Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Rosmarinic acid attenuates TNF-α induced cardiomyocyte injury via regulating miR-344a-3p

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Article Info

Article history:

Received: November 16, 2023
Accepted: January 13, 2024
Published: January 31, 2024

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Abstract

To investigate whether rosmarinic acid protects cardiomyocytes from inflammatory damage through miRNAs, high-throughput sequencing and bioinformatics analysis were performed to identify TNF-α-induced inflammatory damage in cardiomyocytes and miRNAs differentially expressed in TNF-α-induced inflammatory injury in cardiomyocytes, and the bioinformatics analysis shown that the expression levels of 10 miRNAs were significantly up-regulated, and the expression levels of 6 miRNAs were significantly down-regulated. Among them, the expression level of miR-344a-3p was significantly up-regulated in the experimental group, while the expression level of miR-449c-5p was significantly down-regulated in experimental group of cells. The target genes of miR-344a-3p and miR-449c-5p were CCR1 and ATP2B4 respectively. The luciferase reporter system showed that luciferase activity in the WT-CCR1+miR-344a-3p mimic group was significantly decreased, and the expression of CCR1 was significantly decreased at mRNA and protein level after miR-344a-3p was transfected into H9C2 cells, indicating that TNF-α-induced inflammatory injury in cardiomyocytes, rosmarinic acid may up-regulate the expression of miR-344a-3p, thereby inhibiting the expression of CCR1 and ultimately protecting the cardiomyocytes from inflammatory damage. Thus, we thought that CCR1 might be a new therapeutic target for cardiomyocyte injury.

Keywords: miRNAs, Myocardial injury, Rosmarinic acid, TNF-α

1. Introduction

Cardiovascular diseases (CVDs) are one of the leading causes of death around the world, and affect around 18.5 million people, even one-third over 70 ages old die due to this disease [1]. Nowadays, mainly treatment for CVD includes pharmacotherapy, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG), which aims to restore the myocardial perfusion in cardiomyocytes, however, myocardial perfusion in cardiomyocytes might also induce the damage to cardiomyocytes, also known as myocardial ischemia reperfusion injury (MIRI) [2].

A recent study showed that the concentration of inflammatory factors in related to the damage level and number of apoptotic cells, indicating that the inflammatory process plays and important role in the regulation of MIRI [3]. This process would further aggravate the injury of MIRI. TNF-α is a pro-inflammation cytokine, which has been proven to perform various biological functions, including anti-tumor, anti-virus and inflammation response processes, as well as regulation of MIRI [4]. Rosmarinicacid (RA) is a polyphenolic acid that exists in Labiaceae, comfrey and Umbelliferae, and has been widely used in food, medicine and cosmetics and other fields, presents multiple biological functions, including anti-oxidative, anti-apoptosis and anti-tumor [5]. The previous study showed that RA could reduce the accumulation of TNF-α, IL-6 and IL-1β, further increase the activity of superoxide dismutase (SOD), and protects the lung from damage induced by lipopolysaccharide [6]. This study demonstrated that RA protects brain and heart tissues from injury via anti-oxidative stress and anti-inflammation function. miRNA is a family of endogenous non-coding small molecule RNA, and widely exists in flora and fauna, regulates the expression of target genes via degradation or inhibition the translation process of mRNA [7], previous studies have shown that miRNA-133 inhibits the expression of caspase-3, reducing the apoptosis of cardiomyocytes in MIRI site [8]. However, the effect of miRNA in the treatment of MIRI was not clear. In this study, we screened the differential expressed miRNA in the inflammation model of cardiomyocytes induced by TNF-α and found that miR-334a-3p is closely related to the inflammation response of cardiomyocytes via inhibition of CCR1 expression. Thus, we thought CCR1 might be a therapeutic target of MIRI.

2. Material and methods

2.1. Cell culture

H9c2 (GNR 5) cells were purchased from the China Center for Type Culture Collection. Cells were cultured in H-DMEM (11960044, Thermo) cultured medium supplied with 10% FBS (10091, Thermo) under 37°C atmosphere

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.1.28

supplied with 5% CO_2.

2.2. MTT assay

In order to screen a suitable concentration of TNF-α to construct TNF-α stimulation model, MTT (M8180, Solarbio) assay was performed. Briefly, cells were firstly seeded into a 96-well plate at a concentration of 10^5 each well, and divided into seven groups and treated with different concentrations of TNF-α (0, 12.5, 25, 50, 100, 250, 500 ng/mL, T6674, Sigma) for 6 h or followed with different concentration of rosmarinic acid (0, 25, 50, 100 μg/mL, R4033, Sigma) for 48 h. After treatment, cells were incubated with MTT reagent for 4 h, and the OD value at 490 nm was measured with a Multiskan FC microplate reader.

2.3. Detection of CK-MB and Troponin I in cultured medium of H9c2 cells

The concentration of CK-MB was detected using CK-MB (E4608, Biovision) and Troponin I (E4737, Biovision) detection kits. Briefly, cells were divided into three groups including normal control, signal TNF-α treatment group and TNF-α combined rosmarinicacid treatment group. Cells were seeded into a 100 mm plate and then cells were treated with 250 ng/ml TNF-α for 6 h in signal TNF-α treatment group and cells were treated with 250 ng/ml TNF-α for 6 h followed by treatment with 50 μg/ml rosmarinicacid for 48 h in TNF-α combined rosmarinicacid treatment group. Then cultured medium was collected and added into each well of a 96-well plate after treatment. After incubation for 2 h, samples were discarded and the detection antibody was added into each well and incubated at 37°C for 1 h. After washed with washing buffer, samples were incubated with working solution at 37°C for 30 min, and incubated with TMB substrate at 37°C for 30 min away from light. The OD value at 450 nm was measured after the stop solution was added.

2.4. Detection of apoptotic cells using flow cytometry

Cells were seeded into a 100 mm plate and divided into three groups as previously described, and flow cytometry was performed using ANNEXIN V- FITC/PI detection kits (CA1020, Solarbio). Briefly, cells were suspended with binding buffer and then incubated with Annexin V-FITC and PI reagent, and then apoptotic cells were detected.

2.5. Total RNA extraction

RNA extraction was performed using the TRIzol (93289, Sigma) method. Briefly, cells were first seeded into a 100 mm plate and divided into three groups as previously described. Cells were firstly incubated with TRIzol and chloroform, after centrifugated at 12000 rpm for 10 min, the water phase of samples was collected into a new tube and incubated with isopropanol for 10 min. The supernatant was removed after centrifugated at 10000 rpm for 5 min, and RNA samples were collected after resuspending with DEPC-water. Samples were stored at -80°C until the following experiments.

2.6. High-throughput sequencing and bioinformatics analysis of miRNA

High-throughput sequencing was performed using Illumina HiSeq 2500 (Illumina) followed by the construction of DNA library. miRNA was isolated using miRNA extraction kit according to the protocol. After electrophoresis, adapters of 3' and 5' ends were ligated with RNA samples, and the production was used to perform reverse transcription polymerase chain reaction (RT-PCR). Production of PCR sized from 140 to 160 bp was used to construct the cDNA library [9]. In order to acquire clean tags, the results of sequencing were flattered. The tags were aligned with miRNA using miRBase version 21 (http://www.mirbase.org), Rfam12.1 (http://rfam.xfam. org), and piRNAbank (http://pirnabank.ibab.ac.in) to acquire the information of miRNA samples. Mireap (version 2.0) software to screen novel candidate cite of miRNA (in order to remove isomers, hairpin structure, and secondary structure).

2.7. Data analysis and pathway enrichment

P value < 0.05 were set to screen the differential expressed miRNAs and the target genes were predicted using TargetScan (http://www.targetscan.org/) and miRanda (microRNA.org), data of differentially expressed miRNAs were visualized using Cytoscape (version 3.3.0) [10]. Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to analyze the function and pathways of miRNAs using DAVID database (https://david.ncifcrf.gov/).

2.8. Transfection of target genes and detection of luciferase

Cells were cultured as previously described, then cells were transfected with wide type and mutation type of target genes using Lipofectamine 2000 transfection reagent for 48 h. After transfection, the activity of luciferase was detected using the Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System. Briefly, cells were incubated with Dual-Glo reagent at room temperature for 15 min, and the activity was detected using a Multiskan FC microplate reader.

2.9. Detection of target gene expression using real-time quantitative PCR detecting system (qPCR)

Cells were divided into two groups and transfected with wide type or mutation type of target genes of miRNA, and the RNA samples were isolated as previously described. The qPCR experiment was performed using one-step RT-PCR kit (T2240, Solarbio) according to the protocol, the reaction buffer was performed as recommended, and the reaction steps were listed as follows: reverse transcription at 50°C for 20 min, degeneration at 95°C for 3 min, repeat following steps for 50 cycles: degeneration at 95°C for 15 s, annealing at 58°C for 20 s and extending at 72°C for 30 s. Primers used in this experiment are listed in Table 1. Expression of each target gene was calculated using the $2^{-\Delta\Delta Cq}$ method [11].

2.10. Protein sample collection and Western blotting analysis

Cells were divided into two groups and cultured as previously described. Then, cells were lysed with RIPA buffer supplied with a proteinase inhibitor cocktail, and protein samples were collected after centrifuged at 12000 rpm for 10 min. The concentration of protein samples was measured using BCA assay, and 100 μg of protein sample was separated using 10% SDS-PAGE. After electrophoresis, protein samples were transferred onto a PVDF membrane, and followed by incubation with 5% skim milk for 1 h at room temperature. Then, membranes were incubated with

Table 1. List of primers.

Name	Primer sequences
rno-miR-129-5p-F	5′-ACACTCCAGCTGGGCAAAGTGATGAGTAATAC-3′
rno-miR-129-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAGCCAGT-3′
rno-miR-344a-3p-F	5′-ACACTCCAGCTGGGCTCCGGGACGG-3′
rno-miR-344a-3p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCCCAGCC-3′
rno-miR-708-5p-F	5′-ACACTCCAGCTGGGAATGGATTTTTGG-3′
rno-miR-708-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTGCTCC-3′
rno-miR-708-3p-F	5′-ACACTCCAGCTGGGTCCCACTACTTCAC-3′
rno-miR-708-3p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCACAAGT-3′
rno-miR-6215-F	5′-ACACTCCAGCTGGGCTGGGCCCGCGGCGGGC-3′
rno-miR-6215-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCCACGC-3′
rno-miR-3558-5p-F	5′-ACACTCCAGCTGGGGCGGGGCTGGG-3′
rno-miR-3558-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGCGCGCC-3′
rno-miR-222-5p-F	5′-ACACTCCAGCTGGGCAGTGCCTCGGCAGTG-3′
rno-miR-222-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGCTGCA-3′
rno-miR-449c-5p-F	5′-ACACTCCAGCTGGGCGGGCGTGGTGGTG-3′
rno-miR-449c-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACCCCCA-3′
rno-miR-222-3p-F	5′-ACACTCCAGCTGGGTCATTTTTGTGATGTT-3′
rno-miR-222-3p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCTGCAA-3′
rno-miR-92a-1-5p-F	5′-ACACTCCAGCTGGGCACTGGCTCCTTTCTG-3′
rno-miR-92a-1-5p-R	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCTACCCA-3′
U6-F	5′-CTCGCTTCGGCAGCACA-3′
U6-R	5′-AACGCTTCACGAATTTGCGT-3′

primary antibody (CCR1 (Biovision, 3414-100), 1:1000) at 4 ℃ overnight followed by incubation with secondary antibody for 1 h at room temperature. The gray value of protein was detected using chemiluminescent immunoassay.

2.11. Statistical analysis

All the experimental results in this study were statistically analyzed using SPSS 21.0 software. Each experiment was repeated three or five times independently, the measured data were represented as mean standard deviation. In the FACS and WB experiment, the experiment was repeated three times and the other experiment was repeated five times. The one-way ANOVA method was used to compare the differences between groups. P<0.05 was set as a significant difference.

3. Results

3.1. Effect of TNF-α and rosmarinic acid on H9c2 cell viability

As shown in Figure 1A, B, the proliferation of H9c2 cells under 250 ng/ml TNF-α treatment was significantly decreased compared with normal group (P<0.05), and cells under 50 μg/ml of rosmarinic acid treatment was significantly increased compared with normal group (P<0.05). Hence, we chose the 250 ng/ml TNF-α and 50 μg/ml rosmarinic acid treatment to perform the following experiments.

3.2. Detection of model construction

After model construction, we detected characteristic index of myocardial injury in H9c2 cells. The concentration of CK-MB in TNF-α treatment group was significantly increased compared with control group (P<0.05) and the concentration was significantly decreased in rosmarinic acid treatment group (P<0.05, Figure 2A). Besides, the changes in concentration of cTn-T presented a similar trend with CK-MB (Figure 2B). We further detected the apoptosis cells in each group using flow cytometry. The apoptotic cells in TNF-α treatment group were significantly increased compared with control group (P<0.05), while were significantly decreased in rosmarinic acid treatment group (P<0.05). These results proved that myocardial injury model was successfully constructed in H9c2 cells, and RA plays a protective role in cardiomyocytes via inhibition of the cellular apoptosis process (Figure 2C). The expression of CCR1 (CC Chemokine receptor 1) in model and RA treatment groups was also detected, and the expression of CCR1 was significantly increased in the RA treatment group while markedly decreased in model group (Figure 2D).

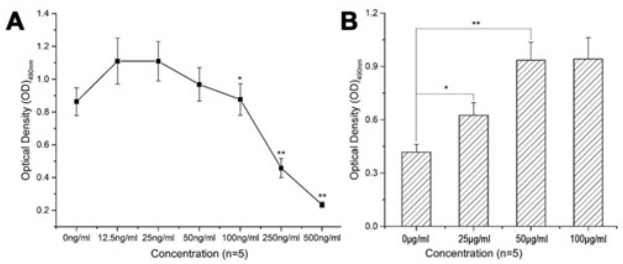

Fig. 1. Proliferation of H9c2 cells under TNF-α and rosmarinic acid treatment. (A) Proliferation of H9c2 cells under different doses of TNF-α treatment. (B) Proliferation of H9c2 cells under different doses of rosmarinic acid treatment. *P<0.05 compared with control group, **P<0.01 compared with control group. One ANOVA analysis was performed to compare the differences between groups.

3.3. Detection of length and distribution of RNA samples

After extraction, we detected the length distribution of the RNA samples. We noticed that most of the RNA samples were 19-25 nt, and the peaking presents at 22 nt (Figure 3). These results showed that the purity and quality of RNA samples were suitable for sequencing analysis.

3.4. Distribution of difference expressed miRNAs between groups

Using bio-information analysis, we noticed that these were 10 miRNAs were significantly increased in RA combined with TNF-α treatment group compared with TNF-α treatment group, including miR-129-5p, miR-344a-3p, miR-708-5p, miR-708-3p, miR-6215, miR-504, miR-193b-3p, miR-1843b-5p, miR-10a-3p, miR-34c-3p, besides, there were 6 miRNAs significantly decreased between these two groups, including miR-3558-5p, miR-222-5p, miR-449c-5p, miR-222-3p, miR-92a-1-5p, miR-146a-5p (Figure 4A). We further detected the expression of these miRNAs in TNF-α treatment group and RA combined with TNF-α treatment group in H9c2 cells, and we found that the expression of miR-344a-3p was significantly increased (P<0.01) and the expression of miR-449c-5p was significantly decreased (P<0.01) (Figure 4B), thus we use these two miRNAs as the target of our present study.

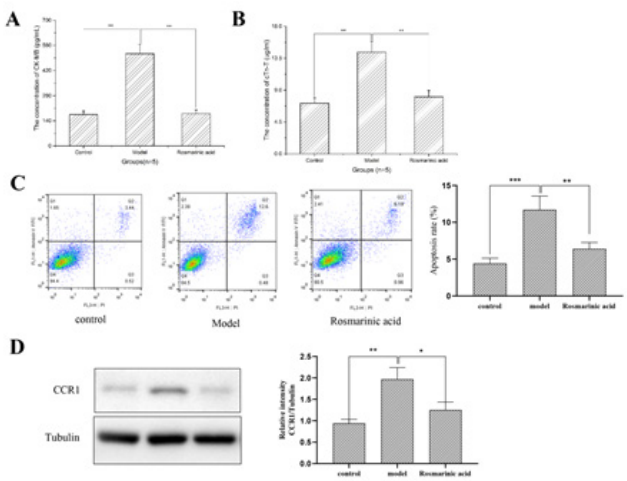

Fig. 2. Detection of markers of cardiomyocytes injury. (A) Expression of CK-MB in each group. (B) Expression of cTn-T in each group. (C) Apoptosis of cells in each group. (D) The expression of CCR1 in each group. *P<0.05 compared with control group, **P<0.01 compared with control group. One ANOVA analysis was performed to compare the differences between groups.

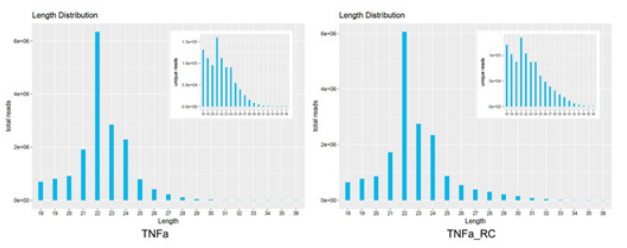

Fig. 3. Length distribution of RNAs in TNF-α and rosmarinic acid treatment. The results showed that the length range of small RNA in both the control group and the experimental group was mostly 19-25 nt, and the peak value was 22 nt, indicating that the purity of the samples in both groups was high, and there were no low-quality sequences in the original sequence of the samples in both groups.

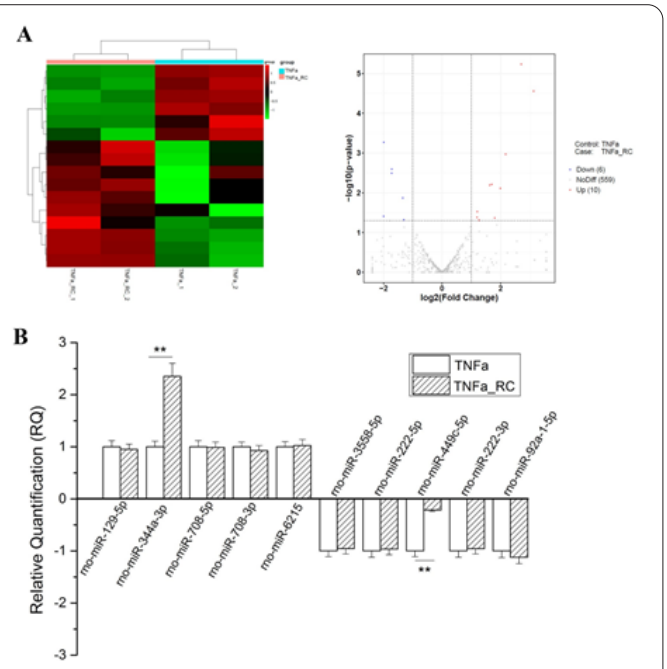

Fig. 4. Differentially expressed miRNAs in TNF-α and rosmarinic acid treatment. (A) Heat map and Volcano plot analysis of differentially expressed miRNAs. The expression of 10 miRNAs was significantly increased, and the expression of 6 miRNAs was significantly decreased. Increased expression: miR-129-5p, miR-344a-3p, miR-708-5p, miR-708-3p, miR-6215, miR-504, miR-193b-3p, miR-1843b-5p, miR-10a-3p, miR-34c-3p; decreased expression: miR-3558-5p, miR-222-5p, miR-449c-5p, miR-222-3p, miR-92a-1-5p, miR-146a-5p. (B) Detection of differentially expressed miRNAs in H9c2 cells. The expression of miR-344a-3p was significantly increased and the expression of miR-449c-5p was significantly decreased. **P<0.05 compared with TNF-α treatment group. Each experiment was repeated for 3 times. One ANOVA analysis was performed to compare the differences between groups.

3.5. Gene ontology (GO) analysis of target miRNAs

Using GO analysis, there were 106 target genes of miR-344-3p, which regulates the function of multiple biological functions such as ribonucleoprotein, protein dimer, and cytoplasmic ribonucleoprotein. Using KEGG pathway analysis, we found that plenty signaling pathways were enriched, including the hedgehog signaling pathway, β3 adrenergic receptor pathway and 5-hydroxytryptamine degradation signaling pathway (Figure 5A). There were 76 target genes of miR-449c-5p, which regulates multiple biological functions such as cellular migration, formation of Golgi apparatus and stimulation of vascular endothelial growth factor. Using KEGG pathway analysis, we found that multiple signaling pathways were enriched, including vascular endothelial growth factor signaling pathway, epidermal growth factor receptor signaling pathway and mitogen-activated protein kinase pathway (Figure 5B).

3.6. Verification of target genes using dual luciferase reporter gene system

Among these target genes, we found that CCR1 was one of the target genes of miR-344a-3p, and ATP2B4 was one of the target genes of miR-449c-5p (Figure 6A). Using dual luciferase reporter gene system, we detected the expression of CCR1 and ATP2B4 in H9c2 cells, and the

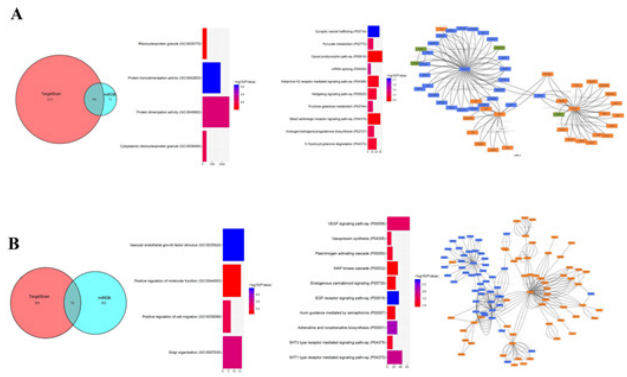

Fig. 5. Bioinformatics analysis. (A-B) GO analysis and KEGG signaling pathway enrichment of miR-344a-3p and miR-449c-5p.

expression of CCR1 was significantly decreased in cells transfected with wide type of CCR1 (P<0.05), while the expression was not significantly changed in cells transfected with mutation type of CCR1 (P>0.05) compared with control group. However, the expression of ATP2B4 was not significantly changed in cells transfected with wide type and mutation type of ATP2B4 compared with control group (Figure 6B).

3.7. Detection of CCR1 expression in H9c2 cells

After transfected with miR-344a-3p mimic, we noticed that the expression of CCR1 was significantly decreased in transfection group compared with control group (P<0.05) at mRNA and protein level (Figure 7A, B).

4. Discussion

Cardiovascular diseases (CVDs), with their high incidence rate, high disability rate, high mortality and serious complications, pose a serious threat to human health around the world. Ischemia of cardiomyocytes is the main pathological change of CVD, resulting in the damage of cardiomyocytes, injury in cardiac function, and even death of patients. RA is a natural pharmacological product, that presents anti-oxidative, anti-inflammation and regulation of immune response function, as well as cardiac protective function [12]. Here, we established a cell model of cardiac injury by inflammation response in H9c2 cells using TNF-α, and found that RA might present a protective role via up-regulation of miR-334a-3p, further down-regulation of CCR1, thus performing a protective role in cardiomyocytes under inflammation stimulation.

RA is synthesized by caffeic acid and 3, 4- dihydroxyphenyllactic acid, which is contained in rosemary, perilla, sage and mint [13]. It is well known that RA regulates the inflammation response process via interaction with complement pathways in vivo and in vitro [14]. A recent study found that RA treatment in a model of sepsis in RAW264.7 cells reduced the expression of TNF-α, IL-6 and high-mobility group box 1 protein via inhibition of NF-κB signaling pathway [15]. Another study found that using an ischemic stroke model in SH-SY5Y, RA reduced the apoptosis and cytotoxicity of cells induced by oxygen-glucose deprivation, and blocked the activation of NF-κB via TNF-α dependent manner [16]. Besides, RA was also proven to reduce the mortality of mice through down-regulation of IL-6, LI-12, TNF-α, IFN-γ and MCP-1 expression [17]. A recent study showed that RA protects cardiomyocytes from hydrogen peroxide-induced DNA damage and apoptosis, as well as attenuates the pressure overload-induced cardiac dysfunction [18], while the exact mechanism was not clear. Using bio-information analysis, we noticed that the expression of miR-344a-3p was significantly increased while the expression of miR-449c-5p was significantly decreased. Combined with the results of luciferase, we thought that miR-344a-3p regulates the expression of CCR1 is the possible mechanism of treatment of RA in cardiac ischemia.

CCR1 is a kind of G protein-coupled receptor predominantly expressed in monocytes, T cells, dendritic cells and neutrophils, and thought to be regulated calcium flux via Gi/o coupled pathway. Besides, CCR1 also regulates tissue damage and inflammation through activation of T cells [19]. Using pharmacological inhibitors of CCR1, researchers found that inhibition of CCR1 expression was useful in treatment of multiple sclerosis, progressive kidney disease, lung injury, fungal asthma and rheumatoid arthritis [20,21], thus CCR1 is considered as a therapeutic target. CCR1 inhibitor such as BX-471 and CP-481715 has been used in clinical trials. However, BX-471 did not present a significant efficacy in Phase II clinical trials in relapsing-remitting multiple sclerosis patients [22], and CP-481715 also failed to present a clinical efficacy in the treatment of patients of rheumatoid arthritis [23]. Here, we noticed that treatment of RA up-regulates the expression of miR-334a-3p, further decreasing the expression of CCR1, resulting in the alleviation the damage of H9c2 cells induced by TNF-α, we thought that miR-334a-3p combined with RA might be a therapeutic method for MIRI.

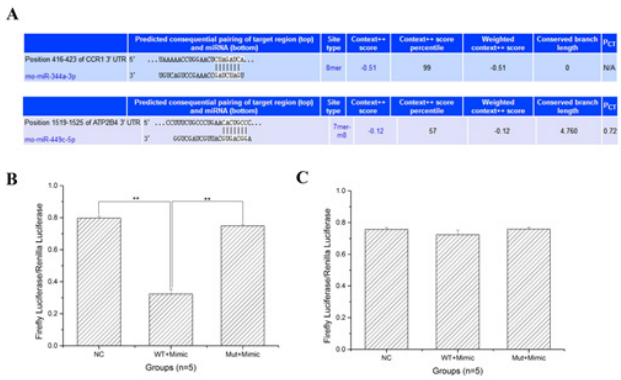

Fig. 6. Dual luciferase reporter gene analysis of target genes. (A) Bioinformatics prediction of binding sites, (B) Fluorescence intensity of CCR1. (C) Fluorescence intensity of ATP2B4. **P<0.05 compared with wide type transfection group. One ANOVA analysis was performed to compare the differences between groups.

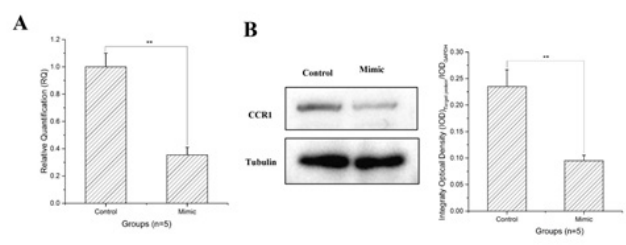

Fig. 7. Expression of CCR1 in H9c2 cells at mRNA. (A) and protein level (B). **P<0.05 compared with control group. Each experiment was repeated 5 times. One ANOVA analysis was performed to compare the differences between groups.

5. Conclusion

From the present research, we thought that CCR1 might be a new therapeutic target of cardiomyocyte injury. However, in the present use of the cell line of rat cardiomyocytes, and in the following experiments, we would use primary cell of rat cardiomyocytes and also collect the clinical sample from patients to explore the detailed mechanism of the present results.

Conflict of interest

All authors declared no conflict of interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Funding

This study was supported by the Medical Innovation Application Research Project of Suzhou Science and Technology Bureau (NO. SKY2022188 and SKY2022131) and Research project of Jiangsu Provincial Health Commission (NO. M2022042).

Acknowledgments

None.

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