Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

The mechanism of miR-525-5p derived from hypoxia and reoxygenation in H9c2 Cardiomyocytes

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ARTICLE INFO

Original paper

Article history:
Received: August 28, 2021
Accepted: October 28, 2021
Published: December 01, 2021

Keywords:
miR-525-5p; ischemia-reperfusion injury; H9c2; mitochondria

ABSTRACT

Ischemia-reperfusion injury (IRI) is associated with ischemic heart disease (IHD) which leads to patients a poor progression. According to Pubmed Datasets, we analyzed different gene and mRNA expressions in IHD patients with IRI. The relevant mRNA expression detected in H9C2 cells undergo hypoxia and reoxygenation, we selected and structured miR-525-5p gene mutation H9C2 cells, the results performed miR-525-5p mutated restored H9C2 metabolism of mitochondria which detected by relevant genes and proteins. At the same time, miR-525-5p silence resisted hypoxia and reoxygenation induced H9C2 cells apoptosis. All the results indicated miR-525-5p maybe protect H9C2 cells without hypoxia and reoxygenation induced injury through regulating the mitochondria metabolism.

DOI: http://dx.doi.org/10.14715/cmb/2021.67.4.3

Introduction

Ischemic heart disease (IHD) still occupies and represents a large burden on individuals' healthy condition worldwide (1). Although the strategies and treatments such as Interventional therapy, bypass surgery, decrease the mortality of IHD (2,3), the negative condition initiated with treatments endangers patients' daily life and clinical outcomes. Ischemia-reperfusion injury (IRI) emerged as a common condition that appears in patients undergoing IHD (4). The classical drugs for the prevention of ischemia include calcium antagonists, β-receptor blockers and angiotensin-converting enzyme inhibitors limited for application, for the side effects and ethical conditions (5). Present days, there remains no effective strategies for IRI.

Micro-RNA a set of endogenous gene encoding RNA, regulate mRNAs' transcription. According to previous reports, microRNA participated in tumors' initiation and progression (6,7), promoted Alzheimer's disease deterioration (8,9), induced Cardiovascular diseases' generation (10,11). The stability of the microRNA 3'UTR sequence maintains the metabolism of the internal environment. This research will investigate the role of miR-525-5p 3'UTR in IRI conditional H9c2 cell's metabolism of mitochondria function and impaction on the H9c2 cells' apoptosis.

Materials and methods

Datasets screened and biological process, molecular function, relative signaling pathway assay

Pubmed database used for screened relative datasets which refer to IRI patients' gene expression-GSE47861, NetworkAnalyst and MetaScape online software analyzed BP, MF and enriched signaling pathway of KEGG and GO, we limited the baseline at a condition:

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Cell culture and treatment

H9C2 cells were maintained with DMEM-high glucose medium with a final concentration 10% FBS and 1% penicillin, streptomycin at 37 ℃ an incubator with 5%CO2. For hypoxia and reoxygenation condition, according to the previous reports, cells incubated at a DMEM medium with 1% FBS maintained in a hypoxia incubator for 2 h as acute hypoxia, the condition of incubator filled with a gas mixture of 5% CO2 and nitrogen to obtain 1% oxygen concentration. After hypoxia, exchanged cells into total medium (DMEM with 10% FBS) and maintained at 37°C in an atmosphere of 21% O2 and 5% CO2 for reoxygenation for 0–360 min.

Micro-RNA 3'UTR mutated cell line's establishment:

Western blotting assay: The expression of mitochondria metabolism was detected by western blotting, the concentration: 30 μg/mL of lysate electrophoresis on SDS-PAGE (10% lower gel and 5% upper gel) for 110min at 100V, PVDF suspended in methanol for transmembrane. 5% BSA used for blocking the none specific binding bits which were maintained at room temperature for 1h, primary antibody incubated the PVDF membrane overnight (1:1000). The HRP-secondary antibody used for combined with primary antibody (1:5000) at room temperature for 1h, ECL system was detected for the protein expression.

Real-time PCR assay

TRIzol lysate was used for assessing and isolating the total RNA, TianGen Quant SYBR Green PCR Kit was used for cDNA assessment. Total concentration 10ng/mL cDNA mixed with SYBR mix, primers, ddH2O for 95°C 30s, 90°C 50s, 72°C 5min maintained 25-30 cycles. The levels of amplification were calculated by the formula: 2–ΔΔCT.

Cell proliferation assay

Incubated cells into 96 wells culture plates maintained with total medium for 24h, 50μg/mL MTT covered with medium for 2h, SDS-lysate solved the compounds and detected at 490nm absorbency by a microplate reader. The proliferation formula: P%= control OD value/ observation OD value×100%.

Apoptosis experiment

FCM assay

Annexin IV/PI probe used for detected cells' apoptosis condition by flow cytometry. Incubated cells into 6 wells culture plates maintained for 24h undergo with hypoxia and reoxygenation, RIPA lysate isolated for the cell suspension, 3-5μL Annexin IV probe-stained suspension for 20min without lights, 3μL PI probe-stained suspension for 15min, FCM detected percentage of apoptosis.

TUNEL stain

Colorimetric TUNEL Apoptosis Assay Kit was used for detected the condition of apoptosis. Incubated cells as the above protocol, and fixed with 4% polyformaldehyde for 10min, final concentration 0.1% Triton-X100 used for knocking membranes. A volume included TdT enzyme, Biotin-dUTP, Biotin-labeled liquid stained cells for 30min at 37°C incubators. Lionheart microscopy detected red fluorochrome density.

Results and discussion

Different gene and micro-RNA expression on GSE47861

Pubmed Datasets database used for screened and selected GSE47861 datasets, which included 20samples, compared with control samples, 56372 genes differed expression in IRI samples, the volcano map performed the condition (Figure 1A), compared with control samples, 12523 micro-RNA different expression in IRI samples, which performed by volcano map (Figure 1B).

The related signaling pathway, biological process (BP) and molecular function (MF) on GSE47861

MetaScape online software analyzed GSE47861 related BP and MF, the results performed 8 metabolisms refers to IRI samples and 4 functions impact on IRI samples, included mitochondria metabolism (Figure 2A, Figure 2B), the results enriched on KEGG and GO databases, 4 molecular signaling pathway assessed include (Figure 2C).

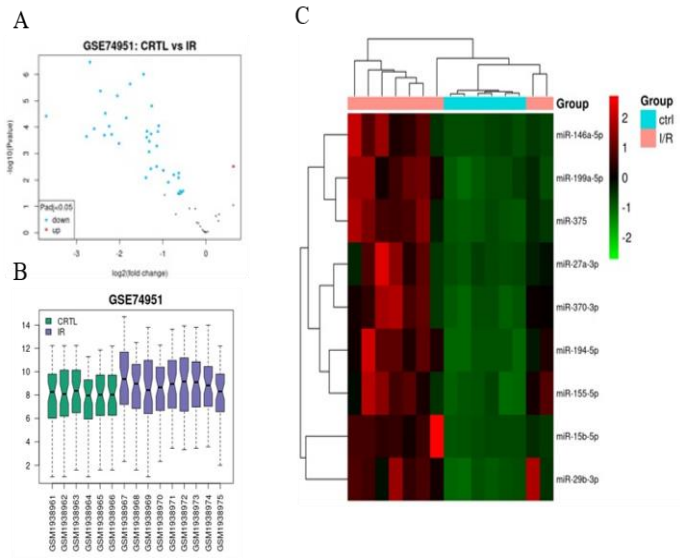

Figure 1. A: GSE74951 datasets screened I/R patients for different miRNA expression, compared with normal. **B**: GSE74951 datasets identified miRNA location in I/R patients, compared with normal. **C**: Top 9 different miRNA regulation in I/R patients, compared with normal.

Figure 2: The related signaling pathway, Biological process (BP) on GSE74951

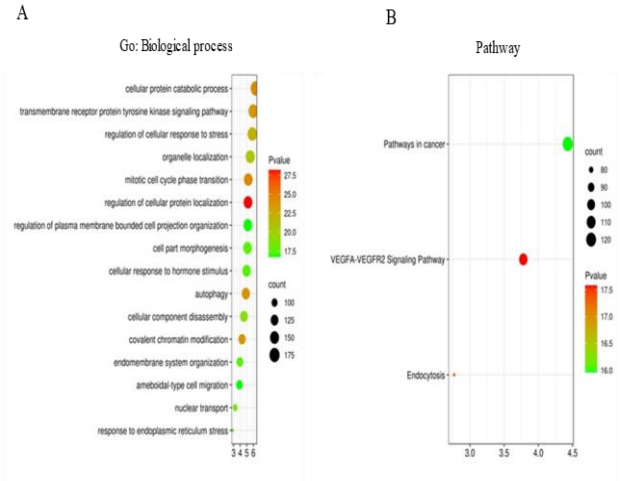

Figure 2. different miRNA Biological process and signaling pathway screened on KEGG and GO database.

The design for miR-525-5p 3'UTR sequence and the effects on cell proliferation

Pubmed database screened the total sequence of miR-525-5p and blasted the primer of miR-525-5p 3'UTR, the 3'UTR sequence performed in Table 1. We structured miR-525-5p mutation H9C2 cell lines (H9C2-mutation, H9C2-NC) which were performed in supplementary Figures and detected the proliferation of the cells at 24h, 48h, 72h, compared with a negative group (H9C2-NC), miR-525-5p mutation-induced H9C2 proliferation (Figure 3).

Table 1. *Design for miR-525-5p 3'UTR*

3´-UTR WT	5´-GGUUGGAAGGUGGGGA-CU CUGGAA-3´
miR-525-5p	3`-UCUUUCACGUAGGGAGACC UC-5´
3´-UTR mut	5´-GGUUGGAGGUGGGGA-CUC AGCAA-3´

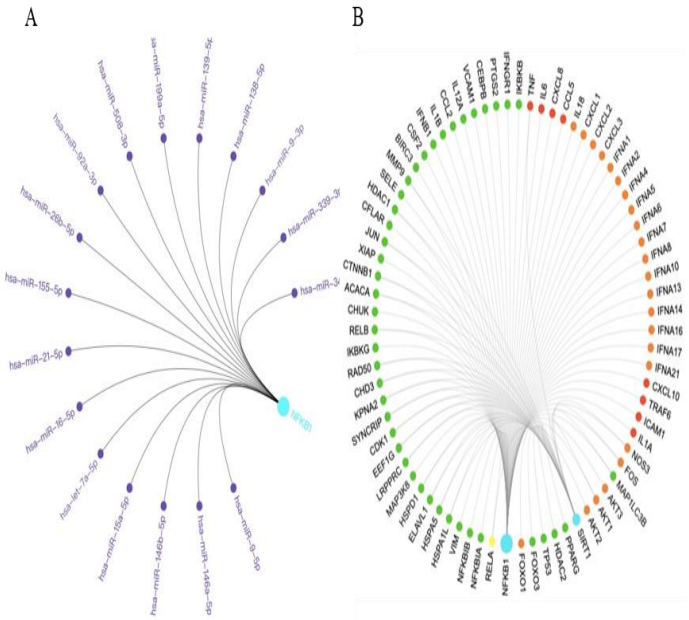

Figure 3. A & B: The prediction of SIRT and NFKB1 protein interaction and relevant miRNA which interacted with NFKB1.

miR-525-5p induced H9C2 cell changes on the function of mitochondria

According to the results (Figure 2), we found that miR-525-5p was associated with mitochondria function and metabolism of energy. Immunoblotting detected the transformation of mitochondria complex proteins and SIRT1 in the miR-525-5p 3'UTR mutated, compared with the negative group, miR-525-5p mutation induce complex I, III, V increased (Figure 4A & B), PCR verified the levels of mitochondria complex with miR-525-5p 3'UTR mutated (Figure 4C).

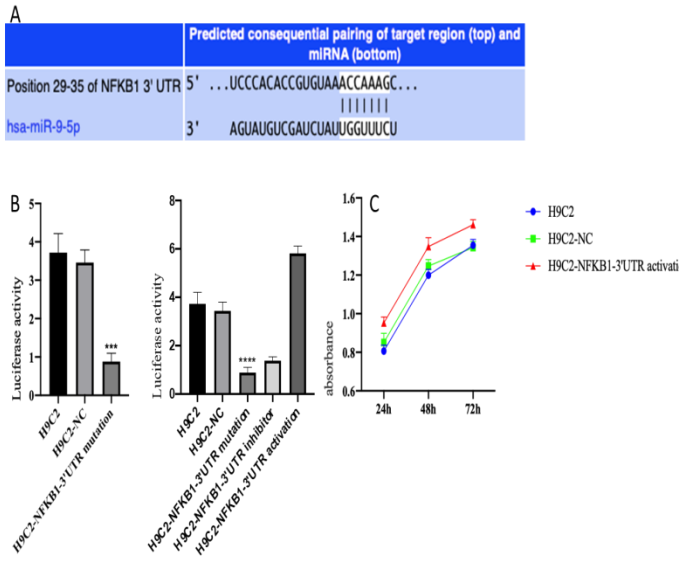

Figure 4. A: TargetScan database predicted hsa_miR_9_5p interacted location with NFKB1 3'UTR. B: Luciferase activity determined NFKB1 3'UTR mutated efficiency, compared with H9C2 and H9C2-NC group, H9C2-NFKB1-3'UTR mutation group, *P<0.01*. C. Cell proliferation analysis detected H9C2 cells, H9C2-NC cells, H9C2-NFKB1-3'UTR mutation cells 24h, 48h, 72h proliferation, compared with H9C2 and H9C2-NC, H9C2-NFKB1-3'UTR mutation cells, *P< 0.01*.

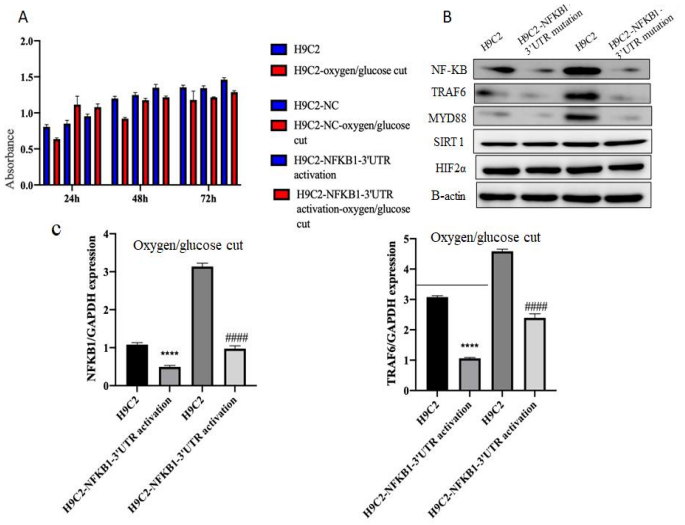

Figure 5. A: Absorbance of H9C2 cells, H9C2-NC cells, H9C2-NFKB1-3'UTR mutation cells undergo oxygen and glucose deprivation. B: The NFKB1/SIRT relevant protein expression of H9C2 cells and H9C2-NFKB1-3'UTR mutation cells. **C**: The NFKB1/SIRT relevant gene expression of H9C2 cells and H9C2-NFKB1-3'UTR mutation cells, compared with H9C2 cells, NFKB1-3'UTR mutation cells, *P<0.05.*

miR-525-5p induce H9C2 cell apoptosis changes

For confirmed miR-525-5p 3'UTR impacts on H9C2 cells anti-apoptosis, FCM (flow cytometry) detected the percentage of H9C2 cells apoptosis on the hypoxia and reoxygenation condition, the results performed H9C2-mutation resisted the injury of hypoxia and reoxygenation compared with negative group and control group (Figure 5A & B), Tunel stain also confirmed the same appearance (Figure 5C).

Ischemia-reperfusion injury (IRI) caused by hypoxia and reoxygenation, the tissues of organs manifested by blood flow-deprived and oxygen-starved organs following the restoration of blood flow (12), according to the previous reports a vast number of molecular mechanisms related to reperfusion injury, such as oxidative stress-induced ROS production, inflammatory signaling pathway activated-NF-kB pathway (13-15). Tissues injury induces the physiological function abnormal could lead to a large burden for the IHD patients. In this study, we analyzed Pubmed Datasets GSE46781, included different gene expressions through high throughput sequencing, the performance indicted 56712 gene expression and 1232 microRNA expression in the IRI samples. The analysis of BP and MF performed, related to the IRI progression, enrichment of KEGG and GO showed 8 molecular signaling pathways, included mitochondria mentalism pathway. According to the previous reports and above results (16), we selected miR-525-5p for the mechanism research.

Micro-RNAs regulate gene transcription through 3'UTR sequence, micro-RNA 3'UTR mutated induces activation/inhibition of disorder progression. (17,18). We established miR-525-5p 3'UTR mutated H9C2 cell line and detected the proliferation of different groups (H9C2-mutated, H9C2-NC, H9C2), the results indicted 3'UTR mutated induced increasing for H9C2 in hypoxia and reoxygenation condition. According to the enrichment of KEGG and GO, we detected the expression protein and gene: mitochondria complex and energy metabolism indictor-SIRT-1, the performance showed the expression of complex I, III, V and SIRT1 restored compared with the control group and negative group. The performance indicted miR-525-5p 3'UTR mutation maybe increase the metabolism and function of mitochondria (19-23). Finally, we examined the impaction of miR-525-5p

3'UTR on H9C2 apoptosis. The results performed 3'UTR mutated induced an anti-apoptosis situation in the hypoxia and reoxygenation. All above results revealed miR-525-5p maybe relate to IRI progression, the mechanism referred to the mitochondrial metabolism and energy metabolism, miR-525-5p 3'UTR may be combined with cell's apoptosis which induces proliferation of cells.

Conclusion

MiR-525-5p protects H9C2 cells without hypoxia and reoxygenation-induced injury through regulating the mitochondria metabolism.

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