Original Article
The upregulation of miR-101 promotes vascular endothelial cell apoptosis and suppresses cell migration in acute coronary syndrome by targeting CDH5

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Abstract: MicroRNAs (miRNAs) have been reported to be of great importance in a wide range of physiological and pathological processes, including acute coronary syndrome (ACS). However, the exact role of miRNAs in the pathogenesis of ACS has not been fully elucidated. In this study, we found that miR-101 was significantly upregulated in the serum samples of patients with acute coronary syndrome compared with healthy controls. In human umbilical vein endothelial cells (HUVECs), the overexpression of miR-101 drastically promoted cell apoptosis and inhibited cell migration. Mechanistically, miR-101 repressed the expression of CHD5 by targeting its 3'-untranslated region (3'UTR). The silencing of CHD5 also induced cell apoptosis and suppressed cell migration in HUVECs. Taken together, our findings suggest that the miR-101-CHD5 axis may play an important role in the biological behaviors of endothelial cells during the pathogenesis of ACS and may afford an effective diagnostic marker and a powerful therapy for this disease.

Keywords: Acute coronary syndrome, miR-101, endothelial cells, apoptosis, migration

Introduction
Acute coronary syndrome (ACS), a life-threatening disease and one of the leading causes of cardiovascular death and disability around the world, is mainly caused by decreased blood flow in the coronary arteries, atherosclerotic plaque rupture, plaque erosion, and nodule calcification [1, 2]. The incidence of ACS has risen over the past few decades, especially in many developing areas. Usually, the risk factors for ACS involve obesity, hypertension, depression, hypercholesterolemia, diabetes, and smoking [3]. Although significant advances in the clinical approach have been found for its treatment and prevention, coronary artery disease is still a major cause of mortality and morbidity in many developing countries. Thus, novel therapeutic strategies are urgently needed to be found to improve clinical outcomes in ACS patients.

The function of vascular endothelial cells (VECs) is frequently impaired in ACS, resulting in plaque erosion and thrombosis formation, which is considered to be a key event for the formation and progression of ACS. Coronary endothelial dysfunction seems to be found in most patients with ACS; however, it’s very difficult to interpret the exact role of VEC dysfunction in the formation and progression of ACS.

MicroRNAs (miRNAs) are a class of endogenous, small (22-24 nt) non-coding RNA molecules that decrease the expression of target genes by combining with the 3'-untranslated region (3'UTR), subsequently triggering the degradation of mRNA or the suppression of translation [4, 5]. Recently, a growing number of studies have shown that miRNAs can regulate the biological behaviors of endothelial cells, inflammation, platelet activation, and aggregation. Moreover, miRNA deregulation influences the initiation, progression, and rupture of atherosclerotic plaques. In addition [6, 7], miRNAs can be secreted into the extracellular matrix, and the blood and urine, which is termed circulatory miRNAs. Circulatory miRNAs can be used as...
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biomarkers for the diagnosis, treatment, and prevention of diseases, including coronary heart disease. However, it is still unclear whether miRNAs can be used as stable biomarkers for ACS and can further evaluate the severity of ACS [8].

Using a high-throughput screening technique, a recent study found that circulating miR-101 is upregulated in young patients with acute coronary syndrome. In this study, we found that miR-101 was clearly upregulated in the serum samples of patients with ACS compared with healthy controls. In HUVECs, the overexpression of miR-101 significantly promoted cell apoptosis and inhibited cell migration. Mechanistically, CDH5 is a direct target of miR-101, and miR-101 repressed the expression of CHD5 via targeting its 3'UTR. CHD5 knock-down also induced cell apoptosis and suppressed cell migration in HUVECs. Our results demonstrate that the miR-101-CHD5 axis may play a critical role in the biological behaviors of endothelial cells during the formation and progression of ACS and may afford an effective diagnostic marker and therapeutic target for ACS treatment.

Materials and methods

Clinical samples

This study recruited adult patients at the Tangshan Workers' Hospital in accordance with the approved guidelines of the Medical Ethics Committee. Informed consent was obtained from all the patients and healthy controls. 14 ACS male patients below 45 years of age, clinically diagnosed with either NSTEMI or STEMI, and 13 gender- and age-matched control subjects were enrolled in this study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

After the sample collection, total RNA from the sera was isolated using the TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). RT-qPCR was performed through two-step reactions. Each RT reaction consisted of 100 ng of RNA, 4 µl of 5X First-Strand Buffer, 2 µl of dNTP Mix (2.5 mM each), 2 µl of DTT (100 mM), 2.5 µl of specific RT primer (20 µM), and 0.5 µl of SMART MMLV Reverse Transcriptase (Takara, Beijing, China), in a total volume of 20 µl. The reactions were performed at 42°C for 60 min, followed by the termination of the reaction by heating at 70°C for 15 min. Real-time PCR was performed using a ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with 20 µl of the reaction mixture including 2 µl of cDNA, 10 µl of 2 × TB Green Premix Ex Taq (Takara), 1 µl of universal forward primers (20 µM), 1 µl of miRNA-specific reverse primer (20 µM), and 6 µl of nuclease-free water. Reactions were incubated in a 96-well optical plate (ABI) at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction was performed in triplicate. The expression of U6 was used as an internal control. The expression levels of the miRNAs were calculated by the 2−ΔΔCt method. The primers used are as follows: Hsa-miR-101 RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAGT; Universal forward: GTGCAAGGGTCCAG; Hsa-miR-101-specific reverse: CTGAAGTCGTATCCAGTGCG. U6-forward: CTCGCTTCGGCAGCACA; U6-reverse and RT: AACGCTTCACGAATTTCGGT.

Western blot analysis

The cells were lysed using the RIPA Lysis Buffer (Beyotime, Shanghai, China) for 15 min at 4°C, and the protein concentration was then determined using an Enhanced BCA Protein Assay Kit (Beyotime). Twenty µg of protein were separated by SDS-PAGE (8% gels) and blotted onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked with 5% non-fat dry milk in TBS at room temperature for 1 h and then incubated with primary antibodies of CDH5 (Abcam, Cambridge, UK; ab232880; 1:1000), BAX (Abcam; ab77566; 1:1000), BAD (Abcam; ab32445; 1:1000), BCL2 (Abcam; ab15184; 1:1000), BCL2 (Abcam; ab196495; 1:1000) and β-ACTIN (Cell Signaling Technology, MA, USA; 4970; 1:5000) overnight at 4°C. The membranes were exposed to anti-rabbit horseradish peroxidase-conjugated secondary antibody (7074; 1:8000, Cell Signaling Technology) for 1 h at 37°C, following 3 washes with TBST for 15 min each. The membranes were detected using the BeyoECL Plus reagent (Beyotime) and quantified by using the ImageJ analysis software (NIH, MD, USA).

Apoptosis assay

Apoptosis assays were carried out using the Annexin V-FITC Apoptosis Detection Kit I (BD
Pharmingen, San Diego, CA, USA) following the manufacturer’s instructions. Briefly, 72 h after transfection, the cells were collected and stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI), followed by flow cytometry analysis in a FACS Canto II machine (BD Biosciences). The apoptotic cells were analyzed by the FlowJo software 7.6.1 (Tree Star Inc, Ashland, OR, USA).

Transwell migration assay

The treated cells (2.5 × 10⁴ cells) were suspended into 200 μL of fresh culture medium without FBS and plated in the top chamber of a 24-well insert (Corning, NY, USA, pore size: 8 μm). A fresh culture medium with 10% FBS was added into the lower chamber. After 16 h of incubation, the migrated cells were stained with 20% methanol and 0.2% crystal violet, and then six photographs of each sample were taken randomly. The stained crystal violet was resolved with 200 μl of 0.05 mM sodium citrate and 0.05 mM citric acid in 50% ethanol and measured at OD570 using an ELISA reader (Molecular Devices, Sunnyvale CA, USA).

Wound-healing assay

The stably transfected cells (1 × 10⁶ per well) were trypsinized and seeded in 24-well plates, and then cultured overnight in the complete medium. The monolayers of cells were scratched using a 200-μl pipette tip. The cells were then washed with the culture medium to remove cellular debris and allowed to culture again up to 24 h in the serum-free medium. The cell migration areas were recorded by microscope every 8 hours (0, 8, and 16 hours).

Dual-luciferase reporter assay

The potential binding site of miR-101 with CDH5 was predicted by the bioinformatics software database TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/). The 3’UTR of CDH5 containing a miR-101 binding site was amplified and cloned into the reporter plasmid pSRII-Check-2 (Promega, Fitchburg, WI, USA). The mutant-type dual-luciferase vector (CDH5-3’UTR MUT) was constructed by using the Site-Directed Mutagenesis System (Beyotime) according to the manufacturer’s instructions. The primers used were as follows: CDH5-3’UTR-WT-forward: 5’-GCGCGATCGCGCGCAGGTCACCT-3’; CDH5-3’UTR-MUT-forward: 5’-GCTCTATATTGCACCCACAGCTGGTCTAGACAGGAAATTTTTTATAC-3’; CDH5-3’UTR-MUT-reverse: GCTATCAAAATTATCATCTTGTATGACACAGTTTGCCGCAATATATAGCA. The luciferase reporter assay was performed as previously described [9].

Quantifying the plasma levels of CRP, IL6, and MCP-1

The plasma levels of CRP (Abcam; ab99995), IL6 (Abcam; ab178013) and MCP-1 (Abcam; ab100586) were measured by the ELISA kits according to the manufacturer’s instructions.

Statistical analysis

All statistical analyses were performed by using SPSS 17.0 (IBM Corporation, Armonk, NY, USA). The differences were analyzed by using the student’s t-test or a one-way ANOVA. A P-value < 0.05 was considered to be statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).

Results

miR-101 is upregulated in the serum samples of patients with ACS

Previous studies have shown that circulating miRNA expression profiles are significantly altered in patients with ACS, including miR-101. To determine the expression of miR-101 in the serum samples of patients with ACS, qRT-PCR was performed. The results showed that circulating miR-101 expression was significantly upregulated in patients with ACS compared with the control groups (P = 0.0004; Figure 1A). Consistently, the concentrations of the ACS-associated cytokines MCP-1 (P = 0.0043, Figure 1B), CRP (P = 0.0153, Figure 1C) and IL6 (P = 0.0153, Figure 1D) were also significantly elevated compared with the controls.

Upregulation of miR-101 contributes to vascular endothelial cell apoptosis

As mentioned above, the dysfunction of vascular endothelial cells plays a critical role in ACS. Thus, to further evaluate the effects of miR-101 on the development of ACS in vitro, we next examined the role of miR-101 in the biological behaviors of vascular endothelial cells. HUVECs were transfected with an miR-101 mimic or a
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Figure 1. The expressions of miR-101, MCP-1, CRP, and IL6 are significantly upregulated in the serum samples derived from patients with ACS. (A) The expression of circulating miR-101 in patients with ACS (n = 14) compared with the healthy controls (n = 13). (B-D) ELSIA analyses were performed to determine the protein levels of MCP-1 (B), CRP (C), and IL6 (D) in the serum samples derived from patients with CAD compared with the healthy controls. All the data were statistically analyzed using Student’s t-test.

matched control. As a result, the miR-101 mimic transfection significantly increased the expression of miR-101 in HUVECs (Figure 2A and 2B). Subsequently, Annexin V and PI double staining assays revealed that cell apoptosis was significantly facilitated by the ectopic overexpression of miR-101 in HUVECs (Figure 2C). The apoptosis assay showed that HUVECs transfected with the miR-101 mimic had a higher percentage of apoptotic cells both in the early phase (Figure 2D) and the late phase (Figure 2E) compared to the control group. Consistent with the flow cytometry analysis, the Western blot analysis also demonstrated that the pro-apoptotic genes BAX, BAD, and BIM were significantly enhanced by miR-101 overexpression, but the anti-apoptotic gene BCL2 was attenuated by the miR-101 overexpression (Figure 2F-H). The above results indicate that miR-101 overexpression promotes apoptosis in HUVECs.

CDH5 is a direct target of miR-101 in HUVECs

To further characterize the biological roles of miR-101 in HUVECs, we performed a transwell assay to evaluate the migration ability of HUVECs transfected with the miR-101 mimic compared with the control. The data demonstrated that the overexpression of miR-101 drastically decreased the cellular migration ability in HUVECs (Figure 3A and 3B). Meanwhile, the influence of miR-101 on HUVEC migration was also assessed by a wound healing assay and a similar result was obtained (Figure 3C and 3D). The results of both of the experiments revealed that high miR-101 expression could suppress the motility and migration of HUVECs.

Silence of CDH5 drastically promoted cell apoptosis and inhibited cell migration in HUVECs

The aforementioned results demonstrated that there is an association between miR-101 and CDH5 in HUVECs. Thus, it was hypothesized...
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Figure 2. The overexpression of miR-101 induced cell apoptosis in HUVECs. (A, B) Semi-quantitative RT-PCR (A) and quantitative RT-PCR (B) to determine the expression of HUVECs transfected with the miR-101 mimic compared with the control. (C-E) Annexin V-FITC/PI staining to evaluate the apoptotic rates of HUVECs transfected with miR-101 mimic compared with the control. (F-H) Western blots to determine the protein expression levels of BAX, BAD, BIM and BCL2 in HUVECs transfected with the miR-101 mimic compared with the control. All the data were obtained from three independent experiments and statistically analyzed by one-way ANOVA. **P < 0.01. ***P < 0.001.

that CDH5 may neutralize the effects of miR-101 on HUVECs. A series of experiments was performed to confirm that CDH5 affects cell apoptosis and migration in HUVECs. These results demonstrated that transfection with CDH5 siRNA (Figure 5A and 5B) promoted cell apoptosis (Figure 5C and 5D) and inhibited cell migration (Figure 5E and 5F) in HUVECs.

Discussion

Cardiovascular events are the leading cause of death and about 20% of these deaths occur from ACS, meaning that ACS is the first cause of human mortality and morbidity all over the world [10]. A growing number of studies have shown that the progression of ACS is a complex, multiple-step process. Endothelial cell dysfunction, which may be affected by endothelial cell proliferation, apoptosis, invasion and migration, is regarded as a major contributor to the pathogenesis of ACS [11]. Up to now, the precise mechanism underlying the pathogenesis of ACS still remains largely unknown. In the present study, we demonstrated the pro-apoptotic and anti-migrated effects of miR-101 in HUVECs. Mechanistically, the effect of miR-101 was mediated through the inhibition of CDH5. Therefore, the miR-101/CDH5 axis may be a novel target for the prevention and treatment of ACS.

Recently, miRNAs are attracting more attention due to their important regulatory roles in the progression of ACS. MiRNAs modulate endothelial functions during the development of ACS. For instance, deregulated expressions of circulating miRNAs such as miR-499 [12], miR-486-3p [13], miR-210 [14], miR-197 [15], and miR-223 [15] have been established in patients with ACS [16], and their roles in the regulation...
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Figure 3. The overexpression of mir-101 suppressed cell migration in HUVECs. A, B. The overexpression of mir-101 inhibited migration in HUVECs transfected with a mir-101 mimic compared with the control, demonstrated by a transwell migration assay. C, D. Determination of the cell mobility of HUVECs transfected with the mir-101 mimic compared with the control, determined by a wound healing assay. The data was calculated in triplicate and are shown as the means ± SEM. Statistical significance, determined by a one-way ANOVA, was indicated by *P < 0.05, **P < 0.01.

Figure 4. CDH5 is a direct target of mir-101 in HUVECs. (A) Schematic view to present a putative mir-101 binding site (wild type and mutant) in the 3'UTR of CDH5. (B) Luciferase reporter assay to assess the effect of mir-101 on CDH5 expression. Data are shown as the means ± SEM (n = 6). Statistical significance, determined by Student’s t-test, was indicated by *P < 0.05, **P < 0.01. (C and D) The overexpression of mir-101 suppressed the expression of CDH5 in HUVECs, as determined by quantitative RT-PCR (C) and western blot (D). Data were shown as the means ± SEM (n = 3). Statistical significance, determined by Student’s t-test, was indicated by **P < 0.01.
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of endothelial function, differentiation of vascular smooth muscle cells, and plaque stabilization leading to the development of these diseases are documented. The objective of our study was to validate the role of miR-101 in patients with acute coronary syndrome and its underlying mechanisms. Our findings demonstrate serum miR-101 could serve as a powerful biomarker of ACS as serum miRNA is stable and easy to measure. Substantial studies demonstrate that miR-101 plays an important role in cardiovascular diseases. For example, Cheng et al. have demonstrated that Geniposide regulates the miR-101/MKP-1/p38 pathway and alleviates atherosclerosis inflammatory injury in ApoE−/− mice [17]. miR-101-3p serves as a therapeutic target that limits endothelial cell death associated with vascular disorders [18]. Dong et al. have shown that the down-regulation of miR-101 contributes to rheumatic heart disease by up-regulating TLR2 [19].

Endothelial cell-cell junctions, comprising the vascular endothelial (VE)-cadherin (CDH5)-catenin complex, function to maintain the monolayer integrity [20]. VE-cadherin (CDH5)-based cell-cell junctions are dynamic and remodeled during several biological processes such as angiogenesis and homeostasis [21]. In this study, CDH5 was identified as a direct target of miR-101 in HUVECs. We propose that upregulation of miR-101 contributes to a decrease of

Figure 5. The silencing of CDH5 promotes cell apoptosis and suppresses cell migration in HUVECs. (A, B) Quantitative RT-PCR (A) and western blot (B) to determine the mRNA and protein levels of CDH5 in the HUVECs transfected with CDH5 siRNA and control siRNA, respectively. (C, D) The silencing of CDH5 elevated the apoptotic cells in HUVECs transfected with CDH5 siRNA compared with the control siRNA, demonstrated by Annexin V/PI double staining. (E, F) The silencing of CDH5 suppressed cell migration in HUVECs transfected with CDH5 siRNA compared with the control siRNA, as determined by the migration assay. The data was calculated in triplicate and shown as the means ± SEM. Statistical significance, determined by one-way ANOVA, was indicated by *P < 0.05, **P < 0.01.
CDH5 leading to the disruption of endothelial cell-cell junction.

Our study has some limitations. First, rescue experiments are lacking to validate the effect of CDH5 on the function of miR-101 in HUVECs. Secondly, no data about in vivo experiments was performed to elucidate the role of the miR-101/CDH5 axis in ACS.

Taken together, our findings suggest the miR-101-CHD5 axis may play an important role in the biological behaviors of endothelial cells in the pathogenesis of ACS and may afford an effective diagnosis marker and therapeutic marker for this disease.

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Disclosure of conflict of interest

None.

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