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Research Paper

MicroRNA-140-5p aggravates doxorubicin-induced cardiotoxicity by promoting myocardial oxidative stress via targeting Nrf2 and Sirt2

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ABSTRACT

Clinical application of doxorubicin (DOX), an anthracycline antibiotic with potent anti- tumor effects, is limited because of its cardiotoxicity. However, its pathogenesis is still not entirely understood. The aim of this paper was to explore the mechanisms and new drug targets to treat DOX-induced cardiotoxicity. The in vitro model on H9C2 cells and the in vivo models on rats and mice were developed. The results showed that DOX markedly decreased H9C2 cell viability, increased the levels of CK, LDH, caused histopathological and ECG changes in rats and mice, and triggered myocardial oxidative damage via adjusting the levels of intracellular ROS, MDA, SOD, GSH and GSH-Px. Total of 18 differentially expressed microRNAs in rat heart tissue caused by DOX were screened out using microRNA microarray assay, especially showing that miR-140-5p was significantly increased by DOX which was selected as the target miRNA. Double-luciferase reporter assay showed that miR-140-5p directly targeted Nrf2 and Sirt2, as a result of affecting the expression levels of HO-1, NQO1, Gst, GCLM, Keap1 and FOXO3a, and thereby increasing DOX-caused myocardial oxidative damage. In addition, the levels of intracellular ROS were significantly increased or decreased in H9C2 cells treated with DOX after miR-140-5p mimic or miR-140-5p inhibitor transfection, respectively, as well as the changed expression levels of Nrf2 and Sirt2. Furthermore, DOX- induced myocardial oxidative damage was worsened in mice treated with miR-140-5p agomir, and however the injury was alleviated in the mice administrated with miR-140-5p antagomir. Therefore, miR-140-5p plays an important role in DOX-induced cardiotoxicity by promoting myocardial oxidative stress via targeting Nrf2 and Sirt2. Our data provide novel insights for investigating DOX-induced heart injury. In addition, miR-140-5p/ Nrf2 and miR-140-5p/Sirt2 may be the new targets to treat DOX-induced cardiotoxicity.

1. Introduction

Doxorubicin (DOX), a kind of anthracycline antibiotics, is widely used in clinical to treat some kinds of tumors because of its high efficiency and wide-spectrum [1]. However, DOX can produce some serious side effects on body after long-term use, especially to cardiotoxicity [2–5]. DOX can lead to left ventricular dysfunction, or even heart failure, and DOX-induced heart injury is closely related to the accumulation in the body [6]. In addition, the risk of DOX-induced cardiotoxicity will be exacerbated in patients as the cumulative dose of DOX is increased. When the accumulation dose of DOX is increased from 400 mg/m^2 to 700 mg/m^2, the incidence of heart failure will rise from 5% to 48% [7–9]. Once the heart failure occurs, the patients' mortality s will be significantly increased. Thus, DOX-caused cardiotoxicity limits its clinical application.

The pathogenesis and prevention of DOX-induced cardiotoxicity has become a hotspot in recent years. Many researches have revealed that DOX-induced myocardial injury may be related to oxidative stress, calcium overload, mitochondrial damage, cardiomyocyte apoptosis and autophagy [10–17]. However, oxidative stress attracts the most attention during DOX-induced myocardial damage. Studies have shown that the semi-quinone DOX, resulting from metabolic process of DOX, can quickly transfer its unpaired electrons to the molecular oxygen, then produce a large number of superoxide anion free radicals (O_2^-), which

Abbreviations: ARE, antioxidant response element; CK, creatine kinase; DAPI, 4′,6′- Diamidino-2-phenylindole; DOX, doxorubicin; ECG, electrocardiograms; FOXO3a, Forkhead box O3; GCLM, glutamate-cysteine ligase modifier subunit; GSH, glutathione; GSH-Px, glutathione peroxidase; GST, glutathione-S- transferase; H&E, hematoxylin-eosin; HO-1, heme oxygenase-1; Keap1, kelch like ECH- associated protein 1; LDH, lactate dehydrogenase; MDA, malondialdehyde; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5 -diphenyl tetrazolium bromide; ncRNA, non-coding RNAs; NQO1, NAD(P)H Quinone Dehydrogenase 1; Nrf2, nuclear erythroid factor 2-related factor 2; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species; RT-PCR, reverse transcriptiton polymerase chain reaction; Sirt2, silent information regulator factor 2-related enzyme 2; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; Tris, hydroxymethyl aminomethane

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can lead to cell damage, mitochondrial dysfunction and DNA injury. In addition, DOX can also combine with iron to destroy the iron homeostasis, and then produce a large number of reactive oxygen species (ROS) [18–20]. Besides, the interaction of ROS with phosphorlipid on the mitochondrial membrane of cells can also lead to mitochondrial dysfunction, and further affect the energy metabolism [21]. Thus, oxidative stress is one potential mechanism of DOX- induced heart damage, and inhibiting oxidative stress may be one effective prevention and treatment method against cardiotoxicity caused by DOX.

MicroRNA (miRNA), belonging to the small non-coding RNAs (ncRNAs), can regulate gene expression through binding with target mRNA [22]. There have growing evidences that miRNAs play key roles in the development of various diseases, suggesting that miRNAs can be considered as the potential drug targets to treat human diseases [23–26]. Some reports have shown that the aberrant expression of miR-499 can lead to irreversible damage of heart via causing arrhythmia and myocardial hypertrophy, indicating that miR-499 may be a potential target for the treatment of heart diseases [27]. In addition, the works of DOX- induced cardiotoxicity have shown that miR-532-3p and miR-208a play important roles in heart diseases [28,29].

Microarrays technology is an effective method for high-throughput screening miRNAs from biological samples, which has been widely used in disease diagnosis, target selection and new drug development. Lu Y et al. assessed the potential role of miRNAs in regulating experimental atrial fibrillation using microarray technology, and found that miR-328 is a potential therapeutic target for the disease [30]. Therefore, microarrays technology is of great importance to pick out differentially expressed miRNAs for deeply investigating molecular mechanisms and drug discovery.

In this study, the differentially expressed miRNAs in DOX-induced heart tissue of rats were tested by using microarrays technology, and then the biological functions of the aimed miRNAs on DOX-induced heart damage were examined, which will provide potential mechanisms and new drug targets for the treatment of DOX-induced cardiotoxicity.

2. Materials and methods

2.1. Chemicals and materials

DOX was purchased from Sigma (Santa Clara, CA, USA). Tissue Protein Extraction Kit was obtained from KEYGEN Biotech. Co., Ltd. (Nanjing, China). The bicinchoninic acid (BCA) protein assay kit, ROS assay kit, cell lysis buffer and phenylmethanesulfonyl fluoride (PMSF) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Creatine kinase (CK), lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-Px) detection kits were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Sodium dodecyl sulfate (SDS), hydroxymethyl aminomethane (Tris) and 4′,6′-Diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was provided by Roche Diagnostics (Basel, Switzerland). Lipofectamin2000, TransZolTM, TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal), TransStart® Top Green qPCR SuperMix were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). SanPrep Column MicroRNA Mini-Preps Kit, MicroRNA First Strand cDNA Synthesis Kit and MicroRNAs Quantitation PCR Kit were purchased from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

2.2. Cell culture

The H9C2 cells were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China), and the cells were maintained in DMEM supplemented with 10% FBS and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C.

2.3. DOX-induced cell injury

DOX was prepared to make a series of working dilutions in serum-free DMEM. The H9C2 cells were plated in 96-well plates at a density of 5×10^4 cells/mL per well for 24 h before challenged with various concentrations of DOX (0–10 μM). After incubation with DOX for 24 h, 10 μL of MTT (5 mg/mL) solution was added to each well. After incubation at 37 °C for 4 h, the medium with MTT was removed. Next, 150 μL of DMSO was added to each well to dissolve the formazan crystals, and the absorbance at 490 nm was measured using a POLARstar OPTIMA multi-detection microplate reader (BioRad, San Diego, CA, USA).

2.4. Measurement of intracellular ROS

The H9C2 cells were plated in 6-well culture plates at a density of 5×10^4 cells/ mL and then treated with DOX (5 μM) for 24 h. After removing the medium, 1.5 mL of DCFH-DA (10.0 μM) was added at 37 °C for 25 min, and then the samples were analyzed by fluorescence microscopy (Olympus, Tokyo, Japan) at 200× magnification.

2.5. Animals and ethical approval

Male SD rats weighing 200 ± 20 g and Male C57BL/6 J mice weighing 20 ± 2 g were obtained from the Experimental Animal Center at Dalian Medical University (Dalian, China) (SCXK: 2013-0003). All experimental procedures were performed in strict accordance with PR China Legislation Regarding the Use and Care of Laboratory Animals, and all experiments involving animals were approved by the Animal Care and Use Committee of Dalian Medical University (Approval number: 20150658). The animals were group-housed with 2–3 rats or mouse per cage on a 12 h light/dark cycle in a temperature-controlled (25 ± 2 °C) room with free access to water and food, and were allowed one week to acclimatize before experiment. Randomization was used to assign samples to the experimental groups and to collect and process data. The experiments were performed by investigators blinded to the groups for which each animal was assigned. Animal studies have been reported in compliance with the ARRIVE guidelines [31,32].

2.6. DOX-induced myocardial damage injury in vivo

Twenty rats and twenty mice were all randomly divided into two groups (n = 10): control group (0.9% saline) and DOX group. The animals in DOX groups were intraperitoneally injected with DOX (15 mg/kg diluted with 0.9% saline for eight days [33,34]), whereas the animals in control groups were intraperitoneally injected with equal volumes of 0.9% saline. After that, the electrocardiograms (ECG) of rats and mice were detected, then all animals were sacrificed and the blood samples were harvested. The serum samples were obtained by centrifugation (3000 r/min, 4 °C) for 10 min, and the heart samples were promptly removed. Part of heart tissue was fixed and the remaining samples were stored at −80 °C for further assay.

2.7. Measurement of electrocardiograms in rats and mice

According to the previous method [35], the animals were anaesthetized and fixed on the table with the supine position. Subcutaneous needle electrodes of the commercial computer-based ECG device (BL-420F Biological Function Experiment System, Chengdu Thai Union Technology Co, Ltd., China) were connected to the animals, and electrocardiograms were recorded.