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MiR-138-5p is downregulated in patients with atrial fibrillation and reverses cardiac fibrotic remodeling via repressing CYP11B2

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Abstract. – OBJECTIVE: To investigate the connection between atrial fibrillation (AF) and miR-138-5p and to further explore the possible mechanism.

PATIENTS AND METHODS: MiR-138-5p expression of right atrial appendage (RAA) tissues in 28 patients with AF and 22 patients with sinus rhythm (SR) was detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Moreover, cell proliferation assay was conducted in AC16 cells which were transfected by miR-138-5p inhibitors or mimics. Furthermore, Western blot assay, luciferase assay, and RNA immunoprecipitation assay were performed to uncover the mechanism.

RESULTS: In the present research, miR-138-5p expression in RAA samples decreased significantly in AF patients than that in SR ones. Moreover, in AC16 cells, higher miR-138-5p expression level suppressed cell growth, while cell growth was promoted after miR-138-5p was knockdown. In addition, further experiments showed that CYP11B2 acted as the main target of miR-138-5p and its expression in AF tissues negatively correlated to miR-138-5p expression.

CONCLUSIONS: All the results above elucidated that cell proliferation of AF could be inhibited by miR-138-5p via suppressing CYP11B2, which may offer a new vision for interpreting the mechanism of AF development.

Key Words:
Atrial fibrillation, MiR-138-5p, CYP11B2.

Introduction

As a common cardiac arrhythmia, atrial fibrillation (AF) contributes to high incidence worldwide especially in elderly patients[1]. Although the mechanism of AF has been studied by many researchers in the last decades, it remains unclear how AF develops.

The development of AF is generally associated with gene expression alterations which could lead to the subsequent abnormal expression of certain protein. Previously, numerous studies indicated that plenty of proteins expression could be regulated by microRNAs through altering the expression of the gene. Furthermore, a number of studies also elucidated that microRNAs are linked to many diseases closely. For instance, miR-214 downregulation acted as a contributing factor to methylglyoxal (MGO)-induced endothelial insulin-resistance[2]. In patients with acute myocardial infarction, miR-379 level in plasma was significantly decreased[3], while miR-298 could improve the myocardial apoptosis after myocardial infarction[4]. In breast cancer, the low miR-597 expression is associated with poor prognosis[5]. MiR-599 expression was significantly downregulated in glioma and could be a useful prognostic marker[6].

Therefore, further exploring the mechanisms of these genomic changes in AF is urgently required. Recently, there were also several studies exploring the links between microRNAs and AF. These researches found out that chronic atrial fibrillation (CAF) increases miR-21 expression which could participate in the CAF-induced downregulation of L-type calcium channel protein[7]. MiRNA-328 (miR-328) expression was found significantly lower in patients with AF[8]. Levels of reactive oxygen species (ROS) in response to autonomic nerve remodeling were increased in AF patients with higher miR-206 expression[9]. Moreover, miR-133 and miR-30 played a key role in controlling structural changes in chronic AF[10].

According to the previous studies, we know that several microRNAs connected closely to the

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development and prognosis of AF. However, there is still no research exploring whether miR-138-5p plays a part in AF or not. In this study, we aimed to find out the connection between AF and miR-138-5p and to further investigate the potential mechanism.

Patients and Methods

Clinical Samples and Cell Lines

A total of 28 AF patients and 22 SR patients were enrolled for right atrial appendage (RAA) tissues who received cardiac surgery in our hospital. Before the operation, written informed consent was obtained. Those patients had no other diseases including pulmonary disease, coronary heart disease, diabetes infective endocarditis, diabetes mellitus, hyperthyroidism, hypertension, active rheumatism, or autoimmune disease. Tissues got from the surgery were stored immediately at -80°C. All tissues were analyzed by an experienced pathologist. This study was approved by the Ethics Committee of Taihe Hospital, Hubei University of Medicine.

AC16 cell line and 293T embryonic kidney cell line (Type Culture Collection, Manassas, VA, USA) were used in this study. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing penicillin and 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C with 5% CO_2.

RNA Extraction and qRT-PCR (Quantitative Reverse Transcriptase-Polymerase Chain Reaction)

Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was used to reverse-transcribe the whole RNA to cDNAs after separated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Thermal cycle was as follows: 95°C for 30 sec, 5 sec for 40 cycles at 95°C, 60°C for 35 sec. U6 was used to normalize the miR-138-5p expression.

Cell Transfection

MiR-138-5p inhibitors and mimics (Genepharma Co., Ltd. Shanghai, China) were utilized to transfect AC16 cells. Non-specific siRNA was used to transfect negative control.

Cell Counting Kit-8 Assay (CCK-8)

A total of 4×10^3 cells were seeded in a 96-well plate per well. All the wells were added with CCK-8 reagent (Dojindo, Tokyo, Japan) respectively at 0, 24, 48, and 72 h according to the instructions. Then, the plate was incubated for 2 h at 37°C. A microplate reader (Bio-Rad, Hercules, CA, USA) was utilized to examine OD (optical density) value.

Western Blot Analysis

Reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after replaced to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-β-actin and rabbit anti-CYP11B2, as well as goat anti-rabbit secondary antibody. A chemiluminescent film was applied for assessment of protein expression with Image J software.

Luciferase Assays

In our study, pGL3 vector (Promega, Madison, WI, USA) was used for the construction of 3′-UTR of CYP11B2 or wild-type (WT) 3′-UTR. Quickchange site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) was used for site-directed mutagenesis of CYP11B2 binding site in miR-138-5p 3′-UTR, mutant (MUT) 3′-UTR. MUT-3′-UTR or WT-3′-UTR and mimics or miR-control was used for cell transfection. 48 h later, the luciferase assay was performed utilizing dual Luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation Assay (RIP)

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was utilized to perform RIP assay. And qRT-PCR was used to detect co-precipitated RNAs.

Wound Healing Assay

Cells were transferred into 6-well plates and then cultured in Dulbecco's modified Eagle medium (DMEM) medium (HyClone, South Logan, UT, USA) overnight. After scratched with a plastic tip, serum-free DMEM was used to culture

cells. At different time sets, wound healing was viewed respectively. Each assay was repeated in triplicate.

Statistical Analysis

Results were statistically analyzed by statistical product and service solutions (SPSS17.0, SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD. Chi-square test, Student *t*-text and Kaplan-Meier method were selected when appropriate. $p<0.05$ was supposed to be statistically significant.

Results

The Expression Level of MiR-138-5p in AF and SR Patients

First, qRT-PCR was conducted for detecting the expression level of miR-138-5p in both 28 AF patients' and 22SR patients' RAA tissues. As a result, when compared with SR patients, miR-138-5p expression level significantly decreased in AF patients (Figure 1).

Cell Proliferation Inhibition of AC16 Cells by MiR-138-5p

MiR-138-5p inhibitor or mimics were utilized to transfect AC16 cells. Then, miR-138-5p expression level was determined by qRT-PCR (Figure 2A). Furthermore, results of CCK-8 assay showed that cell proliferation of AC16 cells was inhibited after miR-138-5p was overexpressed (Figure 2B). And cell proliferation of AC16 cells was promoted after miR-138-5p was knocked down (Figure 2C).

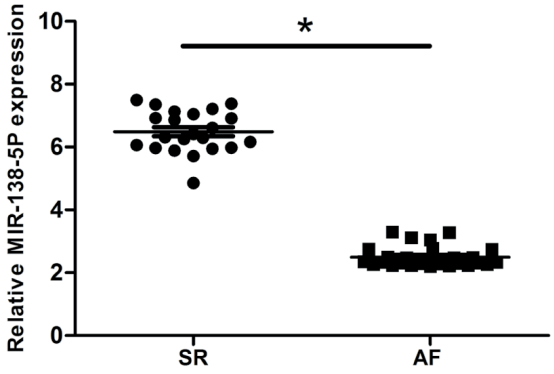

Figure 1. Expression levels of miR-138-5p in RAA tissues. MiR-138-5p expression was significantly decreased in the AF patients compared with SR patients. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

The Expression of CYP11B2 in AF Patients

TargetScan was used to find the miRNAs that contained complementary base with miR-138-5p. Harbor CYP11B2 binding sites were used to predict the target for miR-138-5p (Figure 3A). We further detected CYP11B2 expression in RAA tissues, and found that CYP11B2 expression was significantly higher in AF patients than that in SR patients (Figure 3B).

The Interaction Between CYP11B2 and MiR-138-5p

qRT-PCR results showed that CYP11B2 level of AC16 cells was lower in miR-138-5p mimics group compared with that in control group (Figure 4A). CYP11B2 level of AC16 cells was higher in miR-138-5p inhibitor group compared with that in control group (Figure 4B). Furthermore, the luciferase assay found out that luciferase activity of miR-138-5p -WT cells with CYP11B2 mimics was reduced, while luciferase activity of miR-138-5p-MUT cells was not significantly changed with CYP11B2 (Figure 4C). Meanwhile, RIP assay results demonstrated that by comparison, CYP11B2 could be remarkably enriched in the miR-138-5p series, which suggested that miR-138-5p might work as a CYP11B2 sponge (Figure 4D).

Discussion

Evidence has proved that microRNAs participate in diseases' development. For example, in human retinoblastoma, miR-138-5p inhibits tumor proliferation[11]. MiR-138-5p also takes part in promoting cervical cancer malignant progression[12. And] miR-138-5p was reported to inhibit pancreatic cancer autophagy *via* targeting SIRT1[13].

In the present work, it was found that in RAA samples of AF patients, miR-138-5p was significantly downregulated when compared with SR patients. Furthermore, after the expression of miR-138-5p was upregulated, cell growth in AC16 cells was found to be inhibited. Moreover, knockdown of miR-138-5p could promote AC16 cell growth. These data indicated that miR-138-5p inhibited the proliferation of cardiomyocytes, which was a vital process of cardiac fibrotic remodeling.

Current studies revealed that microRNAs function in diseases' progression by targeting related genes. A meta-analysis which contained a total of 2,758 subjects indicated that polymorphism of

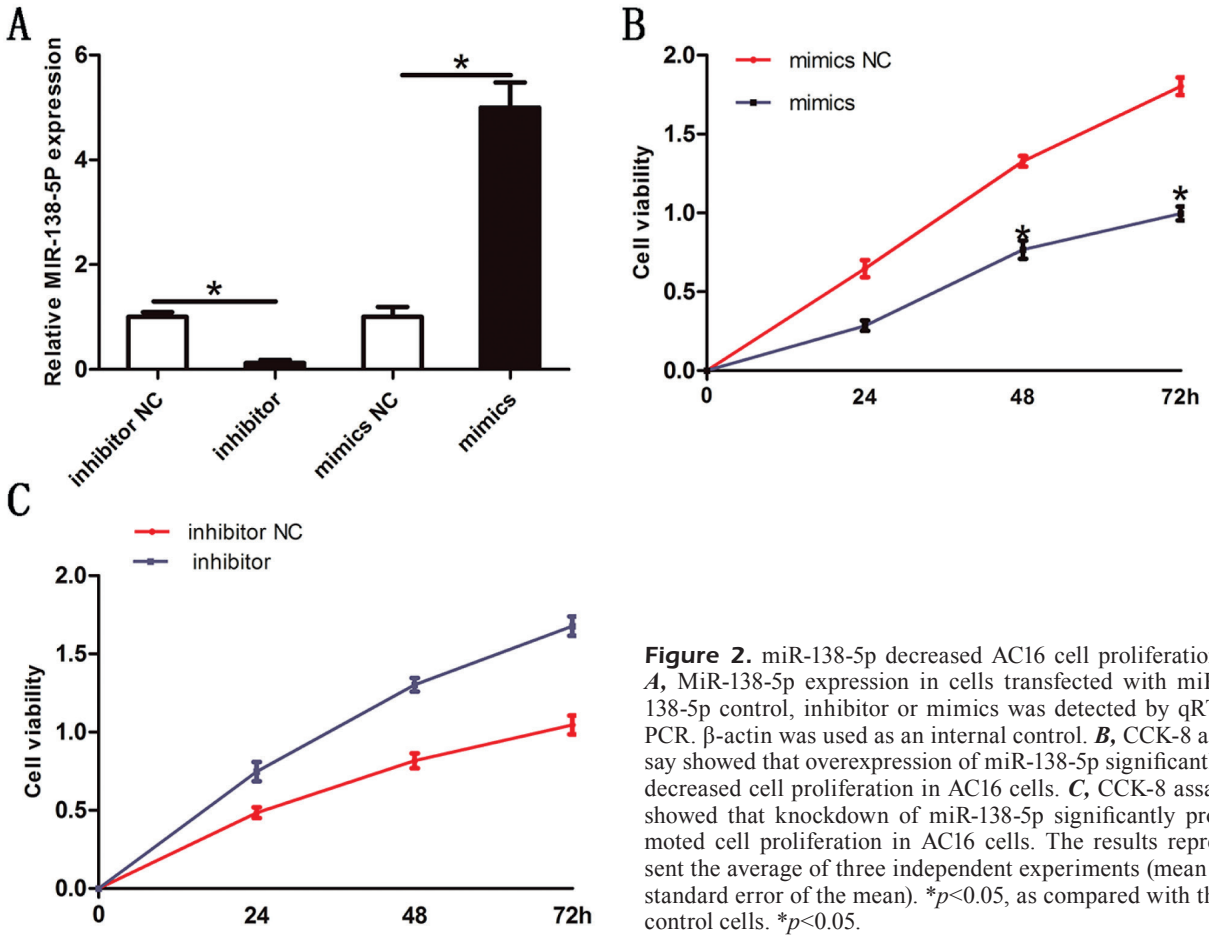

Figure 2. miR-138-5p decreased AC16 cell proliferation. ***A,*** MiR-138-5p expression in cells transfected with miR-138-5p control, inhibitor or mimics was detected by qRT-PCR. β-actin was used as an internal control. ***B,*** CCK-8 assay showed that overexpression of miR-138-5p significantly decreased cell proliferation in AC16 cells. ***C,*** CCK-8 assay showed that knockdown of miR-138-5p significantly promoted cell proliferation in AC16 cells. The results represent the average of three independent experiments (mean ± standard error of the mean). **p*<0.05, as compared with the control cells. **p*<0.05.

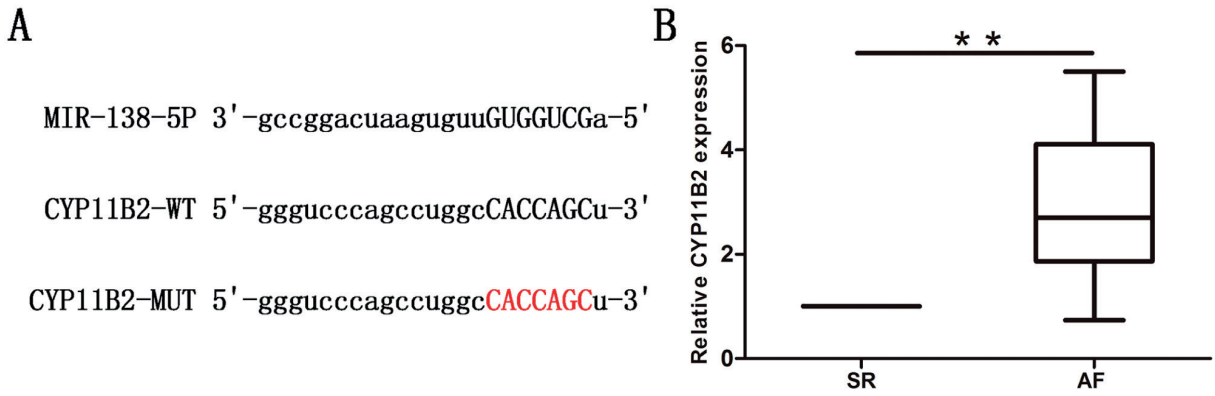

Figure 3. CYP11B2 in AF patients. ***A,*** The binding sites of CYP11B2 on miR-138-5p. ***B,*** CYP11B2 expression of RAA tissues was higher in AF patients than that in SR patients.

CYP11B2 was significantly related to the risk of AF[14]. A study in African Americans elucidates that genotype of CYP11B2 was a remarkable predictor of AF independently[15]. In mice, atrial fibrosis and atrial fibrillation could be prevented after CYP11B2 was inhibited by torasemide[16]. In patients with atrial fibrillation, CYPIIB2 in the atria was also found to act as one of the molecular participating in the possible mechanisms for the progression of atrial interstitial fibrosis[17]. However,

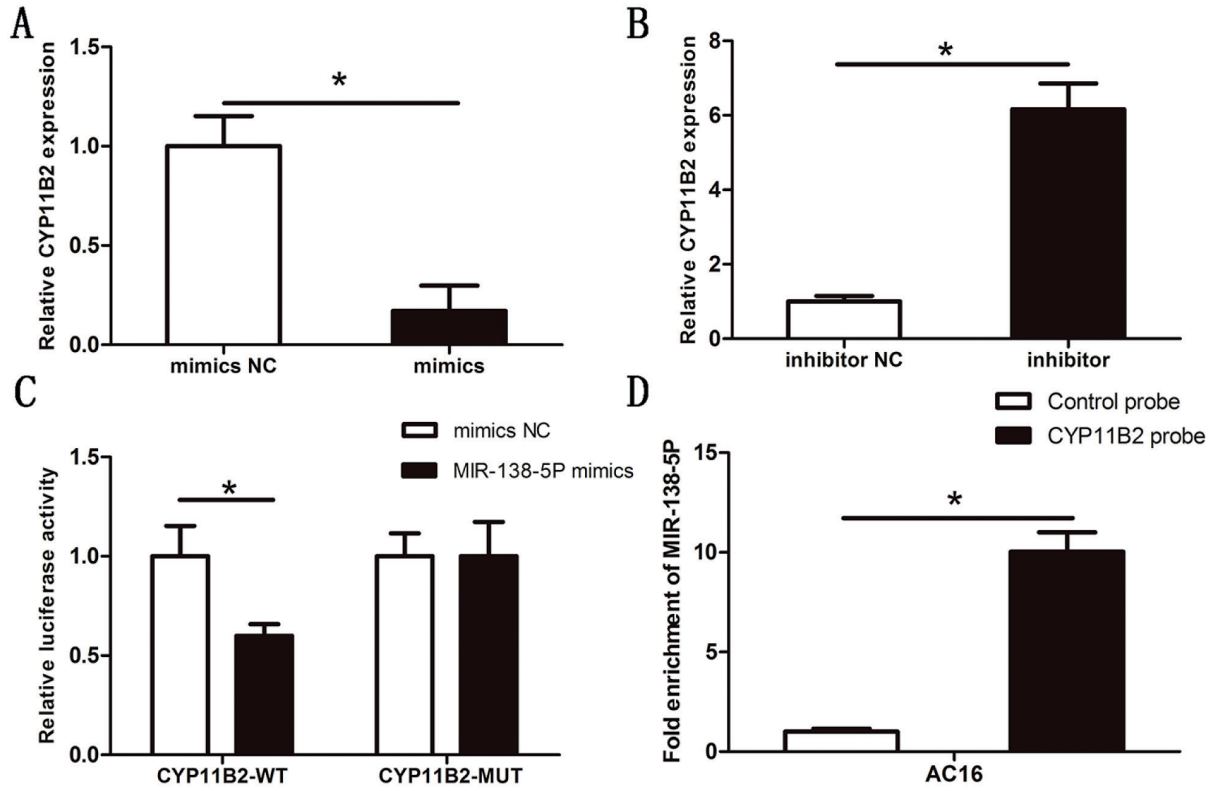

Figure 4. Interaction between miR-138-5p and CYP11B2. ***A,*** CYP11B2 expression was decreased in miR-138-5p mimics group compared with control group. ***B,*** CYP11B2 expression was increased in miR-138-5p inhibitor group compared with control group. ***C,*** Co-transfection of CYP11B2 and miR-138-5p-WT in AC16 cells strongly decreased the luciferase activity, while co-transfection of CYP11B2 and miR-138-5p-MUT did not change the luciferase activity either. ***D,*** CYP11B2 was significantly enriched by RNA immunoprecipitation (RIP) assay in the miR-138-5p group compared with control. The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *$p<0.05$.

there were few researches exploring the underlying mechanism between CYP11B2 and AF. Our study revealed that CYP11B2 was upregulated in AF patients compared with SR patients. Furthermore, CYP11B2 could directly bind to miR-138-5p through a luciferase assay. And CYP11B2 was significantly enriched by miR-138-5p RIP assay. In addition, CYP11B2 expression could be downregulated with overexpression of miR-138-5p. All the results above suggested that miR-138-5p might inhibit the proliferation of cardiomyocytes *via* targetingCYP11B2.

Conclusions

We showed that miR-138-5p could reverse cardiac fibrotic remodeling through repressing CYP11B2. These findings suggested that miR-138-5p might contribute to therapy for AF as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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