MiR-154 promotes myocardial fibrosis through β-catenin signaling pathway

P. DONG¹, W.-J. LIU¹, Z.-H. WANG²

¹Department of Cardiovascular Medicine, Beijing Aviation General Hospital, Beijing, China
²Department of Cardiology, Dalian Friendship Hospital, Dalian, China

Abstract. – OBJECTIVE: To discover the mechanisms of miR-154 affecting myocardial fibrosis.

PATIENTS AND METHODS: Human cardiac fibroblasts (CFs) were cultured in medium containing 10% serum for 48 h. The expression of miRNA-154 in human CFs was detected by Real-time quantitative polymerase chain reaction (qRT-PCR). The miRNA-154 mimics and inhibitors were synthesized and transfected into fibroblasts, respectively. Cell proliferation rate was determined by cell counting kit-8 (CCK8). Collagen I and collagen III, myofibroblast marker (a-SMA) and β-catenin were detected by Western blotting. Transwell migration assay was used to detect the changes of invasiveness of CFs. After the overexpression vector or siRNA of glycogen synthase kinase-3β (GSK-3β) was transfected into fibroblasts, we performed Western blot to detect a-SMA and β-catenin expression.

RESULTS: MiR-154 was overexpressed in cardiomyocytes, and when miR-154 was inhibited, the expression of collagen I, collagen III, a-SMA, β-catenin, and the invasiveness of CFs decreased. Therefore, we considered that miR-154 could promote myocardial fibrosis by inhibiting the expression of GSK-3β.

CONCLUSIONS: MiR-154 can inhibit GSK-3β expression by activating Wnt/β-catenin signaling pathway, which promotes myocardial fibrosis.

Key Words: Myocardial fibrosis, miRNA-154, GSK-3β, β-catenin.

Introduction

Abnormal deposition of collagen fibers in the myocardium leads to a marked increase in the concentration of collagen or an increase in the collagen volume fraction (CVF). The phenomenon of various disorders of collagens and unbalanced proportions is called heart myocardial fibrosis (MF). Many studies² show that MF is a basic disease of many diseases such as heart failure, hypertension, and myocardial infarction. MF is also one of the manifestations of myocardial tissue remodeling. The development of myocardial fibrosis is influenced by many factors including the immune system, cytokines along with RAAS system. To make it more complicated, different types of myocardial fibrosis may exert different pathological conditions and molecular mechanisms³⁴. The formation of MF is also regulated by many factors such as angiotensin, endothelin, nitric oxide, transforming factor, connective tissue growth factor and intracellular calcium ion⁵. Normally, adult cardiac fibroblasts (CFs) are in a relatively static state of equilibrium. Under the pathological conditions, the CFs phenotype is converted into myofibroblasts that are more capable of secreting extracellular matrix (ECM) to express a smooth muscle actin (a-SMA), leading to increased collagen synthesis and secretion. Therefore, in order to effectively prevent and treat myocardial fibrosis, it is necessary to further explore the possible regulatory mechanisms of phenotypic transformation of CFs for target therapy⁶⁷.

MicroRNA (miRNA) is a kind of non-coding small RNA that is relatively conservative in biological evolution. It plays an important regulatory role in the development of diseases by regulating gene expression, transcription and translation. Studies have shown that miRNAs can participate in and regulate the formation of fibrosis through a variety of mechanisms⁸. There’s increasing evidence that miRNA plays an important regulatory role in the development of cardiovascular diseases such as arrhythmia, atherosclerosis and heart failure. Overexpression of miRNA-29, miRNA-101, miRNA-133, etc. can promote fibrosis⁹. However, the biological function of miRNAs is rarely reported in studies of myocardial fibrosis.
Wnt signaling pathway is an important signal transduction pathway that can regulate diverse cellular processes including embryogenesis, cell proliferation, differentiation and metastasis. Many studies confirm that Wnt plays an important role in the pathogenesis of fibrotic diseases such as hepatic, renal, pulmonary and myocardial fibrosis. Some researches implied that Wnt signaling can affect myocardial fibrosis by activation of fibrosis effector cells, but the specific mechanism is still not clear. Wnt signaling pathway mainly includes classic and non-classical pathway, among which Wnt/beta-catenin is the most classic pathway and beta-catenin is the most important signal molecule in this pathway. Glycogen synthase kinase-3β (GSK-3β) and DVL-1 are important components of the Wnt signaling pathway. Once DVL-1 accepts the Wnt signal from the cell membrane, it will pass the signal through its receptor intracellularly. GSK-3β will then be inhibited, further inhibiting the phosphorylation of β-catenin. The phosphorylation causes β-catenin not to be degraded so as to abnormally aggregate, which activates the Wnt signaling pathway. Recent researches have shown that Wnt signaling pathway plays an essential role in the pathogenesis of cardiovascular diseases, but few studies have reported its role in myocardial fibrosis. Studies have indicated that a regulatory relation between miRNA and Wnt signaling pathways may exist in myocardial fibrosis.

However, the specific mechanism of the interaction between Wnt signaling and miRNA is still unclear making it necessary for further studies. Our purpose was to investigate the mechanisms of miR-154 affecting myocardial fibrosis.

**Patients and Methods**

**Clinical Data Collection**

Blood samples from 51 patients with cardiomyopathy were collected from the Department of Cardiology of Dalian Friendship Hospital (Dalian, China) from October 2013 to October 2017. All the patients were confirmed by pathology or imaging examination without any other diseases. Blood samples were also taken from healthy people with confirmation of no cancer and other diseases by physical examinations. The study was approved by the Dalian Friendship Hospital Ethics Committee and informed consent signed by all patients.

**Plasma RNA Extraction**

Cryopreserved plasma samples were placed on ice to melt and then mixed well. TRIzol was added to the mixture and the tube was shaken vigorously for 30 s at vortex and let stand for 5 min at room temperature. 200 μL of isopropanol were then added, inverted, mixed well, shaken at vortex vigorously for 2 min and let stand at room temperature for 5 min. The tubes were centrifuged and then the supernatant was transferred to a new Eppendorf (EP) tube. 0.75 volumes of isopropanol were added to the supernatant, then the mixture in the new tube was inverted and mixed well and let stand at room temperature for 10 min. The tube was centrifuged at 13,000 rpm for 10 min at 4°C to precipitate RNA. The supernatant was discarded and the precipitation was washed with 75% ethanol once; when clear RNA precipitation appeared, 30 μL of diethyl pyrocarbonate were added to dissolve the RNA.

**Transfection of Cells**

Human cardiac fibroblasts (HCF) in logarithmic growth phase were extracted from adult heart tissue and incubated with fibroblasts medium-2 (FM-2, Science Cell) at 37°C, saturated humidity, 5% CO2 in a cell culture incubator. The medium was replaced every 2 days. Once the myocardial fibroblasts were adherent to 80-90%, cells were passaged after tryptic digestion and plated in 24-well plates. Transfection was performed as the cell density reached 50%. The cells in the experiment were divided into seven groups: cardiac fibroblasts control group, cardiac fibroblasts negative control group, miR-154 inhibitor transfection group, miR-154 mimics transfection group, target gene GSK-3β overexpression vector group, GSK-3β siRNA transfection group, co-transfection of miR-154 mimics and GSK-3β overexpression vector group, with three replications per group. The mixture of miR-154 mimic and Lipo2000 or that of miR-154 inhibitor and Lipo2000 was added to 500 μL medium containing CFs cells in a 24-well cell culture plate and was gently mixed by shaking. After culturing for 5-6 h, the medium containing Lipo2000 in the 24-well plate was removed, and then an equal amount of culture medium was added. After 12-24 h, the transfection efficiency of the cells was observed under a fluorescence microscope.

**Cell Proliferation Detection by Cell Counting kit-8 (CCK-8)**

The CFs were plated on 96-well plates at 2 × 104 cells/well. After 12 h, the medium was replaced
with antibiotics-free medium. Empty vector, miR-154 mimic, miR-154 inhibitor were transfected respectively into CFs (0.2 μg plasmid DNA and 0.4 μL Lipo2000 per well) with five replications each group. Lastly, cell proliferation was detected (A450 nm value) according to the kit instructions of CCK-8.

Detection of miR-154 Expression and RNA Changes of α-SMA, GSK-3β by Quantitative Real-Time PCR

After 48 h of transfection, total RNA was extracted by TRIzol method and 500 ng of RNA was used for reverse transcription and quantitative PCR. MiR-154 was detected using miRNA-154 specific reverse transcription primer and qPCR primer (synthesized by Guangzhou Ruibo Co. Guangzhou, China), with U6 as an internal control. Reverse transcription was performed using non-specific primers. GSK-3β specific primers were used for qPCR (reagent was purchased from Shanghai Sangyo Co., Ltd. Shanghai, China) to detect GSK-3β RNA expression with β-actin as an internal control. The reaction conditions were based on the respective kit instructions.

Cell Invasion Experiment

After the CFs grew against the wall of flask, the medium was replaced, and the cells were cultured for another 48 h. After that, they were digested and resuspended in 2×10^5/mL to make single cell suspension with Dulbecco’s Modified Eagle Medium (DMEM). After laying the matrigel in the chamber, we added 100 μL of the cell suspension to the upper chamber and 500 μL of DMEM medium with 2% fetal bovine serum (FBS) in the lower chamber. The whole process was operated on ice. After placed at 37°C, 5% CO₂ incubator for 48 h, cells were harvested. Next, we wiped the unperforated cells on the upper surface of the filter with a cotton swab and stained the permeated cells with eosin, which were observed, photographed and counted under the inverted microscope. The cells counting was performed based on five randomly chosen fields per well. The number of cells passing through the transwell chambers of each group was used as an index to evaluate their invasiveness.

Statistical Analysis

Data were analyzed using statistical product and service solutions 19.0 statistical software (IBM, Armonk, NY, USA). The differential expression of miR-154 in the blood samples of patients was analyzed by t-test and expressed as mean ± SD. The relationship between the expression of miR-154 and the clinical pathological parameters was analyzed by χ²-test; p < 0.05 was considered statistically significant.

Results

The Relationship Between the Expression of miR-154 and the Clinical Features of Patients with Cardiomyopathy

The expression of miR-154 in plasma of 12 patients with cardiomyopathy (Cardiomyopathy, C) and 12 normal subjects (Normal, N) was detected by Real-time PCR; the results showed that the expression of miR-154 in former group was higher than that in the latter, and the difference was statistically significant (Figure 1, p < 0.05). Based on the level of miR-154 in serum, the patients with abnormal expression of miR-154 were divided into two groups: the miR-154 group and the miR-154 ≥ 2 group. Patients with miR-154 level twice higher than the average level were assigned to the miR-154 group, while the patients with miR-154 level lower than that, were assigned to the miR-154 ≥ 2 group. The correlations between the expression of miR-154 with patient’s age, gender, disease type, ejection fraction, smoking history, total cholesterol and lipoprotein-a were analyzed by χ²-test. The results showed that the expression of miR-54 was not obviously associated with the type of cardiomyopathy or patient’s smoking history (p > 0.05, Table I), but was closely related to patient’s age, sex, ejection fraction, total cholesterol, and lipoprotein-a; the difference was statistically significant (p < 0.05, Table I).

The Changes of Cell Proliferation and Invasion Assayed by CCK8 after miR-154 Intervention

The CFs were transfected with negative control (NC), miR-154 mimics and miR-154 inhibitor, respectively. Next, the cells were divided into four groups: control group, NC group, miR-154 mimics group and miR-154 inhibitor group. Cell proliferation rate of these groups was detected at 0 h, 6 h, 24 h, and 48 h. The results showed that the difference of cell proliferation rate between miR-154 mimics group, miR-154 inhibitor group, and control group along with NC group, was statistically significant (p < 0.05), indicating that miR-154 was able to promote the proliferation of CFs significantly (Figure 2A and B). Hyper proliferation and migration of
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CFs are the key to myocardial fibrosis, so transwell assays were used to detect cell migration (Figure 2C and D). The results indicated that miR-154 significantly enhanced the migration ability of CFs. Meanwhile, after transfection of the inhibitor, the migration ability of CFs was significantly weakened ($p < 0.05$).

**Expression of α-SMA, Collagen I and Collagen III Proteins After miR154 Intervention**

48 h after intervention with miR-154 mimics and miR-154 inhibitors, CFs were harvested and total RNA and total protein of CFs were extracted. The mRNA and protein levels of α-SMA, collagen I and collagen III were determined by qRT-PCR (Figure 3A, B and C) and Western blot (Figure 3D and E), respectively. It is found that the expression of the above three proteins in miR-154 mimics group significantly increased compared with the NC or control group, while that in the inhibition group was reduced; the differences between above groups were statistically significant ($p < 0.05$).

**The Impact of miR154 Overexpression on the Expression of GSK-3β**

The miR-154 mimics or inhibitors were transfected into CFs, and the expression of FGF23 protein was detected by Western blot 48 h after transfection. The results showed that miR-154 mimics could decrease the GSK-3β expression, while miR154 inhibitors could significantly up-regulate GSK-3β expression (Figure 4A and B).

**Table I.** The relationship between the expression level of miR-154 and clinical data.

<table>
<thead>
<tr>
<th>miR-154 &lt; 2 (n = 27)</th>
<th>miR-154 ≥ 2 (n = 24)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD</td>
<td>56.0 ± 8.5</td>
<td>72.7 ± 9.0</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>8 (29.6%)</td>
<td>17 (70.8%)</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM</td>
<td>4 (14.8%)</td>
<td>5 (20.8%)</td>
</tr>
<tr>
<td>HCM</td>
<td>6 (22.2%)</td>
<td>4 (16.7%)</td>
</tr>
<tr>
<td>RCM</td>
<td>17 (63.0%)</td>
<td>15 (62.5%)</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>53.8 ± 6.4</td>
<td>47.8 ± 5.4</td>
</tr>
<tr>
<td>History of smoking</td>
<td>7 (33.3%)</td>
<td>6 (25.0%)</td>
</tr>
<tr>
<td>TC (mL/dl)</td>
<td>174.8 ± 20.9</td>
<td>233.0 ± 26.1</td>
</tr>
<tr>
<td>Lp-a (mg/L)</td>
<td>179.4 ± 51.3</td>
<td>332.4 ± 20.4</td>
</tr>
</tbody>
</table>

DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; RCM: restrictive cardiomyopathy; LVEF: left ventricular ejection fractions; TC: total cholesterol; Lp-a: Lipoprotein-a.
Detection of miR-154 Related Target Gene Expression by Western Blot

The results of Western blot showed that miR-154 up-regulated the expression of β-catenin in fibroblasts and down-regulated the expression of GSK-3β after transfection of miR-154 into fibroblasts. On the contrary, the expression of β-catenin decreased significantly in fibroblasts transfected with miR-154 inhibitor, while transfection of GSK-3β overexpression vector resulted in more obvious decrease of α-SMA. In addition, when GSK-3β over-expression vector and miR-154 mimics were co-transfected into CFs, the up-regulation of β-catenin was not conspicuous compared with transfection of miR-154 mimics alone; the differences were statistically significant compared with the control group (p < 0.05, shown in Figure 5A and B). Therefore, our data demonstrated that miR154 can target GSK-3β and up-regulate the expression of β-catenin in CFs, thereby activating the classical Wnt pathway.

Discussion

Cardiac fibroblasts (CFs) are the major cells secreted by extracellular matrix of cardiomyocytes. Myocardial stromal cells account for 2/3 of total cardiac cells and 60-70% of total CFs, most of which are present in the heart mesenchymal and arteriovenous vascular wall. Therefore, they are the main cells for the synthesis of cardiac collagen. Normally, adult CFs are in a state of homeostasis. However, pathological stimuli induced CFs activation, thus leading to the phenotype conversion to myofibroblasts. Some studies
Figure 3. The impact of miR-154 on the expression of α-SMA, collagen I and collagen III was studied. After transfected for 48 h, the expression of α-SMA (A), collagen I (B) and collagen III (C) expression is shown. D, E, α-SMA, collagen I and collagen III protein levels were detected by Western blot. Data are presented as mean ± SD of three different samples. *p < 0.05 and **p < 0.01, respectively, compared with control group.

Figure 4. Effect of miR154 on GSK-3β expression was studied. The expression of GSK-3β was detected by qRT-PCR (C) and Western blot (D) 48 h after transfected with miR-154 mimics or miR-154 inhibitor. Data are presented as mean ± SD of three different samples. *p < 0.05 and **p < 0.01, respectively, compared with control group.
have shown that 50% of myofibroblasts are mainly from CFs, but their ability to synthesize ECM was significantly higher than CFs and α-SMA expression was significantly improved in myofibroblasts. Studies have indicated that the contractility of α-SMA positive myofibroblasts is twice as much as that of the α-SMA negative ones. Therefore, when CFs are activated, their proliferation, migration, ECM synthesis and secretion can promote wound-healing and repair. However, when the ECM is over-accumulated, it can lead to more scarring, contracture and fibrosis. The conversion of CFs into myofibroblasts is an important step in myocardial fibrosis. Therefore, understanding the specific regulation mechanism of phenotypic transformation of CFs is helpful for finding the molecular targets of myofibroblasts as well as for prevention and treatment of myocardial fibrosis.

MicroRNAs (miRNAs) are relatively conservative small, non-coding RNAs that are located in the cell and 22 nt in length, which are involved in many important life processes. It was showed that abnormal expression of miR-1, miR-21 may affect the normal heart operation. It is demonstrated that these miRNAs are involved in the process of fibrosis by directly inhibiting expression of various extracellular matrix proteins and regulating a variety of fibrosis-related signaling pathways, thus leading to the process of myocardial fibrosis in cardiovascular diseases such as hypertension, diabetes and myocardial infarction. Wang et al observed that overexpression of miR-21 causes fibroblast proliferation leading to fibrosis. When myocardial infarction occurs, myocardial cell proliferation in the surrounding area can give rise to myocardial hypertrophy and fibrosis. All of above-mentioned studies make us speculate that miRNA and cardiovascular diseases are closely linked. In this investigation, we also found that serum miR-154 expression of patients with cardiomyopathy was significantly higher than that of normal people. However, the specific mechanism is not yet clear. Therefore, it is of great importance to explore the relationship between miR-154 and CFs.

The relationship between Wnt/β-catenin signaling pathway and miRNA has been widely discussed in multiple studies. It is found that miRNA can regulate key molecules in Wnt signaling pathway. Xing et al also found that upregulated miR-374 significantly increased Wnt-related factors,
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and enhanced epithelial mesenchymal transition and migration, thereby leading to the occurrence of fibrosis and the invasion and metastasis of tumor cells. Zhang et al. found that overexpression of miR-21-5p and miR-135b can promote the activation of Wnt signaling pathway and participate in the development of myocardial remodeling. Based on previous works, we suggested that miR-154 may be involved in Wnt pathway during the process of affecting CFs activation.

Recent researches have reported that when myocardial infarction occurs, migration and proliferation of cardiac fibroblasts are involved in the healing of infarct area, and Wnt pathway is closely related to this process. Hypertension is a common and frequently occurring disease, with left ventricular hypertrophy (LVH) appeared in 20-40% of hypertensive patients. Although the exact mechanism of myocardial interstitial fibrosis is unclear, it is currently thought that the imbalance between protein kinase and protein phosphatase may be related to the fibrosis. Glycogen synthase kinase-3β (GSK-3β) and DVL-1 are important components of the Wnt signaling pathway. In this report, through the study of the impact of miR-154 on protein GSK-3β, we provide experimental basis for further investigation on the effect of Wnt signaling pathway in the pathogenesis, prevention and treatment of myocardial fibrosis.

Conclusions

We showed that miR-154 can inhibit GSK-3β, promote Wnt/β-catenin activation, thus promoting myocardial fibrosis. The exact role of miR-154 and GSK-3β in myocardial fibrosis requires further study in order to provide guidance for the prevention and treatment of myocardial fibrosis.

Conflicts of Interest

The Authors declare that they have no conflict of interests.

References


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