Network construction of aberrantly expressed miRNAs and their target mRNAs in ventricular myocardium with ischemia–reperfusion arrhythmias

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Abstract

Background: Increasing evidences have verified that microRNAs (miRNAs) play an important role in formation and progression of various cardiac diseases including arrhythmias. Existing research has showed that certain miRNAs exhibit significantly different expressions and effects in arrhythmias. However, the effect of miRNAs in the progression of hypothermic ischemic–reperfusion arrhythmias (RA) and its potential mechanism remains to be further discussed. **Methods:** By utilizing a model for hypothermic ischemia–reperfusion of rats, miRNAs expression profiles of ventricular myocardium with global hypothermic ischemia–reperfusion and those of ventricular myocardium with hypothermic ischemia–RA were established through high-throughput sequencing. Furthermore, the aberrantly expressed miRNAs in myocardium with and without hypothermic ischemia–RA were screened and verified. By applying RNAhybrid, MiRanda, and TargetScan software, the target genes of these aberrantly expressed miRNAs were predicted. Based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, the mRNA targets associated with these miRNAs were determined and the miRNA–mRNA interaction during the progression of cardiovascular diseases was explored. The aberrantly expressed miRNAs related to hypothermic ischemia–RA were validated by employing quantitative fluorescence polymerase chain reaction (PCR). **Results:** Eight significantly aberrantly expressed miRNAs were identified (novel-miR-1, novel-miR-16, novel-miR-17, novel-miR-19, novel-miR-30, novel-miR-43, rno-miR-122-5p, rno-miR-429), in which six were up-regulated and two were down-regulated. Moreover, target genes and signaling pathways associated with these aberrantly expressed miRNAs were predicted and analyzed. According to miRNA–mRNA interaction network graph, it was revealed that GJA1 gene was considered as the target of novel-miR-17. **Conclusions:** Aberrantly expressed miRNAs were possibly associated with the formation mechanism of hypothermic ischemia–RA. Specific miRNAs, such as novel-miR-17 and rno-miR-429 are probably new potential targets for further conducting functional study on hypothermic ischemia–RA.

Background

Reperfusion arrhythmia (RA) refers to arrhythmia induced by recovering myocardial perfusion after occurrence of coronary occlusion or blocking of myocardial blood flows. It is one of characteristics of myocardial ischemia–reperfusion injury (MIRI). It mainly appears as various ventricular arrhythmias including ventricular premature beat, ventricular tachycardia (VT), and ventricular fibrillation. RA can even trigger hemodynamic disorder to cause sudden cardiac death (SCD) [1-3]. In recent years, with the development of various technologies such as anesthesia and cardiopulmonary bypass (CPB), the effect of cardiac surgical procedures has been greatly improved. However, RA is still the major complication during heart resuscitation through open heart surgery under cardiopulmonary bypass (CPB), which directly influences whether the surgery can be successfully conducted or not and patients' prognosis. Therefore, investigating the formation mechanism of RA after hypothermic ischemia exerts significance on preventing this kind of complications and also provides new targets and directions for clinical treatment.

MicroRNAs (miRNAs) play a crucial role in pathogenesis and progression of various cardiac diseases. They can lead to the development of certain cardiac diseases by regulating associated target genes [4]. Multiple miRNAs participate in the reconstruction of electrophysiology and ion channel by regulating gene expressions of cardiomyocyte during arrhythmias [5]. Clinical research showed that miRNA-1 in patients' serum greatly rises after conducting CPB [6]. Moreover, Bostjancic *et al.* [7] validated that two target genes (GJA1 and KCNJ2) are speculated from the nucleotide sequence at 5' end of miRNA-1, which are coded as Cx43 and potassium channel subunits Kir2.1, respectively. The over-expression of mi-RNA-1 can inhibit the expressions of GJA1 and KCNJ2, resulting in the electrolyte disorder of cardiomyocyte to further trigger arrhythmias. In hypothermic ischemia–RA, whether there are miRNAs affecting RA by regulating GJA1 and KCNJ2 or not has not been known.

Gap junction (GJ), as the basic cardiac electrophysiological structure, can sustain normal coupling in electrocardiogram (ECG) signals and mechanical coupling by mediating intercellular electrochemical communication. Connexin (Cx) is the basic unit of GJ [8], and its normal expression is crucial for guaranteeing normal electric coupling and conduction between cardiomyocytes. Cx43 is subjected to lateralization and dephosphorylation during ischemia–reperfusion injury, thus leading to lateral conduction, formation of reentry and electrical uncoupling to further trigger RA [9, 10]. By observing myocardium with ischemia–RA, it can be also found that the expression, distribution and phosphatization states of Cx43 are all changed at different degrees to slow down conduction velocity of myocardium and increase the formation probability of RA [11]. However, the specific correlation between Cx43 and miRNAs in hypothermic ischemia–RA has not been clarified.

Therefore, miRNAs related to hypothermic ischemia–RA were screened by utilizing high-throughput sequencing. By applying bioinformatics analysis methods including GO and KEGG databases, the miRNA–mRNA interaction was further analyzed. On this basis, the potential functions of aberrantly expressed miRNAs and the possible mechanism of interaction between the miRNAs and hypothermic ischemia–RA through genes were determined. It is expected to provide new targets and directions for preventing hypothermic ischemia–RA.

Methods

Ethical approval The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All protocols were approved by the Ethics Committee of Guizhou Medical University(No.1800451).

Preparation of models of isolated rat hearts. Sixteen healthy male SD rats of 2~3 months old weighing 300~400 g at clean grade were provided by the Experimental Animal Center, Guizhou Medical University (Guiyang, Guizhou Province, China). Heparin (3%, batch number: 51606118, Jiangsu Wanbang Biochemical Medicine Co., Ltd.) of 500 lu/kg was intraperitoneally injected for anticoagulation. After administration for 10 min, 300 mg/kg of 10% chloral hydrate (batch number: 20180429, Tianjin Kermel Chemical Reagent Co., Ltd.) was intraperitoneally injected for anesthesia. The chest of the rats was

opened fast after the anesthesia took effects to take the heart, which was then placed in the Krebs–Henseleit (K–H) solution at 4 °C immediately to trim and expose the aorta. The aorta was fixed on the Langendroff perfusion equipment (Shanghai Alcott Biotech Co., Ltd.) to conduct acyclic retrograde perfusion at constant temperature (37 °C) and constant pressure (8.65 kPa) with the K–H solution saturated with 95% O₂ and 5% CO₂. The model for Langendroff perfusion of isolated rat hearts was regarded successfully prepared if the heart rhythm (HR) was recovered within 3 min after balance perfusion and HR was higher than 180 times/min at the end of the balance perfusion.

Experimental treatments. Sixteen prepared models for Langendroff perfusion of isolated rat hearts were divided randomly into two groups (n=8 in each group): a control group (C group) and an ischemia reperfusion (IR) group. The former was subjected to perfusion continuously for 120 min with K–H solution at 37 °C. As for the latter, after balance perfusion with K–H solution at 37 °C for 30 min, Thomas solution of 20 ml/kg at 4 °C was injected at the root of the aorta to allow 60 min of cardiac arrest. The periphery of the heart was protected with K–H solution at 4 °C. Then, Thomas solution of 10 ml/kg at 4 °C was perfused again after cardiac arrest lasted for 30 min and K–H solution at 37 °C was re-perfused for 30 min after 60 min of cardiac arrest.

Experimental grouping. The arrhythmias during reperfusion were observed and then quantified according to the Curtis and Walker as well as Lepran grading systems. Rat hearts with scores higher or not higher than 3 were graded to have high risk of IR (IR-H) or low risk of IR (IR-L). Then, the hearts were divided into three groups based on the arrhythmia degree: a C group, an IR-L group, and an IR-H group, each containing specimens of ventricular myocardium of four rats. Myocardium of ventriculus sinister was removed immediately after the perfusion and the hearts were frozen and transferred to a refrigerator to be stored at -80 °C.

High-throughput sequencing and analysis of aberrant expression of miRNAs. The Trizol method was used to extract the total RNA of the specimens and the RNA was treated with Dnase I to eliminate the DNA pollution. All these procedures strictly followed the instructions. Through the use of the micro-nucleic acid protein tester (Agilent Company), formaldehyde denaturing gradient gel electrophoresis (DGGE), and capillary electrophoresis, the extracted total RNA was tested to ensure that its concentration and completeness reached the requirements for sequencing. The Beijing Genomics Institute (BGI) was entrusted to perform high-throughput sequencing for the above samples on the BGISEQ-500 platform. The expression of miRNA of the samples was standardized as transcripts per million (TPM). For given transcripts, the gene expression was estimated by aligning the number of fragments in a gene region. The edgeR software was used to analyze changes in the expression of the gene transcripts of the three groups of samples. After obtaining the value of *P*, multiple hypothesis testing was conducted and the corrected *P* was represented by false discovery rate (FDR), satisfying FDR<0.05. If the fold change in expression was equal to or larger than 2, the gene was deemed as aberrantly expressed.

Prediction of target genes of aberrantly expressed miRNAs. RNAhybrid, miRanda, and TargetScan [12] databases were used to predict the target genes of aberrantly expressed miRNAs and the predicted ones

were mapped to each terms in the gene ontology (GO) database to count the number of genes that mapped to each term. Then, hypergeometric distribution was applied to compute the value of P . If $P \leq 0.05$, a GO term was regarded to have significantly enriched target genes of aberrantly expressed miRNAs. Then, by using Kyoto Encyclopedia of Genes and Genomes (KEGG) database, target genes of aberrantly expressed miRNAs were mapped and the same calculation method was used to obtain the analysis results of KEGG. Finally, the mRNA–miRNA interaction network graph was preliminarily established.

Analysis of genes associated with the progression of cardiovascular diseases. All of the target mRNAs of differently regulated miRNAs were screened using the TargetScan 7.1. Then, the mRNA–miRNA process was comprehensively analyzed to build networks of the physiological system and pathophysiology. GO and KEGG cardiac function databases were taken to select the mRNAs that participated in pathophysiological process of cardiovascular diseases (CVDs), including hypertrophy, fibrosis, conduction abnormality, and arrhythmia.

Screening of the target miRNAs. Based on gene function analysis, KEGG pathway analysis, and mRNA–miRNA interaction, the bioinformatics analysis was carried out on transcripts of miRNAs related to KCNJ2/GJA1 genes. The target miRNAs regulating expressions of KCNJ2 or GJA1 in the hypothermia ischemic–RA were preliminarily screened.

Fluorogenic quantitative PCR validation. The Trizol method was used to dissociate and extract the RNA. The ultraviolet spectrophotometer was used to detect the purity of total RNA and for quantification. After subpackage, the RNA was preserved at -80°C . The reverse transcription was performed according to the instructions of ThermoScript™ RT-PCR System (Invitrogen, the United States) and the obtained cDNA was preserved at -20°C for later use. The primer was synthesized by Wuhan Biofavor Biotechnology Services Co., Ltd. The SYBR Premix Ex Taq™ reagent was used and 20 μl of reaction system contained cDNA (2 μl), 2×Master Mix (10 μl), 20×TaqMan@ probe and primer mixture (1 μl), and double distilled water (7 μl). The polymerase chain reaction (PCR) was performed under the following conditions: initial denaturation for 5 min at 94°C , followed by 40 cycles at 94°C , 60°C , and 72°C , respectively for 45 s, and finally extending for 7 min at 72°C . The fluorogenic quantitative PCR detecting system was used for carrying out PCR amplification and the solubility curves were drawn. The final data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method.

Statistical analysis All data are presented as the means $\pm$ standard errors of the mean. The incidence was compared with Fisher's exact test, and different experimental groups were compared by Student's t test and analysis of variance using SPSS 22.0 (IBM SPSS Statistics, USA). $P < 0.05$ was considered to indicate a statistically significant difference among the all groups.

Results

Aberrant expression of miRNAs. Some 85 significantly aberrantly expressed miRNAs commonly existing in the three groups were predicted using the miRDeep2 software(**Fig.1a**). Among the miRNAs with

significant aberrant expressions, expressions of 46 miRNAs were up-regulated in the C and IR-H groups while those of 9 ones were down-regulated(**Fig.1b**). In the C and IR-L groups, 32 miRNAs were found to have up-regulated expressions while 9 ones showed down-regulated expressions(**Fig.1c**). As for the IR-H and IR-L groups, 28 miRNAs had up-regulated miRNAs and 20 ones exhibited down-regulated miRNAs(**Fig.1d**) .

Screening of miRNAs associated with hypothermia ischemic–RA. According to the principle of fold change ≥ 2 and FDR < 0.05, 8 miRNAs most closely associated with the hypothermia ischemic–RA were selected from the 85 aberrantly expressed miRNAs (Table 1). A comparison of expressions of the miRNAs revealed that the three groups had discrepancies in the expressions of novel-miR-1 and novel-miR-16, and IR-H and IR-L groups showed difference in expressions of novel-miR-16 and novel-miR-30. The RA groups and the C control also displayed differences in rno-miR-122-5p expression. A significant increase in the expression of novel-miR-17 was observed in the IR-H group compared with the IR-L and C groups. Although the expressions of novel-miR-1 and novel-miR-16 did not exhibit significant differences in the three groups, they had higher expressions in the IR-H group.

Predicting target genes of aberrantly expressed miRNAs. RNAhybrid and miRanda were used to predict target genes of aberrantly expressed miRNAs. On this basis, it was found that each miRNA had multiple predicted target genes. Totally 2,810 target genes were predicted for the 8 aberrantly expressed miRNAs. These target genes are probably the targets related to hypothermia ischemic–RA.

GO and KEGG data analysis. Three GOs separately described the biological process (BP), molecular function (MF), and cellular component (CC) of genes. In the current research, aberrantly expressed miRNAs were enriched in numerous CCs, included cell junction, proteinaceous extracellular matrix–cell projection–dendrite–basolateral plasma membrane(**Fig.2a**). Likewise, the molecular function ,delayed rectifier potassium channel activity–RNA polymerase II core promoter proximal region sequence–specific DNA binding–protein kinase binding–chromatin binding.were also influenced(**Fig.2b**). The biological process under influences included potassium ion transport across cell membranes, delayed rectifier potassium channels, and regulation of heart rate by cardiac conduction(**Fig.3a**). Then, enrichment of target genes of aberrantly expressed miRNAs in KEGG pathways was further analyzed and the results showed that the target genes took part in 6 signaling pathways correlated with cardiac diseases(**Fig.3b**). These signaling pathways mainly included those of epinephrine, arrhythmogenic right ventricular cardiomyopathy (ARVC), and cardiac hypertrophy.

Bioinformatics data. Totally 151 mRNAs showed miRNA–mRNA interactions (**Fig. 4**). We observed the correlations of these miRNAs with various cardiac pathophysiological processes and the process that they were likely to trigger RA or finally developed to RA (**Fig.5**). The relationship between the two analyses displayed that 19 of the 151 mRNAs were associated with the development of cardiac diseases (**Fig. 6**). The target gene GJA1 of novel-miR-17 participated in the proarrhythmia and the associated pathways, which is consistent with previous research results. The target gene KCNQ4 of rno-miR-429 and novel-miR-17 was associated with the delayed rectifier potassium channel and the potassium ion transport across

cell membranes. Through screening using the pathway analysis software, no target gene associated with the electrocardiological mechanism regulated by novel-miR-1, novel-miR-13 and novel-miR-43 was found.

RT-qPCR validation of aberrantly expressed miRNAs. Four miRNAs that were closely associated with the RA formation mechanism were selected from the 8 miRNAs and then validated through the use of qRT-PCR. It was found that these four miRNAs were novel-miR-17, novel-miR-30, no-miR-122-5p, and no-miR-429. The results revealed that the change trend of expressions of the four miRNAs agreed with the results of high-throughput sequencing (Fig.7).

Discussion

The miRNAs regulate the gene expression by inhibiting the translation process of protein-coding mRNAs through combination with the 3' untranslated region (3'UTR) of specific mRNAs [13-15]. Research in recent years has revealed that miRNA molecules played a significant role in the progression of cardiac diseases such as MIRI and arrhythmia [16, 17]. Human intervention on the expression of some miRNAs affects the progression of RA, the formation mechanism of which is closely associated with the electrophysiological reconstruction. The miRNAs are key molecules in arrhythmia, so their intervention and regulation has become a new target for treatment of diseases [18, 19]. The target genes of the four miRNAs newly discovered in the research are mainly found in the ARVC pathway, while effects of these miRNAs in biological and pathological research have not been reported yet. Therefore, clarifying whether these miRNAs have important influences on the RA by regulating relevant signaling pathways provides more possible directions for the research on hypothermia ischemic-RA.

Aberrantly expressed miRNAs and their target protein-coding RNAs consist of a complicated network, which participates in the regulation of the formation and progression of RA. Previous research has reported that miRNAs and Cx43 take part in the formation of RA [20], while the correlation mechanism between the two is rarely investigated in detail. Apart from ventricular arrhythmia, upward shift of the S-T segment, and QT prolongation, the ECG also exhibited prolonged QRS interval after ischemia reperfusion, all of which are associated with a slower intermyocardial conduction velocity after ischemia reperfusion [21]. GJ, as the basic structure of cardiac electrophysiology, maintains the normal coupling in ECG signals and mechanical coupling by mediating intercellular electrochemical communications. The normal expression of Cx43 that is coded by gene GJA1 is crucial for the electric coupling and conduction between cardiomyocytes [22]. We also found that the expression, distribution, and phosphorylation state of Cx43 in the myocardium of ischemic-reperfusion arrhythmia all changed at different degrees, thus slowing down the conduction velocity of myocardium and increasing the formation of RA [23, 24]. Therefore, regulating the expression of Cx43 is one of the key factors for the formation of hypothermia ischemic-RA. In the research, GJA1 was the target of novel-miR-17. Whether novel-miR-17 can regulate Cx43 protein by acting on GJA1 and therefore influence the formation of hypothermia ischemic-RA will be a future research topic.

Through GO functional and KEGG pathway enrichment analyses, it was found that target genes of the eight significantly aberrantly expressed miRNAs took part in the potassium ion transport across cell membranes and the delayed rectifier potassium channels. Therefore, it can be assumed that aberrantly expressed miRNAs are capable of regulating the target genes to participate in the action mechanism of hypothermia ischemic–RA via the above channels. After suffering hypothermic ischemic injury, the electrophysiological changes in irregular myocardium repolarization including MAPD prolongation and increasing transmitted drug resistance (TDR) in the myocardium indicated that the potassium channel possibly took part in the formation of RA [25, 26]. According to the regulation network of the aberrantly expressed miRNAs and their mRNAs, it was revealed that the relevant target genes KCNQ4 and KCNA6 of rno-miR-429 and novel-miR-17 were deemed associated with the potassium ion transport across cell membranes and delayed rectifier potassium channels. Therefore, they can lead to the formation of RA by participating in the regulation of the ion channels, and the specific regulation mechanism will be an important topic in future research. Moreover, the miRNA–mRNA interaction also indicated that seven mRNAs (Cacna1c, Itgb3, Ctnnb1, Gja1, Itgav, Cacnb3, and Tcf7l1) are probably the targets of the candidate miRNAs that take part in the progression of CVDs. This provides more possible targets and directions for the future research.

Conclusion

In conclusion, Novel-miR-17 probably has effects on the CJA1 gene, thus regulating the formation of RA. Meanwhile, the biological analysis reveals that novel-miR-17 and rno-miR-429 probably regulate their target genes to influence the ion channels and affect the formation of RA. This indicates that novel-miR-17 is possibly a potential biological marker. We will further study the functions of these miRNAs, so as to broaden our knowledge on the mechanism of action of hypothermia ischemic–RA.

Abbreviations

RA—reperfusion arrhythmias—Cx43: Connexin 43; GO—Gene Ontology—KEGG—Kyoto Encyclopedia of Genes and Genomes—MIRI—myocardial ischemia–reperfusion injury—VT—ventricular tachycardia—SCD—sudden cardiac death—CPB—cardiopulmonary bypass—GJ—Gap junction—ECG—electrocardiogram

Declarations

Ethics approval and consent to participate

The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All protocols were approved by the Ethics Committee of Guizhou Medical University (No. 1800451).

Consent for publication

Not applicable.

Availability of data and materials

All raw data is available upon request and the corresponding author, Prof. Hong Gao (E-mail: 2169617@qq.com) should be contacted if someone wants to request the data.

Competing interests

The authors declare no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Authors' contributions

HG designed the research/study. JT performed the research/study. FYR and WGL collected the data. JS, HYQ and LYQ analysed the data. All authors read and approved the final manuscript.

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Table

Tab. 1 Eight microRNAs with the highest expression among 85 differentially expressed microRNAs

Mature-ID	Fold change	P value	Regulation	FDR
novel-miR-1	1.57	0.0000045	up	<0.001
novel-miR-16	4.32	0.0000562	dpwn	<0.001
novel-miR-17	2.57	0.0000765	up	<0.001
novel-miR-19	5.87	0.0000694	up	<0.001
novel-miR-30	6.63	0.0000425	up	<0.001
novel-miR-43	7.13	0.0000238	up	<0.001
rno-miR-122-5p	2.38	0.0000758	up	<0.001
rno-miR-429	5.34	0.0000346	down	<0.001

Figures

Figure 1

The plots of differentially expressed microRNAs. A. Group names are listed at the bottom. The number of the significantly ($p < 0.1$) expressed microRNAs are shown on the left. Significant differential expression of 85 microRNAs: green, downregulation; red, upregulation. B. volcano plot of differentially expressed microRNAs from high-throughput sequencing. (C vs IR-DW). The horizontal lines correspond to 2-fold up and down, respectively, and the vertical line represents a FDR. So the red and the green points in the plot represent the differentially expressed microRNAs with significant difference. C. volcano plot of C vs IR-GW. D. volcano plot of IR-DW vs IR-GW.

Figure 2

GO Analysis of differentially expressed MicroRNAs. A. GO analysis for differentially expressed (DE) mRNA-Cellular component (CC). Bar plot explanation (Enrichment Score): the bar plot shows the top ten Enrichment Score value of the significant enrichment terms. B. GO analysis for differentially expressed (DE) mRNA-Molecular function (MF). Bar plot explanation (Enrichment Score): the bar plot shows the top ten Enrichment Score value of the significant enrichment terms.

Figure 3

GO Analysis of differentially expressed MicroRNAs. A. GO analysis for differentially expressed (DE) mRNA-Biological process (BP). Bar plot explanation (Enrichment Score): the bar plot shows the top eleven Enrichment Score value of the significant enrichment terms. B. Pathway analysis of target genes of differentially expressed (DE) microRNAs. Target genes were enriched into different pathways about heart disease based on KEGG pathway database, the top 6 pathways were shown above.

Interaction of miRNAs with pathophysiological processes related to RA. Green Box – cardiac pathophysiological processes; red symbols - miRNAs. cytoscape software data.

Figure 6

Interaction of mRNAs with pathophysiological processes associated with RA. Red symbols - mRNA; blue symbols- cardiac pathophysiological processes. cytoscape software data.

Figure 7

Expression of miRNAs(novel-miR-17, novel-miR-19, rno-miR-429, rno-miR-122-5p) among control, IR-DW and IR-GW groups. Group names are listed at the bottom. Relative expression was quantified using the comparative CT.

Supplementary Files

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