oxidative stress *in vitro* model

Lastly, we used FOXO3 inhibitor to analyze the role of FOXO3 in the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model. As showed in Figure 8, FOXO3 inhibitor reduced the protein expression of FOXO3, reduced SOD levels, and increased MDA levels *in vitro* model of CHD by miR-339 down-regulation,

compared with miR-339 down-regulation group.

Discussion

CHD is a common disease among the elderly population in China, which is affected by the rapid social economical development and accelerating aging process (3). The morbidity of CHD shows an increasing trend (12,13). At present, CHD has become a frequently occurring disease threatening social public psychological and physiological health. Patients may develop symptoms like chest pain,

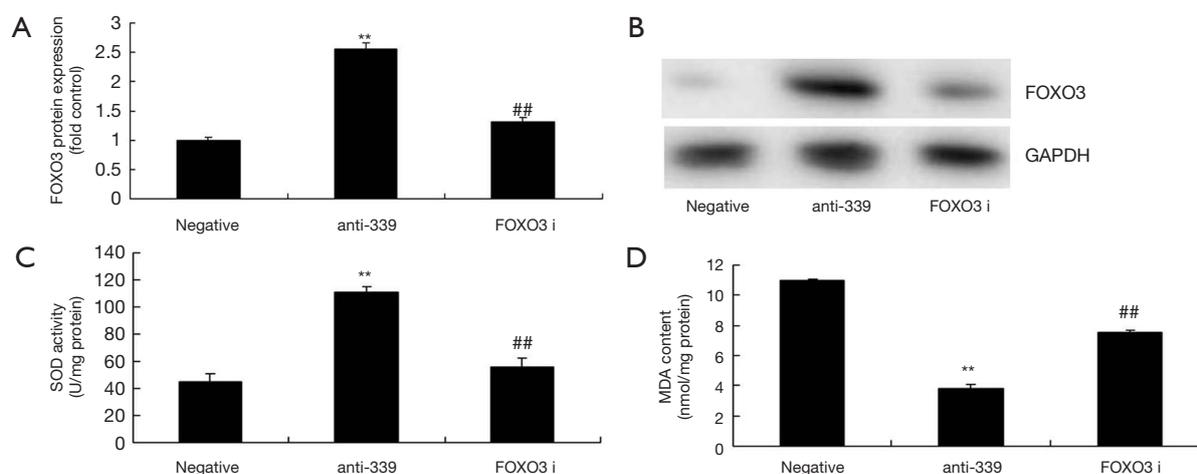


Figure 8 The FOXO3 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model. FOXO3 protein expressions by statistical analysis (A) and western blot analysis (B), SOD (C), MDA (D). Negative, negative mimics group; anti-339, down-regulation of miR-339 group; FOXO3 i, down-regulation of miR-339 and FOXO3 inhibitor group. **, $P < 0.01$ compared with negative mimics group; ##, $P < 0.01$ compared with anti-339 group. SOD, superoxide dismutase; MDA, malondialdehyde; FOXO3, forkhead box protein O3.

palpitation, and weakness (13). In some severe cases, patient will suffer from acute myocardial infarction (12). Therefore, it has caused great damage to the life health and daily living of patients. On this account, active prevention of CHD to reduce the disease-related injury has become a focus of current medical research (13). In this paper, we firstly found that the serum of miR-339 expression was increased in patients or rats with CHD. Saddic *et al.* showed that acute human left ventricular ischemia upregulated miRNAs miR-339-5p and miR-483-3p and the downregulated miRNA miR-139-5p (14).

Endothelial structural and functional impairments are also involved in the formation and qualitative changes of atheromatous plaque (15). The excessive activation of the oxidative stress response is an important step leading to endothelial injury (5). ROS is an important catalyzing enzyme in the body, which can catalyze the production of oxygen free radicals and mediate the activation of oxidative stress response (16). High ROS expression can increase the production of oxygen free radicals and act on the endothelial cell (17). On the one hand, it can result in increased production of lipid oxidation product MDA. On the other hand, it can result in elevated production of endothelial injury marker ET-1 (15). Sirt2 and SOD are the antioxidants exerting protective effect during endothelial oxidative injury (18). The former can reduce the production of oxygen free radical through the Nrf2/

FOXO3 pathway, while the latter can scavenge the oxygen free radical through reduction reaction (19). Our findings implied that over-expression of miR-339 increased oxidative stress and not affected on ROS levels *in vitro* model.

SIRT2 participates in multiple pathophysiological processes, including aging, energy restriction, oxidative stress, cell apoptosis and inflammatory response (7). Besides, it also plays a role in mitochondriogenesis and biological circadian rhythm regulation. The latest study finds that SIRT2 is related to the genesis of multiple tumors, nervous system-related degenerative disease, aging-related disease and cardiovascular disease (20). It exerts its function through signal pathways such as FOXO transcription factor, Nrf2, PEPCK1 and p65 (19). Our present data showed that over-expression of miR-339 suppressed Sirt2, Nrf2, HO-1 and FOXO3 protein expressions *in vitro* model. Meanwhile, the Sirt2 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model. Wang *et al.* suggested that miR-339/Sirt2/NF- κ B/FOXO1 was keeping in the effects of acupuncture in SH-SY5Y cells and rat PC12 cells (21).

Recently, reports have reported the biological function of SIRT2 in oxidative stress. Oxidative stress will up-regulate SIRT2 expression in NIH3T3 cells due to the elevated hydrogen peroxide level (20). In addition, it can activate the FOXO3 gene through deacetylation, which can thereby

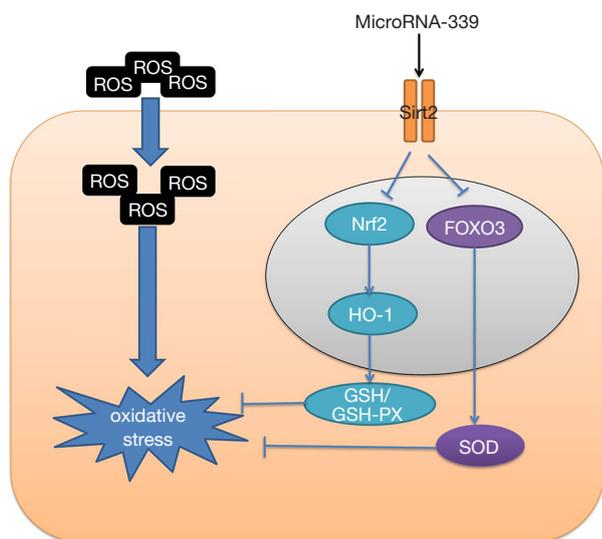


Figure 9 MiR-339 is a Potential Biomarker of CHD to aggravate oxidative stress through Nrf2/FOXO3 targeting Sirt2. CHD, coronary heart disease; Nrf2, nuclear factor E2-related factor 2; Sirt2, silent information regulator 2 protein; FOXO3, forkhead box protein O3.

up-regulate MnSOD and reduce the ROS level (19). In mouse macrophage-like cell lines, the pep-1-sirt2 protein with cell permeation function can induce lipopolysaccharide and H_2O_2 related death in mouse cells (22). FOXO3 is an important protein family system for regulating cell apoptosis, cell cycle, DNA damage repair, oxidative stress, energy metabolism, longevity, cancer genesis and development, and other cellular functions (22). Our data also showed that FOXO3 inhibitor reduced the protective effect of miR-339 down-regulation on oxidative stress *in vitro* model.

Nrf2 is a critical transcription factor for regulating the expression of multiple antioxidant genes. For instance, HO-1 is an enzyme with antioxidation and cell protection. The activation of the Nrf2/HO-1 signal can protect the cell from oxidative injury (23). HO-1 is an important antioxidant that mainly catalyzes the decomposition and metabolism of heme into ferrous, carbon monoxide and biliverdin (23). On the one hand, heme group degradation contributes to preventing its oxidation promoting effect (24). On the other hand, the by-product biliverdin, together with the reduced form bilirubin, has effective ROS scavenging activity (24). They can defend against peroxide, peroxyxynitrite, hydroxyl, and superoxide free radical (24). Thus, our data demonstrated that the Nrf2 inhibitor reduced the protective

effect of miR-339 down-regulation on oxidative stress *in vitro* model (Figure 9).

Therefore, our study provided novel insight into the pathological mechanisms of CHD, and miR-339 is a potential biomarker of CHD to aggravate oxidative stress through Nrf2/FOXO3 targeting Sirt2. Although our study confirmed miR-339 is a potential biomarker of CHD, the target genes of miR-339 or other differentially expressed are also necessary to be carefully tested for CHD.

Conclusions

Our study has revealed that Over-expression of miR-339 increased oxidative stress and not affected on ROS levels. MiR-339 is a Potential Biomarker of CHD to aggravate oxidative stress through Nrf2/FOXO3 targeting Sirt2. MiR-339/Sirt2 may be considered as the new targets for the treatment of CHD.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/apm-20-603>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/apm-20-603>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-20-603>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Beijing Chaoyang Hospital, Capital Medical University (No.: 2016-6-4-1). Experiments were performed under a project license granted by ethics board of Beijing Chaoyang Hospital, Capital Medical University (approval NO: F2016-025), in compliance with institutional guidelines for the care

and use of animals.

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