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miR-28 promotes cardiac ischemia by targeting mitochondrial aldehyde dehydrogenase 2 (ALDH2) in mus musculus cardiac myocytes

S.-P. LI[1], B. LIU[1,2], B. SONG[3], C.-X. WANG[4], Y.-C. ZHOU[1]

[1]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong Province, China
[2]Guangzhou Institute of Cardiovascular Disease, the Second Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong Province, China
[3]Shandong Tumor Hospital and Institute, Jinan, Shandong Province, China
[4]Department of Oncology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong Province, China

Suping Li and Bin Liu contributed equally to this study

Abstract. – OBJECTIVE: Aldehyde dehydrogenase 2 (ALDH2) is a crucial enzyme involved in protecting the heart from ischemic. MicroRNAs (miRNAs) are involved in gene down-regulation. However, this mechanism is unclear. The aim of this study was to investigate the role of miR-28 in the regulation of ALDH2 and to explore the mechanism of miR-28 in musculus of myocardial ischemia.

MATERIALS AND METHODS: To evaluate the role of miR-28, we assessed cellular apoptosis. In addition, the regulation of ALDH2 by miR-199b was evaluated by Western blotting and luciferase assay.

RESULTS: MiR-28 was up-regulated, while ALDH2 expression decreased in a time-dependent manner under normoxic conditions. The miR-28-transfected cells showed a significant decrease in the cellular apoptosis. Compared with the negative control 1 precursor molecules, miR-28 over-expression caused about 55% increase in myocardial apoptosis under hypoxic conditions, and miR-28 silencing by anti-miR-28 attenuated a 41% decreasing in apoptosis. MiR-28 and pGL3-ALDH2 vector-transfected cells showed that ALDH2 protein expression was suppressed and luciferase activity was reduced.

CONCLUSIONS: These findings suggest that miR-28 promotes myocardial ischemia through the inhibition of ALDH2 expression in mus. miRNAs is as a probable index in identification of myocardial ischemia after acute myocardial infarction.

Key Words:
MicroRNA, aldh2, Cellular apoptosis, Cardiac ischemia.

Introduction

Acute myocardial infarction (AMI) with subsequent left ventricular dysfunction and heart failure is a major cause of morbidity and mortality in the Western world[1]. Clinical interventions such as angioplasty or thrombolytic agents have been effective in disrupting the occlusion and re-establishing the coronary flow. However, these treatments to reduce the injuries during the ischaemic period or reperfusion are not available. A large number of studies have confirmed that mitochondrial enzyme aldehyde dehydrogenase 2 (ALDH2) is a crucial enzyme involved in protecting the heart from ischemic by mediating both the detoxification of reactive aldehydes such as acetaldehyde and 4-hydroxy-2-nonenal (4-HNE) and the bioactivation of nitroglycerin (GTN)[2,3].

Over-expression of ALDH2 wild-type enzymes can confer multiple beneficial effects to the heart tissue and cardiac functions. ALDH2 plays a role in protecting the heart from aldehyde toxicity and against myocardial damages caused by acute ethanol toxicity[4,5]. Moreover, ALDH2 knockouted transgenic mice lacked any detectable ALDH2 enzyme activity and thus accumulated a high level of acetaldehyde after ethanol gavages[6,7]. So it was significantly more sensitive to alcohol and acetaldehyde-induced toxicity and damage than wild type mice[6,7]. Previous experimental and clinical investigations have uncovered that ethanol consumption and acetaldehyde inhalation are higher risk to ALDH2-inactive humans than those with

Corresponding Author: Chuanxi Wang, MD; e-mail: chuanxiwang@126.com
Yingchun Zhou, MD; e-mail: ychchou@126.com

functional ALDH2. ALDH2-deficient humans who habitually consume alcohol have a high cancer risk[8-10]. However, the actual regulatory mechanism of ALDH2 wild-type gene is not clear.

MicroRNAs (miRNAs) inhibit translation or induce mRNA degradation in general by binding to the complementary sequences in 3'-untranslated regions (3'-UTR) of target mRNAs[11]. Whether miRNAs are involved in this ALDH2 gene regulation is unclear. Many of the protein-coding genes regulated by miRNAs are presently not defined. And bioinformatic approaches may help to recognize them. A search for miRNAs regulatory targets by bioinformatic tools led to the identification of ALDH2 as a possible target of mmu-miR-28 (miR-28). But miR-28 regulation ALDH2 expression has not yet been reported in mus musculus. This study was to investigate the role of miR-28 in the regulation of ALDH2 and to explore the mechanism of miR-28 promotes cardiac ischemia in mus musculus.

Materials and Methods

Cell lines and reagents

Mouse cardiac myocytes was established from the heart of mice 1-4 days after birth, human embryonic kidney cells-derived cell lines 293T (American Type Culture Collection (ATCC) number: CRL-11268), were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and penicillin/streptomycin (Sigma, St Louis, MO, USA)). All cells were cultured at 37°C in a humidified chamber supplemented with 5% CO_2. Cells were grown under hypoxic (1% O_2) or normoxic (21% O_2) conditions at 37°C/5% CO_2. Mmu-miR-28, anti-mmu-miR-28, and the negative control were purchased from Ambion (Applied Biosystems, Foster City, CA, USA). Anti-ALDH2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MiRNA Target Prediction

The analysis of potential mmu-miR-28 targets was performed by the algorithms TargetScan 5.1 (http://targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), and miRanda (http://microRNA.org). To identify the genes, we predicted by the 3 different common algorithms. Results of the forecast targets were intersected by miRGen (http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html) website tools[12].

Vector construction

Mus musculus mitochondrial aldehyde dehydrogenase 2 (ALDH2) 3'UTR target site was amplified by PCR using the primers 5'-TCTAGAGCTTTTTCTTAATTTCATT-3' (forward) and 5'-TCTAGATTTTAAAATGATGCTACTGC-3' (reverse). DNA products were then cloned into pUC18 (TaKaRa). After verification by DNA sequencing, the ALDH2 3'UTR was digested by *Xba* I and *Xba* I, then cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA) which was with the same restriction enzyme. This construct, named pGL3-ALDH2, was used to transfect for 293T cell lines. To make mmu-miR-28 binding site delete, we used the two-step PCR approach where the seed sequences were deleted in the primers used for PCR reactions.

Transfection with mmu-miR-28

The day before transfection, 293T cells were seeded in antibiotic-free medium. Transfection was carried out using 100 nM of mmu-miR-28, anti-mmu-miR-28 and negative control 1 precursor miRNAs using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, Carlsbad, CA, USA). The level of mmu-miR-28 expression in transfected 293T cell line was assessed by real-time RT-PCR (Taqman MicroRNA Assays, Foster City, CA, USA) 24 h after transfection as described above.

Luciferase Activity Assay

293T cells were transfected with 0.1 μg of pGL3-ALDH2 along with 0.01 μg of pRL-TK vector (Promega) containing *Renilla* luciferase and 30 pmol of mmu-miR-28 or negative control 1 or anti-mmu-miR-28 oligonucleotides, and split into 12-well plates in duplicates. After 24 h, the cells were lysed. And luciferase activity was measured by the assay kit from Promega. pGL3 was cotransfected and used for normalization. Each transfection was repeated twice in triplicate.

Real-time RT-PCR analysis of miRNA and mRNA

Total RNA was isolated by Trizol reagent (Invitrogen). The detection of expression levels of mature mmu-miR-28 through the stem-loop method was performed by the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA), as described[13]. Relative expression levels of mmu-miR-28 were calculated by the

Δ*CT* method and normalized with internal control *U*6 RNA (Applied Biosystems). To detect relative levels of ALDH2 mRNA, Taqman real-time qRT-PCR (Invitrogen) was performed by Cyber Green method. For normalization, we used β-actin. PCR primers were ALDH2 sense, 5'-CCCCTCACCCAACGAAAAATTAC-3', and ALDH2 antisense, primer 5'-GATCCT-GAATCTGGGGCATGG-3'. All qRT-PCRs were performed in triplicate, and the data are presented as means ± standard errors of the means (SEM).

Western Blot

Mouse cardiac myocytes and 293T cells were transfected with 100 pmol of mmu-miR-28 or negative control 1 in 6-well plates. After transfection, cells were cultured at 37°C. And intermediate samples at 24 and 48 h were collected and analyzed by Western blot to assess ALDH2 expression. The cells were incubated overnight with polyclonal antibody against anti-ALDH2 alpha (BD Biosciences, San Jose, CA, USA) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. And they were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP). The signals were detected by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). Digital images of auto-radiographs were acquired by Fluor-S MultiImager, and band signals were quantified using a Quantity-one build-in specific densitometric algorithm (Quantity-one; Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). Values were expressed as the mean ± SEM. Differences/correlations between groups were calculated by Student's τ test. $p < 0.05$ was considered to be statistically significant.

Results

miR-28 promotes cardiac ischemia

To investigate whether miR-28 promote myocardial ischemia in musculus, the effect of acceleration of miR-28 on myocardial damage was examined in vitro. We found that the miR-28 expression of cells increased, while ALDH2 expression reduced in a time-dependent manner under normoxic conditions (Figure 1A-C). The miR-28-transfected cells showed a significant decrease in the cellular apoptosis. Compared with the negative control 1 precursor molecules, miR-28 silencing by anti-miR-28 attenuated about 41% myocardial apoptosis under hypoxic conditions ($p = 0.017$, *t*-test). miR-28 over-expression caused a 55% increasing in apoptosis. (Figure 1D). In comparison with negative control 1 precursor miRNAs, transfection of miR-28 into cardiomyocytes caused a 54% decrease of ALDH2 protein levels (Figure 1E). This finding suggests that ALDH2 is downregulated by miR-28 in cardiac cells.

ALDH2 is a Target of miR-28

The role of miR-28 in promoting myocardial ischemia was unclear. To identify the potential role of miR-28 in myocardial cells, we identified putative target genes by a bioinformatic analysis: microRNA, TargetScan, and PicTar algorithms. At least 2 of the algorithms showed that ALDH2 was a potential mmu-miR-28 target gene (Figure 2A, B).

Mouse myocardial cells were used to determine whether miR-28 down-regulated ALDH2 expression. In comparison with negative control 1 precursor miRNAs, transfection of miR-28 into myocardial cells caused a 53% decrease in ALDH2 protein levels. On the contrary, anti-miR-28-transfected cells exhibited a 1.8-fold increase in ALDH2 protein levels, compared with negative control 1 miRNA inhibitors (Figure 2C). This finding suggests that ALDH2 is down-regulated by miR-28 in mouse myocardial cells.

MiR-28 Interacts with 3'UTR of ALDH2 mRNA

To determine whether the 3'untranslated region of ALDH2 mRNA was a functional target of miR-28, we cloned a 106-bp ALDH2 3'-UTR, which includes a potential target site for miR-28, into the downstream of the luciferase reporter gene to generate the pGL3-ALDH2 vector. This vector was cotransfected into 293T cells along with miR-28 or negative control 1. A *Renilla* luciferase vector (pRL-TK) was used to normalize the differences in the transfection efficiency. As is shown in Figure 3, miR-28 suppressed the luciferase activity in 293T cells by 60% compared with the negative control ($p < 0.001$, *t*-test). So, immunoassay and luciferase results provided strong indications that ALDH2 is a target of miR-28 in mouse myocardial cells.

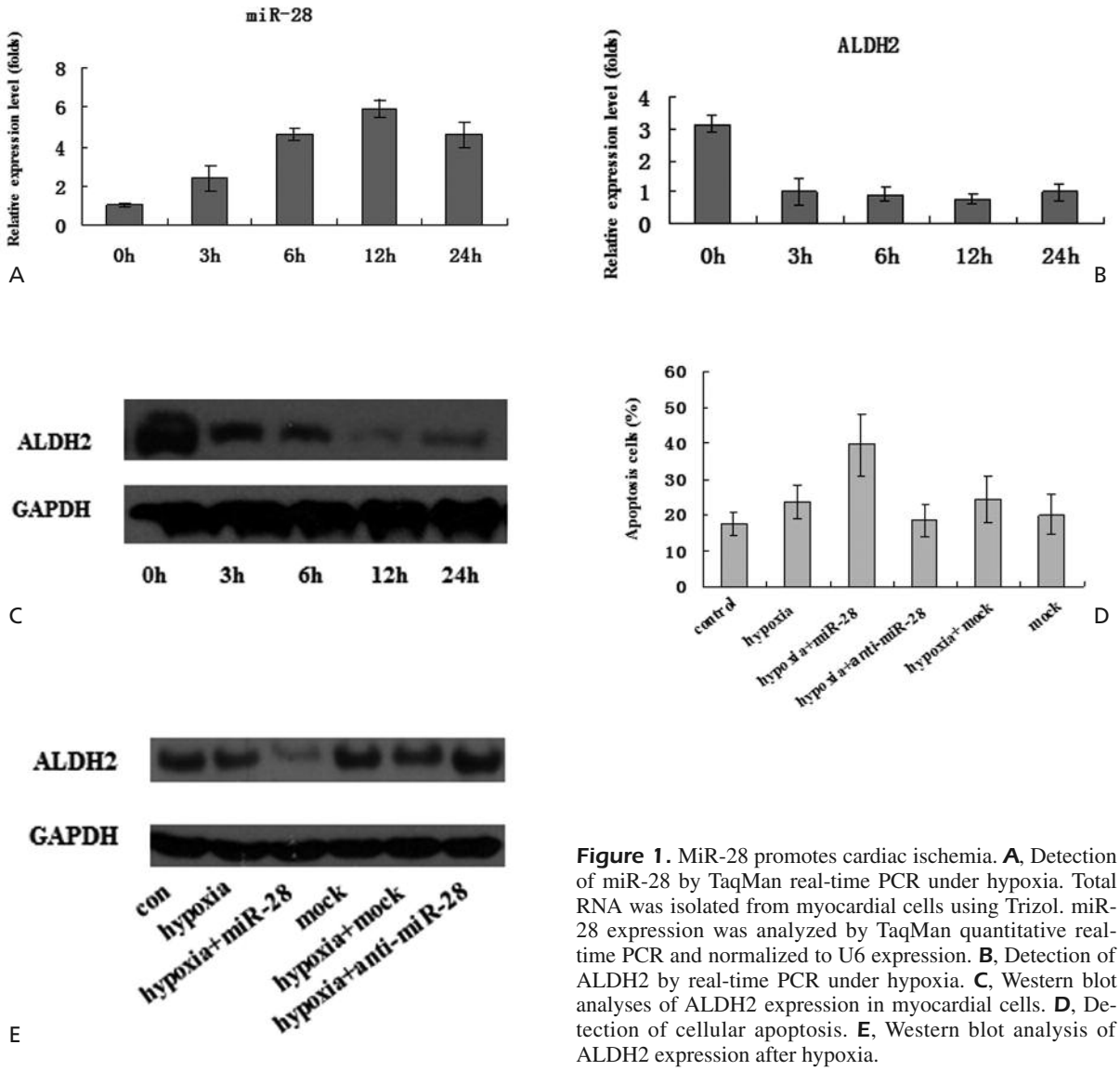

Figure 1. MiR-28 promotes cardiac ischemia. ***A***, Detection of miR-28 by TaqMan real-time PCR under hypoxia. Total RNA was isolated from myocardial cells using Trizol. miR-28 expression was analyzed by TaqMan quantitative real-time PCR and normalized to U6 expression. **B**, Detection of ALDH2 by real-time PCR under hypoxia. **C**, Western blot analyses of ALDH2 expression in myocardial cells. **D**, Detection of cellular apoptosis. **E**, Western blot analysis of ALDH2 expression after hypoxia.

Discussion

Cardiac ischemia is a major cause of cardiac diseases-related death. Hence, identifying the role of miRNAs in promoting cardiac ischemia has direct clinical implications. Although it is now known that miRNAs might have a key role in diverse cellular processes including differentiation, proliferation and apoptosis[14], the underlying mechanism in cardiac ischemia is largely unknown. We show here that the over-expression of mus-miR-28 promotes myocardial ischemic damage in vitro, and identify ALDH2 as a direct functional target for miR-28 through interacting with their 3'UTR. More importantly, ALDH2 is expressed in mitochondrial but barely detectable in the blood. It is known to us, miRNAs expression can very easily and quickly detected in tissues and serum[15,16]. Therefore, we hypothesis that miRNAs seems to be as a probable index in the identification of cardiac ischemia after acute myocardial infarction. Similar to our results, Nana Sinkan and Croce[17] found the potential of miRNA biology for both the molecular pathogenesis of cancer and the inherent complexities in translating its biology to clinics. We are recognizing the potential for miRNA biology to clarify both the molecular pathogenesis of cancer and the inherent complexities. An increasing evidence has indeed proved the importance of miRNAs in cancer, suggesting their possible use as diagnostic biomarkers and miRNA-based anticancer therapies[18].

Target gene	microRNA prediction	Predicted consequential pairing of target region(top) and miRNA(bottom)	mirSVR score
ALDH2	mmu-miR-28 Position of ALDH2 3' UTR	3' gaguuaucugacacUCGAGGAa 5' mmu-miR-28 145:5' cuggugggggggaaAGCUCCUg 3' Aldh2	-0.1755

Target gene	microRNA prediction	Predicted consequential pairing of target region(top) and miRNA(bottom)	seed match
ALDH2	Position 159-165 of Aldh2 3' UTR mmu-miR-28	5' ...GCUGGUGGGGGGGAAAGCUCCUG... 3' GAGUUAUCUGACACUCGAGGAA	7mer-m8

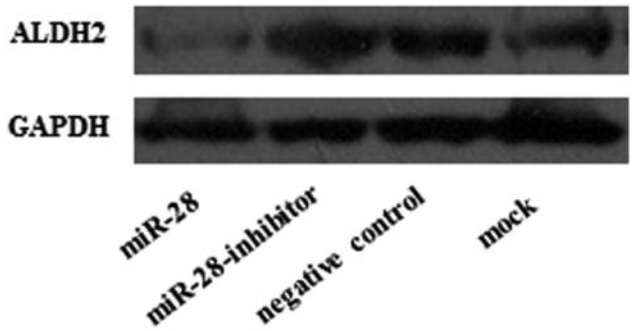

Figure 2. ALDH2 is a target of miR-28. **A**, Putative binding site of miR-28 in ALDH2 3′-UTR region as detected by microRNA. **B**, Putative binding site of miR-28 in ALDH2 3′-UTR region as detected by TargetScan. **C**, Western blot analysis of ALDH2 expression after miR-28 transfection. Lane 1, myocardial cells treated with miR-28; lane 2, cells treated with anti-miR-28; lane 3, cells treated with negative control; lane 4, mock.

The mitochondrial ALDH2 is one of 19 members of the ALDH gene family, which plays a crucial role in the oxidation and detoxification of reactive aldehydes involved in protecting the heart from ischemic injury[4,19]. ALDH2 is a key metabolic enzyme in catalyzing the oxidation of acetaldehyde to acetic acid in ethanol metabolism and detoxification of other reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE). In addition to its dehydrogenase activity, ALDH2 has an esterase activity that catalyses the conversion of nitroglycerin (glyceryl trinitrate, GTN) to 1,2 glyceryl dinitrate (1,2-GDN), and thus mediates bioactivation of GTN. Activation of ALDH2 could significantly reduce ischemia/reperfusion damage by attenuating cardiac aldehydes, creatine kinase isoenzyme MB and protein carbonyl formation. In addition, ALDH2 also alleviated ischemia/reperfusion-induced myocardial contractile function impairment by improving maximal velocity of pressure development and decline, left ventricular developed pressure and heart rate[20]. On the contrary, a functional ALDH2 decrease causes constitutive myocardial apoptosis and injury[21]. Therefore, ALDH2 down-regulation by miR-28 may contribute to ischemia/reperfusion (I/R) injury. To gain further insight into whether miR-28 promotes cardiac ischemia, we transferred miR-28 precursor or anti- miR-28 inhibitor into musculus myocardial cells. The data in this study shows that the miR-28 expression increased, while ALDH2 expression reduced in a time-dependent manner under normoxic conditions. The miR-28-transfected myocardial cells showed a significant decrease in the cellular apoptosis. Compared with the negative control 1 precursor molecules, miR-28 over-expression increased

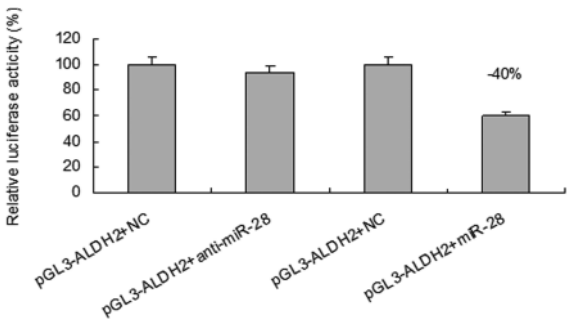

Figure 3. ALDH2 3′-UTR regulates luciferase activity depending on the presence of miR-28. Expression of the firefly luciferase reporter activity is significantly reduced when pGL3-ALDH2 vector, containing part of the 3′-UTR of the ALDH2 gene, is cotransfected together with miR-28 ($p < 0.001$ versus negative control 1, t test). Firefly luciferase activity was normalized on Renilla luciferase activity of the cotransfected pRL-TK vector.

about 55% myocardial apoptosis under hypoxic condition. On the contrary, the effect of cardiac ischemia was almost abolished by anti-miR-28. The myocardial protective effect of ALDH2 against ischaemia/reperfusion injury possibly through detoxification of toxic aldehyde and the differential regulation of autophagy through AMPK- and Akt-mTOR signaling during ischaemia and reperfusion were previously reported in knockout mice[22]. In this article, we describe miR-28 as a direct regulator of ALDH2 expression in myocardial cells, and show a new mechanism responsible for ALDH2 regulation. Our data suggest that the myocardial damaging effect of miR-28 is involved in miR-28- ALDH2 signal during ischaemia.

Conclusions

Our results suggest that miR-28 promotes myocardial ischemia through the inhibition of ALDH2 expression in mus. Our results also indicate that miRNAs is as a probable index in the identification of myocardial ischemia after acute myocardial infarction.

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Conflict of interest

The Authors declare that there are no conflicts of interest.

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