Screening and Function Analysis of MicroRNAs Involved in Exercise Preconditioning-Attenuating Pathological Cardiac Hypertrophy

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Summary

Exercise preconditioning (EP) attenuates pathological cardiac hypertrophy by increasing the functional capacity of the cardiovascular system; however, the underlying molecular mechanisms remain unclear. MicroRNAs (miRNAs) play important roles in various physiological and pathological processes by regulating the expression of the targeted gene. In this study, we aimed to screen the miRNAs involved in EP-attenuating pathological cardiac hypertrophy. The histological and echocardiographic parameters assessment showed that pathological cardiac hypertrophy induced by transverse aortic constriction (TAC) was significantly alleviated in EP treated rats. The left ventricular tissues (n = 3) from Sham, TAC and EP + TAC groups were subjected to small RNA deep sequencing. A total of 570 known mature miRNAs and 530 putative novel miRNAs were detected. DEGseq analysis showed that there were 37 and 88 differentially expressed miRNAs in the comparisons of TAC versus Sham and EP + TAC versus TAC, respectively. Among them, EP treatment could relieve the expression changes of 32 miRNAs, which were supposed to be involved in EP-attenuating pathological cardiac hypertrophy. After miRNAs target genes prediction by miRDB algorithm, pathway analysis showed that the most frequently represented pathways were involved in Calcium signaling pathway and MAPK signaling pathway. The results would provide valuable clues to finding therapeutic targets for the treatment of pathological cardiac hypertrophy.

Key words: Bioinformatics analysis, Deep sequencing, Function prediction

Pathological cardiac hypertrophy, as occurs with overload pressure or hypertension, induces a reduction in the cardiac function and is known to be a precursor for heart failure. Ischemic precondition was reported to be an effective way of alleviating pathological cardiac hypertrophy by increasing the functional capacity of the cardiovascular system. In addition to ischemia, pretreatment with hypoxia, short-term hypertrophic stimulation, or certain drugs can induce this protective effect of preconditioning. Our previous studies also demonstrated that exercise preconditioning (EP) could attenuate pressure overload-induced pathological cardiac remodeling and cardiac dysfunction. Cardiac hypertrophy is involved in the complex pathological process of multiple signaling pathways; however, the underlying molecular mechanisms of EP-attenuating pathological cardiac hypertrophy are still poorly understood.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, and they play important roles in various pathological and physiological processes by translational inhibition or mRNA cleavage of the targeted genes. Many miRNAs had been found abnormally expressed in a hypertrophic heart, and they were proved to be involved in cardiac hypertrophy regulation. Recent reports showed that many miRNAs would be dynamically regulated by exercise. However, it had not been determined yet whether these miRNAs participated in the improvement of the cardiac function by exercise training. In the present study, we screened the differentially expressed miRNAs during EP-attenuating pathological cardiac hypertrophy through high throughput small RNA deep sequencing, and hoped to provide valuable clues to finding therapeutic targets for the treatment of pathological cardiac hypertrophy.

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Methods

Animal models: The rat models of pressure overload cardiac hypertrophy were established by transverse aortic constriction (TAC) as described previously. Thirty male Sprague Dawley rats, weight 200-250 g, were randomly divided into 3 groups (n = 10 for each group): Sham group, TAC group, and EP + TAC group. EP was performed by moderate-intensity exercise (approximately 60% of their maximal aerobic velocity) for 4 weeks.

The institutional review board of the local university approved the animal work performed in this study, and we carried out the experiment protocols according to the guidelines for the care and use of laboratory animals established by the US National Institutes of Health.

Hematoxylin and eosin staining: The rat heart was collected carefully and was weighed after humane sacrifice of the rats. Paraffin-embedded left ventricular tissue was sectioned at 4-6 mm thickness and was stained with hematoxylin and eosin for routine examinations and was photographed using a light microscope.

Small RNA library and deep sequencing: The total RNA was isolated from rat left ventricular tissues by miRNeasy Kit (Qiagen) according to the manufacturer’s instructions. About 10 μg of the total RNA were ligated with proprietary adapters, were reverse transcribed to cDNA, and were amplified by PCR. Subsequently, the PCR products were purified by RNA gel electrophoresis, and were validated for library construction. Finally, the libraries were deep sequenced using HiSeq 4000 (Illumina) at Shanghai OE Biotech Co., Ltd. MultiExperiment Viewer software was applied for the comparison of the miRNA expression values, the preparation of the heat-map and the hierarchical clustering analyses (fold change ≥ 1.5 or ≤ 0.66; P < 0.05; q-value < 0.01).

Real-time quantitative PCR (qRT-PCR): The total RNA was used to generate cDNA by using PrimeScript RT reagent Kit (TAKARA) with special stem-loop primer for miRNA and oligo-dT or random primer for mRNA. Real-time quantitative PCR (qRT-PCR) was performed on a LightCycler 480 II PCR system (Roche) by using SYBR Green (TAKARA). U6 and GAPDH genes were used as reference genes for the miRNAs and gene expression respectively.

Target prediction and functional analysis: The potential target genes were predicted by the miRDB algorithm (http://www.mirdb.org). Gene ontology (GO) functional analysis and pathway analysis were performed in the standard enrichment computation method based on the Database for Annotation, Visualization and Integrated Discovery (DAVID) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). DIANA-miRPath v.3.0 software was used to perform the enriched KEGG pathway analysis. Only KEGG pathways with P-value < 0.05 were retained.

Statistical analysis: SPSS version 21.0 was used to perform all the statistical analyses. The quantitative data were analyzed by using One-Way ANOVA followed by the post-hoc tests of Tukey. P-values less than 0.05 were considered statistically significant difference.

Results

EP attenuated pathological cardiac hypertrophy induced by pressure overload: The same as our previous findings, EP could attenuate pressure overload-induced pathological cardiac hypertrophy. The parameters for assessing cardiac hypertrophy, including heart weight/body weight (HW/BW), LV posterior wall thickness in the end diastole (LVPWd) and the expression of MYH7, were significantly decreased in the EP + TAC group, compared with the TAC group (Figure 1A-D).

Overview of small RNA deep sequencing data in rat hearts: It had been proved that EP could attenuate pressure overload-induced cardiac hypertrophy. In this study, Illumina Solexa sequencing was applied to detect the differential expression of miRNAs induced by EP during cardiac hypertrophy. A range of 6,676,300-8,775,284 raw reads for all the groups was obtained. After filtering out low quality and meaningless reads, about 5 million clean reads (length between 15 and 30 nt) were obtained for each sample in the 3 groups (Table). Size distribution assessment showed that small RNA sequence length was mainly concentrated at 20-24 nt, and the length of 22 nt was the maximum size (Figure 2A). The unique small RNA reads were mapped to the chromosome by blasting with rat genome. The results showed that most of the reads could be perfectly mapped to the rat genome. Moreover, they were mainly located at chromosome 1 (10.50%), 2 (9.35%), 4 (6.63%), and 5 (6.41%) (Figure 2B). According to their biogenesis and annotation from Rfam databases, the unique sequences were categorized into different groups, including rRNA, miRNA, snoRNA, and tRNA. There were 155,357 (14%) miRNAs sequences in the Sham group, 158,765 (14%) in the TAC group, and 122,071 (14%) in the EP + TAC group (Figure 2C).

Characterization of known and putative novel miRNAs: To identify the known miRNAs from the rat heart, the miRBase database (http://www.mirbase.org/) was applied. A total of 570 known mature miRNAs were detected in the rat hearts. There were 454, 445, and 459 known miRNAs in the Sham group, the EP + TAC group and the TAC group respectively, of which 409 known mature miRNAs were expressed in all the 3 groups. Figure 3 showed the description of the top 20 abundant known miRNAs in each group. Among them, miR-143-3p and miR-1-3p were the most abundant expressed in the heart. There was no significant difference in the highly expressed miRNA species among the 3 groups. Meanwhile, 530 putative novel miRNAs were also predicted from the unannotated reads by miRDeep2 analysis (Supplemental Table I). A BLASTN search from organism of Rattus norvegicus in miRbase database revealed all the putative novel miRNAs shared little or no homology.

Differentially expressed miRNAs during EP-attenuating cardiac hypertrophy: Differentially expressed miRNAs were identified by DEGseq analysis. Consequently, 37 differentially expressed miRNAs were detected in the comparisons of the TAC versus Sham groups, including 28 up-regulated and 9 downregulated miRNAs. Meanwhile, 88 differentially expressed miRNAs were detected in the comparisons of the EP + TAC versus...
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Figure 1. EP attenuated the pressure overload-induced pathological cardiac hypertrophy. A: Representative image of hematoxylin and eosin-stained horizontal-section (upper panel) and longitudinal-section (lower panel) in rat hearts from Sham (n = 10), TAC (n = 7) and EP + TAC (n = 8) groups. B: Heart weight/body weight (HW/BW) was measured in rats from each group. C: LV posterior wall thickness in end diastole (LVPWd) were measured by echocardiographic analysis. D: The relative expression of MYH7 mRNA was determined by qRT-PCR. *P < 0.05, and **P < 0.01.

Table. Summary of Data Generated from Deep Sequencing

<table>
<thead>
<tr>
<th></th>
<th>Sham group</th>
<th>TAC group</th>
<th>EP + TAC group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Raw reads</td>
<td>8,340,526</td>
<td>6,676,300</td>
<td>7,083,696</td>
</tr>
<tr>
<td>Clean reads</td>
<td>6,042,507</td>
<td>4,696,975</td>
<td>4,664,283</td>
</tr>
</tbody>
</table>

TAC groups, including 34 up-regulated and 54 downregulated miRNAs (Supplemental Table II). Further analysis showed that 24 miRNAs which were up-regulated in the comparisons of TAC versus Sham were found downregulated (n = 11) or unchanged (n = 13) in the comparisons of EP + TAC versus TAC, and 8 miRNAs which were downregulated in the comparisons of TAC versus Sham were found up-regulated (n = 4) or unchanged (n = 4) in the comparisons of EP + TAC versus TAC (Figure 4A). Then, 6 miRNAs were selected for validation by real-time PCR. All the miRNAs showed a consistent expression pattern with the results from deep sequencing (Figure 4B), indicating high reliability of the analysis. These 32 miRNAs might be involved in the regulation of EP-attenuating pathological cardiac hypertrophy.

Prediction and annotation of miRNA target genes: A total of 7592 target genes were predicted for the 32 miRNAs. Then, GO functional analysis and pathway analysis were performed with these putative targets. It was found that the most enriched GO was correlated with positive regulation of transcription from RNA polymerase II promoter in the biological process analysis, and the majority of the genes were proved to be related to the cytoplasm region in the cellular component analysis and ATP binding in the molecular function analysis (Figure 5A-C). The biological functions of these target genes were investigated further through the KEGG pathway analysis. The most frequently represented pathways were involved in the Calcium signaling pathway and in the MAPK signaling pathway (Figure 5D).
Figure 2. Summary of small RNA deep sequencing data in rat hearts. A: Read length distribution (15-30 nt) and abundance of small RNAs sequences. The y-axis represented the count number. B: Distribution of unique small RNAs sequences on rat chromosome. The unique small RNA reads were mapped to chromosome by BLASTing with the rat genome; MT indicates mitochondrial transcriptions. C: Frequency of unique small RNA distribution among the different categories. The unique sequences were subjected to searches for the types and numbers of sRNA using the Rfam databases.

Figure 3. Description of top 20 abundant known miRNAs in rat heart tissues from each group. There was no significant difference in highly expressed miRNA species among the 3 groups, and miR-143-3p and miR-1-3p were the most abundant expressed miRNAs.
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Figure 4. Screening of miRNAs involving in EP-attenuating pathological cardiac hypertrophy. A: Hierarchical clustering analysis on differentially expressed miRNAs (P < 0.05) in comparison of Sham, TAC and EP + TAC groups. Red indicated upregulation; green, downregulation; and black, no change. B: Validation of differentially expressed miRNAs by qRT-PCR. *P < 0.05 and **P < 0.01 versus Sham group. #P < 0.05 and ##P < 0.01 versus TAC group.

Discussion

It had been reported that EP could provide cardio-protective benefits to tolerance to cardiac injury, including ischemia-reperfusion, pressure-overload, and myocardial infarction. However, the mechanism involved in EP-induced cardio-protection still remains controversial, such as increased heat shock protein production, increased cardiac antioxidant capacity, and improvement in ATP-dependent potassium channel function. In the present study, we focused on the differentially expressed miRNAs during EP-attenuating cardiac hypertrophy, and we analyzed the function annotation of their potential target genes. The result would provide valuable clues for the internal mechanism of miRNA-mediated regulation of cardiac hypertrophy.

Although the enlargement of the heart by pathological and physiological hypertrophy has similar morphological appearances, the outcomes of the two types of hypertrophy are strikingly different. Exercise-induced physiological hypertrophy leads to a considerable improvement in the quality of life, whereas pressure overload-induced pathological hypertrophy is associated with the progressive deterioration of heart functions. Previous reports had proved that aerobic exercise training induced physiological cardiac hypertrophy accompanied the differential expression of the miRNAs. In the present study, according the expression profile of the miRNAs among the Sham, TAC and EP + TAC groups, 32 miRNAs were identified to participate in the regulation of EP-attenuating pathological cardiac hypertrophy.

Increasing evidence had proved that miRNAs acted as important regulators in cardiac hypertrophy. In our study, 37 miRNAs were differentially expressed in the TAC group, including 28 up-regulated and 9 downregulated miRNAs. Among them, most of the downregulated miRNAs (miR-9a-5p, miR-182, miR-26a-5p, miR-101a-3p, miR-1-3p, miR-29a-3p, and miR-133a-3p) had been reported to have cardio-protective and anti-hypertrophic effects, while some up-regulated miRNAs (miR-451-5p, miR-221-3p, miR-222-3p, miR-21-5p, miR-132-3p, miR-499-5p, miR-214-3p, and miR-365-3p) had been reported to have pro-hypertrophic effects. However, some differentially expressed miRNAs’ roles on cardiac hypertrophy were still unclear. Further studies would help to understand the function and molecular mechanism of miRNA in future.

Only a few studies which investigated the impact of
Figure 5. The function annotation of the potential target genes of miRNAs. A-C: The GO analysis was performed based on DAVID. D: The pathway analysis was performed based on KEGG databases. The potential target genes of miRNAs were predicted by miRDB algorithm.
EP on miRNAs expression in heart tissues were available. Cardiac-enriched miR-1-3p was up-regulated in mice trained by swimming for 4 weeks (1 hour twice a day), but was found downregulated in mice trained by increased load swimming for 8 weeks (30 minutes at 1st week, increase by 10 minutes per week). So far, most of the studies of human EP miRNAs focused on the circulating levels. It was reported that miR-1-3p was moderate negative correlated with fractional shortening in marathon athletes, whereas miR-133a was positively related to the thickness of the intraventricular septum. However, Baggish, et al. found that miR-133a-3p displayed no significant expression changes immediately following exhaustive exercise. It might be suggested that the miRNAs profiles in the heart tissue or in circulating might be associated with the intensity of EP.

In our previous study, HSF1 and NF-κB p65 were found to participate in the process of EP-attenuating pathological cardiac hypertrophy. HSF1 and NF-κB, recognized as ubiquitous transcription factors (TF), played important roles in cardiac remodeling and dysfunction. Among the 32 differentially expressed miRNAs, the expression of miR-214-3p and miR-146a-5p were reported to be regulated by HSF1, while miR-146a-5p and miR-26a-5p were reported to be involved in the regulation of the NF-κB signaling pathway. The network of the TF-miRNA-mRNA interactions will help to validate the experimental observation and to develop new therapeutic approaches.

Small RNA deep sequencing could detect the mRNA expression profile at the universal level, and it had become a useful tool for detecting functional miRNAs. One advantage of small RNA sequencing is the opportunity to discover novel miRNAs. By using miReap algorithm based on miRNA biogenesis, 530 identified clusters were analyzed for the miRNA precursor characteristics. A BLASTN search revealed little or no homology to known miRNA of Rattus norvegicus. However, the confirmatory and functional analysis of these novel putative miRNAs remain to be explored in future.

In conclusion, we detected the differential expression of miRNAs induced by EP during cardiac hypertrophy by deep sequencing, and identified 32 potential miRNAs involved in the regulation of EP-attenuating cardiac remodeling. Further studies are needed to explore their regulatory mechanisms, which would help to develop novel therapeutic targets for heart disease.

Disclosures

Conflicts of interest: None.

References

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Supplemental Files
Supplemental Table I, II
Please see supplemental files; https://doi.org/10.1536/ihj.17-498