MicroRNA-663 participates in myocardial fibrosis through interaction with TGF-β1

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Abstract. MicroRNA-663 (miRNA-663) regulates the expression of transforming growth factor β1 (TGF-β1), which participates in the pathogenesis of myocardial fibrosis. Therefore, miRNA-663 may also serve a role in myocardial fibrosis. The present study aimed to determine whether miRNA-663 participates in myocardial fibrosis via interaction with TGF-β1. In the present study, the expression of miRNA-663 was significantly downregulated, whereas that of TGF-β1 was significantly upregulated in the endomyocardial biopsies of patients with myocardial fibrosis compared with those in control necropsies. Pearson's correlation analysis revealed that the expression levels of miRNA-663 were negatively correlated with those of TGF-β1 in patients with myocardial fibrosis, but not in the controls. Receiver operating characteristic analysis demonstrated that the downregulation of miRNA-663 distinguished patients with myocardial fibrosis from controls. In the AC16 human cardiomyocyte cell line, miRNA-663 overexpression resulted in downregulated TGF-β1 expression, whereas exogenous TGF-β1 treatment exhibited no significant effects on miRNA-663 expression. These results indicate that miRNA-663 may participate in myocardial fibrosis, possibly through interaction with TGF-β1.

Introduction

Cardiac fibrosis is a cardiac disorder induced by excess deposition of extracellular matrix in the cardiac muscle (1). This pathological change causes pressure or volume overload, which in turn leads to maladaptive cardiac remodeling (2). Maladaptive cardiac remodeling is usually followed by the development of MYOCARDIAL stiffness, which is characterized by wall thickening and increased left ventricular mass; once myocardial stiffness occurs, electrical conduction is impaired, and the risk of arrhythmias and heart failure is increased (3).

The occurrence and development of myocardial fibrosis requires the involvement of multiple signaling pathways (4). Transforming growth factor β1 (TGF-β1) serves an important role in fibroblast proliferation and collagen synthesis in the myocardium, which contributes to the development of myocardial fibrosis (5). Therefore, activation of TGF-β1 signaling is frequently observed in patients with myocardial fibrosis (5). A growing body of literature has demonstrated that inhibition of TGF-β1 may serve as a potential therapeutic target for myocardial fibrosis (6-8). TGF-β1 achieves signal transduction not only through proteins, but also by interacting with non-coding RNAs, such as microRNAs (miRNAs) (9-11). miRNAs are a group of short non-coding RNAs with crucial functions in physiological and pathological processes (12). During the development of myocardial fibrosis, miRNAs directly target a large number of downstream target genes to participate in the development of myocardial fibrosis, and regulation of miRNA expression has exhibited potential in the clinical treatment of myocardial fibrosis (4). miRNA-663 directly targets TGF-β1 expression in multiple human diseases (13,14), but its involvement in myocardial fibrosis is currently unknown. The present study aimed to determine whether miRNA-663 participates in myocardial fibrosis through interaction with TGF-β1.

Materials and methods

Cell line and patients. AC16 human hybrid cardiomyocytes (cat. no. SCC109) were purchased from EMD Millipore. Cells were cultivated under in sodium bicarbonate buffered Medium 199 (Cellgro M-199, Mediatech, Inc; Corning) under the conditions of 37°C, 5% CO2 and 95% humidity. For TGF-β1 treatment, 104 cells cells were cultured in 2 ml sodium bicarbonate buffered Medium 199 containing 0, 10, 20 or 40 ng/ml TGF-β1 (Sigma-Aldrich; Merck KGaA) for 24 h prior to subsequent experiments.

Endomyocardial biopsies were obtained from 34 patients with myocardial fibrosis (patient group) admitted to the Second Hospital of Lanzhou University between January 2015 and May 2018. Necropsies were obtained from 19 individuals (control group) recently deceased following a car accident, in

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whom no cardiac lesions were observed. Inclusion criteria of the patient group: i) Patients diagnosed by endomyocardial biopsies; ii) patients willing to join the study. Exclusion criteria: i) Patients diagnosed with multiple diseases; ii) patients who received treatment within 90 days before admission. The patient group comprised 20 males and 14 females, and their age ranged from 30 to 67 years (mean age, 48.8±6.1 years). The control group comprised 11 males and 8 females, and their age ranged from 29 to 66 years (mean age, 48.0±5.6 years). This study was approved by the Ethics Committee of the Second Hospital of Lanzhou University. The subjects or their families signed informed consent. General information on the two groups of participants is presented in Table I.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA and miRNA were extracted from tissue and cells using a MPure™ Total RNA Extraction kit (cat. no. 117022160; MP Biomedicals, LLC) and miReasy Mini kit (cat. no. 217004; Qiagen China Co., Ltd.), respectively. Total RNA was reverse transcribed into cDNA using Tetro Reverse Transcriptase kit (Bioline Inc.) with the following conditions: 52°C for 20 min and 75°C for 10 min. PowerUp SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.) was used to prepare qPCR mixtures and PCR was performed using a 7500 Fast Real Time PCR System (Applied Biosystems) with β-actin or U6 as the endogenous control. Detection of miRNA-663 and U6 was performed using TaqMan assays (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 50 sec at 95°C, followed by 40 cycles of 12 sec at 95°C and 32 sec at 57°C. The primers used were: β-actin forward, 5'-GACACCACCTTCAATATG-3' and reverse, 5'-TGGTTGATCATCCACCTTG-3'; TGF-β1 forward, 5'-TACCATGCCAACTTCTGCTGGGA-3' and reverse, 5'-ATGTTTGACAACGTCTCCACCTTG-3'; miRNA-663a forward, 5'-AGCCGGGCGCGCAGGAGC-3'; miRNA-663a reverse primer and U6 primers were included in the kit. Data were processed using the 2^ΔΔCq method (15).

Cell transfection. MISSION® miRNA Mimic hsa-miR-663 (cat. no. HM0895) and MISSION® miRNA Negative Control 1 (cat. no. HM0002) were purchased from Sigma-Aldrich (Merck KGaA). Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) was used according to manufacturer’s instructions to transfect miRNAs into AC16 cells at a concentration of 15 nM. Untransfected cells were used as control cells. Cells transfected with MISSION® miRNA Negative Control 1 were negative control cells. An miRNA-663 overexpression rate of 200% was considered to indicate a successful transfection.

Western blotting. Total protein was extracted from successfully transfected AC16 cells using CelLytic™ MEM Protein Extraction kit (Sigma-Aldrich; Merck KGaA). Protein was quantified using a BCA kit (Sangon Biotech Co., Ltd.) followed by electrophoresis using 10% SDS-PAGE (30 µg per lane) and gel transfer (PVDF membranes). membranes were incubated with rabbit anti-human TGF-β1 (cat. no. ab92486; 1:1.200; Abcam), plasminogen activator inhibitor-1 (PAI-1; cat. no. ab28907; 1:1.200; Abcam), TIMP metalloproteinase inhibitor 1 (TIMP-1; cat. no. ab109125; 1:1.200; Abcam) and GAPDH (cat. no. ab9485; 1:1.200; Abcam) primary antibodies (4°C for 18 h), followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (cat. no. MBS435036; 1:1,200; MyBioSource, Inc.) goat secondary antibody (24°C for 2 h). Signals were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Densitometry was performed using Image J v1.46 software (National Institutes of Health). TGF-β1, PAI-1 and TIMP-1 expression was normalized to GAPDH.

Expression levels of miRNA-663 and TGF-β1 are altered in endomyocardial biopsies of patients with myocardial fibrosis. Expression levels of miRNA-663 and TGF-β1 mRNA in endomyocardial biopsies of patients with myocardial fibrosis and necropsies of control subjects were detected by RT-qPCR. Compared with the control group, miRNA-663 was significantly downregulated (P=0.0124; Fig. 1A), whereas TGF-β1 was significantly upregulated (P=0.0111; Fig. 1B) in endomyocardial biopsies of patients with myocardial fibrosis.

Table I. General information of the two groups of participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n=34)</th>
<th>Controls (n=19)</th>
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<tbody>
<tr>
<td>Sex (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>30-67</td>
<td>29-66</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>48.8±6.1</td>
<td>48.0±5.6</td>
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</table>

Expression of miRNA-663 is negatively correlated with TGF-β1 in the patient group, but not in the control group. Correlations between expression levels of miRNA-663 and TGF-β1 were analyzed by Pearson correlation coefficient. A
significant negative correlation between the expression levels of miRNA-663 and TGF-β1 was observed in the patient group (Fig. 3A). However, no correlation was observed between the expression levels of miRNA-663 and TGF-β1 in the control group (Fig. 3B).

Overexpression of miRNA-663 mediates the downregulation of TGF-β1 and myocardial fibrosis markers in AC16 cells. Cells of the AC16 human cardiomyocyte cell line were successfully transfected with miRNA-663 mimic (Fig. 4A). The expression of TGF-β1 was detected by western blotting; compared with the control and negative control groups, TGF-β1 protein expression was significantly reduced in AC16 cells transfected with miRNA-663 mimic (P=0.0123; Fig. 4B). By contrast, treatment with 10, 20 and 40 ng/ml TGF-β1 did not significantly change the expression of miRNA-663 in AC16 cells (Fig. 4C). In addition, overexpression of miRNA-663 led to the reduced expression of myocardial fibrosis markers PAI-1 (P=0.0292) and TIMP-1 (P=0.0189) (1) compared with the control and negative control groups (Fig. 5), which further supported the hypothesis that miRNA-663 exhibits inhibitory effects on myocardial fibrosis.

Discussion

miRNA-663 is a well-characterized tumor suppressor miRNA in various types of human malignancies, including glioblastoma, papillary thyroid carcinoma and lung cancer (13,14,16), whereas its involvement in other human diseases is largely unknown. The present study demonstrated that miRNA-663 may regulate the expression of TGF-β1 to participate in myocardial fibrosis. The development of myocardial fibrosis is accompanied by changes in expression levels of a large number of genes, including miRNAs (17). Altered expression levels of certain miRNAs may reflect the progression of myocardial fibrosis (18). As a tumor suppressor, miRNA-663 is downregulated in different types of cancer, and the downregulation of miRNA-663 promotes cancer cell proliferation and tumor growth (13,14,16). In the present study, significantly reduced expression of miRNA-663 was observed in patients with myocardial fibrosis compared with controls. The development of myocardial fibrosis involves the abnormal proliferation of cardiac fibroblasts (19); therefore, miRNA-663 downregulation may be involved in the dysregulation of the proliferation of cardiac fibroblasts. Further studies are required to test this possibility.
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The activation of TGF-β1 promotes fibroblast proliferation and collagen synthesis in the myocardium, which in turn contributes to the development of myocardial fibrosis (5). TGF-β1 is overexpressed in the majority of patients with myocardial fibrosis (20). Consistent with previous studies, the present study observed significantly increased TGF-β1 mRNA expression levels in patients with myocardial fibrosis compared with those in controls. Previous studies have demonstrated that the expression of TGF-β1 in human diseases can be regulated by miRNAs (9-11). In the present study, a significant reverse...
Correlation between TGF-β1 and miRNA-663 was observed in patients with myocardial fibrosis. The results of the in vitro experiments using the AC16 human cardiomyocyte cell line also demonstrated that TGF-β1 may act upstream of miRNA-663 as an inhibitor in cardiomyocytes due to the following observations: i) miRNA-663 reduced TGF-β1 expression; ii) exogenous TGF-β1 treatment failed to significantly alter the expression of miRNA-663. However, the expression levels of TGF-β1 and miRNA-663 were not significantly correlated in control groups. This is potentially due to the in vitro overexpression system not fully reflecting the in vivo conditions. In addition, disease-related factors may mediate the regulation of TGF-β1 expression by miRNA-663. Overexpression of miRNA-663 also led to the downregulated expression of myocardial fibrosis markers PAI-1 and TIMP-1, which suggests that miRNA-663 exhibits inhibitory effects on myocardial fibrosis.

In conclusion, miRNA-663 was significantly downregulated, whereas TGF-β1 was significantly upregulated in patients with myocardial fibrosis. miRNA-663 overexpression led to downregulated TGF-β1 expression in cardiomyocytes. Therefore, miRNA-663 overexpression may serve as a potential therapeutic target for myocardial fibrosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions


Competing interests

The authors declare that they have no competing interests.

References


