Jin He MSc*
Yan Yue PhD*
Chunsheng Dong PhD
Sidong Xiong PhD

*These authors contributed equally to this study.

Jiangsu Key Laboratory of Infection and Immunity, Institutes of Biology and Medical Sciences, Soochow University, Suzhou, P. R. China.

MiR-21 confers resistance against CVB3-induced myocarditis by inhibiting PDCD4-mediated apoptosis

Abstract

Purpose: The participation of microRNAs (miRNAs) in cardiovascular diseases suggests them as potential targets for novel preventive and therapeutic strategies. In this study, the key myocardial miRNA, miR-21, was identified in the murine coxsackievirus B3 (CVB3)-induced myocarditis model and its contribution to disease progression was explored.

Methods: Myocardial microRNA expression changes in CVB3-infected mice were analyzed by real-time PCR and miR-21 was found to be the miRNA whose expression was significantly reduced. Mice were injected with plasmid encoding miR-21 (pMDH-miR-21) at day 1 post CVB3 infection and myocarditis severity was evaluated 7 days post-infection. The underlying mechanism of miR-21 in viral myocarditis was also investigated.

Results: Myocardial miR-21 expression was negatively related to viral myocarditis severity. Recovery of miR-21 expression, by injecting with pMDH-miR-21, significantly relieved CVB3-induced myocarditis as shown by increased body weight, reduced myocardial injury, lowered myocarditis score and increased survival rate. Further study showed that miR-21 could protect myocardial apoptosis by specifically inhibiting its target programmed cell death 4 (PDCD4) expression.

Conclusion: miR-21 administration efficiently alleviated CVB3-induced myocarditis by repressing PDCD4-mediated apoptosis. Our study not only helps to better understand the pathogenesis of viral myocarditis, but also proves the potential of miR-21 as a novel therapeutic target for treatment of CVB3-induced myocarditis and other apoptosis-mediated cardiovascular diseases.

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Correspondence to:
Sidong Xiong, Ph.D.
Professor and Director
Institutes of Biology and Medical Sciences, Soochow University,
199 Ren-Ai Road, Suzhou, Jiangsu, 215123, P. R. China.
Email: sdxiongfd@126.com

Coxsackievirus B3 type (CVB3), a small single-strand RNA virus belonging to the picornavirus family, is often associated with heart disease and has been involved in up to 45% of the human cases of myocarditis or dilated cardiomyopathy [1-3]. Although much knowledge about CVB3 characteristics [4-6], as well as the control measures [7-10], has been gained using murine CVB3-induced myocarditis models, there are still no effective preventative or therapeutic strategies available in the clinic. The major reason for this is that the pathogenesis of viral myocarditis is not well understood; therefore, there is an urgent need to explore further mechanisms underlying the interaction of CVB3 with the host and to find new potential targets for viral myocarditis therapy.

MicroRNAs (miRNAs) are a class of endogenous small single-strand non-coding RNAs that act as important post-transcriptional gene regulators. As part of a multi-protein complex known as RNA-induced silencing complex (RISC), miRNAs can silence the expression of multiple targets by partially complementing the 3' untranslated region (UTR) of target mRNA. Emerging evidence suggests that miRNAs are involved in many fundamental myocardial physiological and pathological processes [11-14] by regulating apoptosis [15, 16], vascularity [17] or cardiogenic differentiation [18]. Altered microRNA expression patterns could be applied as a new biomarker and diagnostic tool [19], and manipulating miRNAs might be an attractive therapeutic strategy [20, 21], since they target genes specifically and are easy to manipulate with specific mimics or inhibitors. In support of this, recent studies have revealed miRNA-based treatments on pathological cardiac remodeling [22], ischemic heart disease [15] and heart failure [23].

Given the important role of miRNAs in myocardial physiopathology, myocardial miRNA expression patterns may change in CVB3-induced myocarditis. Identification of the key miRNA that specifically impacts the severity and prognosis of viral myocarditis may provide not only a better understanding of the disease pathogenesis, but also clues to develop novel therapeutic regimes against infectious and inflammatory heart diseases.

Methods

Animals

Male BALB/c (H-2^d) mice, 6 weeks of age, were bought from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P. R. China) and bred in the specific pathogen-free facility. All animal experiments were carried out in strict accordance with the recommendations in the Guide

for the Care and Use of Medical Laboratory Animals (Ministry of Health, P. R. China, 1998). The protocol was approved by the Ethical Committee of Soochow University.

Virus

CVB3 (Nancy strain) was maintained by passage through HeLa cells (ATCC number: CCL-2). Virus titer was determined by a 50% tissue culture infectious dose (TCID₅₀) assay on HeLa cell monolayer and calculated by the Reed-Muench method. Mice were infected by an intraperitoneal injection of 0.1 ml of PBS containing 10³ TCID₅₀ CVB3.

In vivo delivery of pMDH-miR-21 and pcDNA3.1-PDCD4

Plasmid pMDH-miR-21, encoding the murine precursor miR-21 and GFP protein, was a gift from Prof. Jinping Zhang at the Institutes of Biology and Medical Sciences, Soochow University, China. Mice were intraperitoneally infected with 10³ TCID₅₀ CVB3 at day 0, and given 100 µg pMDH-miR-21 by hydrodynamic injection 1 day later. Myocarditis severity was evaluated at day 7 post-infection. For rescue of CVB3-induced programmed cell death 4 (PDCD4) down regulation, each mouse received 100 µg plasmid pcDNA3.1-PDCD4 kindly provided by Dr. Myung Haing Cho at Seoul National University, by hydrodynamic injection at 1 day post-infection. The other two groups were treated with empty vector pcDNA3.1 or PBS as controls. Day 7 post-infection, the apoptosis of heart cells were measured by Annexin-V (eBioscience) using a FACS caliber instrument (Becton Dickinson).

Quantitation of viral load in heart tissues

Seven days after CVB3 infection, hearts were collected and homogenized. Total RNA of the heart tissue was reversely transcribed to cDNA using a specific primer (5'-CACCGGATGG CCAATCCA-3') and then the cDNA was subjected to SYBR green real-time PCR using CVB3-specific primers (5'-ATCAA GTTGCGTGCTGTG-3' and 5'-TGCGAAATGAAAGGA GTGT-3'). Expression of the virus RNA load was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Histopathological analysis and myocarditis scoring

Hearts were collected from infected mice at day 7, fixed in 10% buffered formalin solution and embedded in paraffin. Sections 5 µm thick were cut and stained with hematoxylin and eosin. The extent of myocardial lesions was quantified and scored as previously described [24]. Pathological scores were defined

TABLE 1. Stem-loop RT primers

	Primer sequence (5'-3')
mmu-miR-21	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACTCAACA
mmu-miR-29b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACAACACT
mmu-miR-92a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACCAGGCC
mmu-miR-126-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACCGCGTA
mmu-miR-199a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACGAACAG
mmu-miR-210	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACTCAGCC
mmu-miR-320	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACTCGCCC
mmu-miR-499	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACAAACAT

based on the following semi-quantitative scale: 0, no inflammatory infiltrates; 1, one to five inflammatory cells in each detected microscopy field and the total inflammatory infiltrate area was less than 5% of the field; 2, more than five inflammatory cells in each detected microscopy field or the total inflammatory infiltrate area was between 5% and 20%; 3, inflammatory infiltrate area was more than 20% and there was no necrosis; 4, diffuse inflammatory infiltrates with necrosis. Analysis was performed double-blind by a trained pathologist.

Real-time PCR

The total RNA of heart tissues was extracted with miRNeasy MiniKit (Qiagen) and then reverse transcribed with miRNA-specific stem-loop RT primers (Table 1) into cDNA. Real-time primers for microRNAs (miR-21, -29b, -92a, -126-5p, -199a, -210, -320, -499) and house-keeping gene GAPDH were designed and sequences were shown in Table 2. Gene expressions were detected by SYBR green real-time PCR and quantification of data was analyzed using the $2^{-\Delta\Delta C_t}$ method.

FACS analysis of annexin-V⁺ myocardiocytes

Hearts were collected from CVB3-infected mice at day 7 and a single-cell suspension of myocardiocytes was prepared. Following staining with fluorescein-isothiocyanate (FITC) conjugated annexin-V, cells were subjected to a flow cytometry assay using FACS calibur and CellQuest software (Becton Dickinson).

Immunohistochemistry

To evaluate the myocardial expression of PDCD4, sections of heart tissue were heated by microwave in 0.01% citrate buffer and treated with 3% H₂O₂ for 15 min to inhibit endogenous peroxidase activity. After blocking with 3% BSA-PBS for 30 min, sections were incubated with the rat anti-mouse PDCD4 antibody (Cell Signaling Technology) at 4°C overnight, then with biotinylated rabbit anti-rat antibody (Dako) and a streptavidin-peroxidase-based detection kit (Dako) before revealing with diaminobenzidine/hydrogen peroxidase substrate and hematoxylin counterstaining.

TABLE 2. Real-time PCR primers

		Primer sequence (5'-3')
sense	mmu-miR-21	GCGCTAGCTTATCAGACTGA
	mmu-miR-29b	TAGCACCATTGTGAAATCAGTG
	mmu-miR-92a	TATTGCACTTGTCCCGGC
	mmu-miR-126-5p	CATTATTACTTTTGGTACGCG
	mmu-miR-199a	CCCAGTCTTCAGACTACCT
	mmu-miR-210	CTGTGCGTGTGACAGCGGCTGA
	mmu-miR-320	AAAAGCTGGGTGAGAGGGC
	mmu-miR-499	TTAAGACTTGCAGTGATGTTT
anti-sense		GTGCAGGGTCCGAGGT

FIGURE 1. Myocardial miRNA expression in CVB3-induced myocarditis. Mice ($n=5$ per experimental group) were injected intraperitoneally with 10^3 TCID₅₀ CVB3. Seven days later the hearts were collected and miRNA expression was detected by real-time PCR. Results were presented by the relative expression level of myocardial miRNAs in CVB3-infected mice compared with naive mice with the level of expression of miRNA in naive mice set as one. The experiments were repeated three times with similar results.

Statistical analysis

Data are presented as mean $\pm$ SEM. Differences between experimental groups were analyzed for statistical significance by ANOVA test using GraphPad Prism version 4.01. A value of $P<0.05$ was considered statistically significant.

Results

Myocardial miR-21 expression was dramatically reduced in CVB3-induced myocarditis

Mounting evidence shows that miRNAs play an important role in infectious [25] and myocardial diseases [11] and we propose that miRNAs may participate in the development of CVB3-induced myocarditis. To address this hypothesis, the expression of several previously reported myocardium-associated miRNAs were determined in CVB3-infected heart tissues. As shown in Fig. 1, expression of three miRNAs (miR-21, -199a, -320) was downregulated in CVB3-infected mice compared with naive mice, with the most dramatic decline in miR-21 (about 3.6 fold). Meanwhile, the expression of five miRNAs (miR-29b, -499, -210, -126-5p, -92a) was increased in CVB3-infected heart tissues.

FIGURE 2. Myocardial miR-21 expression in CVB3-infected mice with mild or severe myocarditis. Mice ($n=5$ per group) were infected with 10^3 TCID₅₀ CVB3, and seven days later hearts were collected and divided into two groups based on myocarditis pathological scores: mild myocarditis group with pathological scores of 1 or 2, severe myocarditis group with pathological scores of 3 or 4. Myocardial miR-21 expression was detected in these two groups by real-time PCR. Each group contains five mice. The experiments were repeated three times with similar results. *, $P<0.05$, ***, $P<0.001$.

Considering that the downregulated miRNA may exert protective effects on CVB3-induced myocarditis, the myocardial miR-21 expression levels in mice with mild and severe viral myocarditis were compared. It was found that miR-21 expression was much lower in mice with severe myocarditis than those with benign myocarditis (Fig. 2), suggesting that miR-21 may take a protective part in CVB3-induced myocarditis.

MiR-21 treatment significantly alleviated CVB3-induced myocarditis

To clarify the role of miR-21 in CVB3-induced myocarditis, mice were given a hydrodynamic injection of pMDH-miR-21 encoding the miR-21 precursor at day 1 postinfection. By day 7, there was a 2.3-fold increase in expression in the pMDH-miR-21 injection group (Fig. 3A), confirming successful *in vivo* delivery of miR-21. In contrast to the dramatic drop in body weight in control and empty vector pMDH-treated mice, a slight increase in myocarditis was shown in pMDH-miR-21-treated mice (Fig.3B), accompanied by reduced myocardial inflammation (Fig.3C), lower myocarditis score (Fig.3D), improved survival rate and increased heart viral load (Fig.3E and 3F). These data indicated that miR-21 administration substantially improved CVB3-induced myocarditis despite of promot-

FIGURE 3. MiR-21 treatment alleviated CVB3-induced myocarditis. Mice ($n=5$ per group) were injected with pMDH-miR-21 at day 1 post-infection, and myocardial miR-21 expression was detected by real-time PCR on day 7 (A). Meanwhile, myocarditis severity was evaluated on day 7 by indices of body weight loss (B), myocardial pathological observation (C) and myocarditis score (D) as well as survival rate (E). Heart viral load was also determined on day 7 (F). For survival rate evaluation, each group contained ten mice. The experiments were repeated three times with similar results. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$.

FIGURE 4. MiR-21 treatment significantly reduced CVB3-induced myocardial apoptosis. Mice (n=5 per group) were given pMDH-miR-21 at day 1 post-CVB3 infection, on day 7 single-cell suspension of myocardiocytes was prepared, stained with FITC-labelled annexin-V antibody and subjected to FACS analysis. (A) Representative FACS data of annexin-V⁺ apoptotic myocardiocytes in mice. (B) Statistical analysis of the frequency of annexin-V⁺ apoptotic myocardiocytes in mice. The experiments were repeated three times with similar results. **, P<0.01.

ing virus replication.

MiR-21 treatment robustly decreased CVB3-induced myocardial apoptosis

Previous studies have suggested that miR-21 could influence disease by regulating cell apoptosis, miR-21's effect on CVB3-induced myocardial apoptosis was analyzed using flow cytometry. There was a 2-fold decrease in the percentage of apoptotic myocardiocytes in pMDH-miR-21 treated group compared with that of the control group (52.11% and 21.03%, respectively, Fig. 4A and 4B), indicating that miR-21 functioned as an anti-apoptotic factor of myocardiocytes in CVB3 infection. As expected, no obvious change in myocardial apoptosis was shown between the pMDH treated and control mice.

MiR-21 performed its anti-apoptotic effect on CVB3-induced myocarditis by targeting PDCD4

PDCD4 plays an important role in regulating cell apoptosis and is a direct target gene of miR-21. To verify whether PDCD4 is involved in CVB3-induced myocardial apoptosis,

FIGURE 5. MiR-21 treatment obviously reduced myocardial PDCD4 expression in CVB3-induced myocarditis. (A) Mice (n=5 per group) were treated with pMDH-miR-21 at day 1 post-infection, hearts were collected at day 7 and subjected to immunohistochemistry assays for PDCD4 detection. Representative immunohistochemistry observation of each group was shown. (B) Mice were treated with pcDNA3.1-PDCD4 at day 1 post-infection, apoptotic myocardiocyte percentage was detected by FACS assays. The experiments were repeated three times with similar results.

its expression in CVB3-infected heart tissues was investigated using immunohistochemistry staining assays. As shown in Fig. 5A, CVB3 infection significantly elevated myocardial PDCD4 expression, while this increase could be robustly abolished by pMDH-miR-21 administration evidenced by the obviously fewer PDCD4 positive cells in hearts. In addition, when PDCD4 expression in CVB3-infected mice was rescued by pcDNA3.1-PDCD4 administration, the apoptotic myocardiocyte frequency was significantly increased (67.7%) compared with mice receiving empty vector pcDNA3.1 (44.1%) or no treatment (41.2%) (Fig. 5B), indicating that miR-21 exerted its anti-apoptotic effect on viral myocarditis by reducing target PDCD4 expression.

Discussion

In this study, the altered myocardial expression of several myocardium-associated miRNAs in CVB3-induced myocarditis is described. Three downregulated (miR-21, -199a, -320) and five upregulated miRNAs (miR-29b, -499, -210, -126-5p, -92a) were identified, suggesting that miRNAs could be modulated by CVB3 infection and may participate in the pathogene-

sis of viral myocarditis. Among these miRNAs, miR-21 has been proven to play a role in infectious [26] and inflammatory heart disease [27] and was the miRNA whose expression dropped most significantly. In addition, the altered miR-21 expression was more likely due to CVB3-induced myocarditis because no significant reduction in miR-21 was seen at the early stages of infection, with the maximum viral replication (day 3), but was observed at the late infection stage, with the maximum myocardial inflammation (day 7). For this reason, the next steps in the study focused on miR-21.

Myocardial miR-21 expression was significantly reduced in CVB3-infected mice, and the more pronounced the myocarditis was, the greater the decrease in myocardial miR-21 levels was, indicating a negative correlation between miR-21 expression and myocarditis severity (Fig.2). To further evaluate the role of miR-21 in CVB3-induced myocarditis, pMDH-miR-21 was administered to the infected mice and led to significantly alleviated myocarditis. It is known that apoptosis and necrosis both contribute to the cell death in myocarditis [3]; however, a change in degree of necrosis was not seen with miR-21 levels over the course of infection indicating the protection role of miR-21 in CVB3-induced myocarditis might depend on regulation of apoptosis. It has been reported that CVB3 infection can dramatically induce myocardial apoptosis via death receptor-mediated and mitochondrial-mediated signaling pathways [28-30], and apoptosis is also often evidenced in patients of acute myocarditis [31]. Additionally, anti-apoptosis approaches significantly improved CVB3-induced heart injury [32, 33], further confirming the pathological role of apoptosis in the acute myocarditis. Our results were in line with previous studies that show miR-21 targeting PDCD4 to produce anti-apoptotic effect; however, these studies are mainly focused on the fields of tumor [34-36] and vascular diseases [37, 38]. Only a few studies report that miR-21 exerts an anti-apoptotic effect by targeting PDCD4 in heart diseases. Furthermore, all of these studies report a role of miR-21 in non-infectious heart diseases, such as myocardial ischemia-reperfusion injury [39, 40] and H₂O₂-induced myocardial injury [41]. In the present study, CVB3 infection significantly reduced the expression of myocardial miR-21, which protected infected myocytes from apoptosis. This is the first time miR-21 has been shown to perform its anti-apoptotic effects by targeting PDCD4 in viral myocarditis and thereby extending its anti-apoptotic effects to the field of infectious heart diseases. Nevertheless, the possibility that miR-21 may also perform its protective effect on CVB3-induced myocarditis by other pathways like promoting myocyte outgrowth [42] and upregulating important cardioprotective proteins [43] cannot be excluded.

MiRNAs exert their biological functions by regulating their multiple target genes. It has been reported that the tumor suppressor PDCD4 is an important functional target of miR-21 [44] and participates in the apoptosis of many cell types [45, 46]. Recently Cheng *et al.* [41] reported that miR-21 suppressed the H₂O₂-induced myocardial apoptosis via targeting PDCD4. miR-21 may, therefore, perform an anti-apoptotic role in viral myocarditis by inhibiting PDCD4. As illustrated by the immunohistochemistry results, the number of PDCD4-positive myocytes was significantly reduced in mice receiving pMDH-miR-21, indicating that miR-21 efficiently inhibited its target PDCD4 expression and decreased the subsequent myocardial apoptosis. Other apoptosis-associated targets of miR-21, such as tumor suppressor phosphatase and tensin homolog (PTEN) [47] and Fas ligand and metalloproteinase inhibitor 3 (TIMP3) [48], may also participate in CVB3-induced myocarditis, and further studies need to be conducted for validation.

In this study, myocardial miR-21 expression was significantly decreased in CVB3-induced myocarditis and negatively associated with the disease severity. MiR-21 treatment could significantly relieve viral myocarditis by inhibiting PDCD4-mediated myocardial apoptosis. This study may help us better understand the pathogenesis of myocarditis and represent a new therapeutic strategy against CVB3-induced myocarditis.

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