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Original Article

Differential plasma microRNA expression after remote ischemic conditioning in healthy volunteers: identification by high-throughput sequencing

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Abstract: Background: Remote ischemic conditioning (RIC), 3 to 4 cycles of transient episodes of ischemia/reperfusion on limbs, may protect against myocardial ischemia reperfusion injury markedly. MicroRNAs are important humoral factors, which may play a key role in RIC. However, no systematic studies on changes in the plasma miroRNA expression profile in RIC have been carried out. This study aimed to investigate the plasma miRNA profiles in five healthy subjects after RIC. Methods: High-throughput sequencing technique was applied to detect the expression profiles of plasma microRNAs in five healthy subjects after RIC. The results were validated by using qRT-PCR. The target genes of these differentially expressed miRNAs were analyzed by GO and KEGG pathway analysis. Finally, possible pathway of target genes in heart was detected in a rat model. Results: After the sequencing, 37 miRNAs were significantly differentially expressed after RIC ($P<0.05$). The GO and KEGG analysis showed that the Wnt, RNA transport, and endocytosis signaling pathways might be involved in the regulation of RIC. By using a rat model, we found that RIC could activate the phosphorylation of GSK-3β, the key molecule in the Wnt signaling pathway. Conclusion: RIC modifies the plasma microRNA expression profiles significantly. MicroRNAs might play important roles in the mechanism of cardioprotection conferred by RIC, providing a potential therapeutic target for the clinical treatment of myocardial ischemia/reperfusion injury in future.

Keywords: Remote Ischemic conditioning (RIC), microRNA expression, high-throughput sequencing technique, cardioprotection

Introduction

Early reperfusion to restore the myocardial blood supply is the most effective treatment method for myocardial infarction. However, reperfusion itself can cause ischemia reperfusion (IR) injury, which seriously affects the short- and long-term prognosis of patients [1]. Although the traditional ischemic preconditioning or postconditioning can effectively alleviate the IR injury, it is still an invasive procedure and may cause adverse consequences, such as thrombosis and aggravated myocardial ischemia [2]. Therefore, its clinical application is limited. Remote ischemic conditioning (RIC), i.e., 3 to 4 cycles of transient episodes of 5 min ischemia/5 min reperfusion on upper or lower limbs by using a non-invasive blood pressure cuff before/after prolonged lethal myocardial ischemic injury, could have a powerful cardioprotective effect [3-5]. In 1993, Przyklenk et al. first [6] reported the cardioprotective role of remote ischemic preconditioning (RIPC), one kind of RIC, performed before prolonged ischemia. However, in >80% of cases, percutaneous coronary intervention (PCI) is carried out ad hoc, promptly after diagnostic coronary angiography, which seriously limited application of RIPC, as accurate information about the timing of index ischemia is uncertain in vasoocclusive emergencies. Therefore, remote ischemic postconditioning (RIPostC), applied immediately after prolonged myocardial ischemia, overcoming the limitation of RIPC above, making clinical application more possible, has been gradually emphasized in recent years [7].

RIC has gained attentions of researchers due to its advantages of simplicity and noninvasiveness. However, the mechanism of cardioprotection conferred by RIC remains unclear. According to the literature, RIC may induce changes in various peptide/protein factors in plasma, such as IL-10, nitrite and SDF-1 (stromal derived factor-1) [8-11], which could play key roles in the cardioprotective effect of RIC. Moreover, RIC has been reported to induce the modification of plasma proteome in healthy volunteers in 2013 [12]. MicroRNAs are important humoral regulatory factors, and miR-1, miR-21, and miR-144 have been shown to be of paramount importance in RIC [13, 14]. However, no systematic studies on changes in the plasma miroRNA expression profile in RIC have been done. Further research on targeting microRNAs and their roles in RIC should be carried out to explain the mechanism of cardioprotection conferred by RIC and ultimately to guide clinical treatment. Therefore, the present work aimed to investigate the plasma miRNA profile in five healthy subjects after RIC by using a next generation high-throughput sequencing technique.

Materials and methods

RIC protocol

This study on human subjects was approved by the Peking University Third Hospital Ethics Committee (no. 2016236-02) and all the participants provided their informed consent. The five healthy adult subjects were 30.4±1.1 years old. They were fasted and water-deprived from 22:00 the day before the trial until the end of the trial on the next day. Four cycles of 5 min ischemia/5 min reperfusion were applied on the upper limbs with the use of a blood pressure cuff. Briefly, the limb was bundled with a sphygmomanometer cuff, and the blood pressure was increased to 200 mmHg to cause limb ischemia for 5 minutes. Then the cuff was released, and the limb was reperfused for 5 minutes. A total of 4 cycles were performed. Venous blood (10 ml) was collected from each subject before RIC (control group, Con) and 10 minutes after the procedure (RIC group, RIC). The blood samples were stored in EDTA anticoagulant tubes at 4°C in a refrigerator and processed within 2 hours; i.e., the samples were centrifuged at 4000 g and 4°C for 10 mins, and supernatants were removed and stored at -80°C [12, 14, 15].

High-throughput microRNA sequencing and data analysis

Plasma RNAs of the five volunteers were extracted by using Trizol reagent according to the manufacturer's protocol (Invitrogen) and sent to the BGI (The Beijing Genomics Institute) to construct small RNA library: Con Library (RNA from the plasma of control group) and RIC Library (RNA from the plasma of RIC group). Then the cDNA libraries were constructed and microRNAs were sequenced by using a next generation high-throughput sequencing technique. Briefly, Illumina HiSeq 2000 instrument (BGI) was applied to obtain the raw sequencing data, then these data were mapped to human miRNAs database (miRBase 21), and differentially expressed miRNAs were screened out between RIC Library and Con Library. The target genes of differentially expressed miRNAs were predicted and used to carry out GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) bioinformatics analysis [16].

Real-time quantitative PCR

The total RNAs were transcribed to cDNAs, then qRT-PCR assay was performed according to manufacturer's instruction (Qiagen). The differential expressions of miRNA before and after RIC were compared the use of the $2^{-\Delta\Delta CT}$ method.

Establishment of myocardial IR and RIPostC in rat and TTC (2,3,5-Triphenyltetrazolium chloride) staining

The study on animals was done in accordance 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). The protocol was approved by the Committee on the Ethics of Animal Experiments of Peking University Third Hospital. Healthy adult male Sprague Dawley rats were anesthetized by intraperitoneal injection, and their chests were open at the third and fourth left intercostal space 0.5 cm away from the median sternal line along the intercostal. Vessel ligation was applied 2 cm below the origin of the left anterior descending coronary artery between the right margin of the left atrial appendage and the left margin of the pulmonary artery for 30 minutes. Then the ligature was removed, fol-

Table 1. Summary of reads from raw data to cleaned sequences for small RNAs in Con and RIC libraries

	Con Library (counts, % of total)	RIC Library (counts, % of total)
Total reads	13933653	13622791
High quality	13863101 (100%)	13554121 (100%)
3' adaptor null	61584 (0. 44%)	60853 (0.44%)
Insert null	20892 (0. 15%)	17825.8 (0.11%)
5' adaptor contaminants	211213 (1.67%)	45777.8 (0.32%)
Smaller than 18 nt	92051 (0.62%)	93063.2 (0.59%)
Poly (A)	179 (0.00%)	47 (0.00%)
Clean reads	13477182 (97.12%)	13336554 (98.53%)

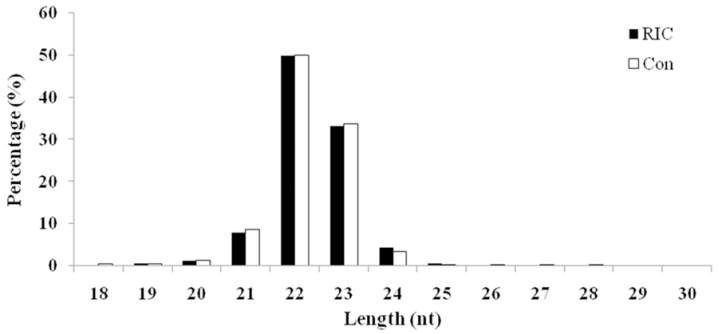

Figure 1. Length distribution of all small RNAs obtained by sequencing. Con: Control.

lowed by reperfusion for 180 minutes. RIPostC treatment (4 cycles of 5 min ischemia/5 min reperfusion of lower limb) was applied at the start of the reperfusion. The heart was removed after reperfusion and immediately stored at -20°C in a refrigerator for 20 minutes. About five slices of heart tissue, with a thickness of about 1 mm, were cut along the apex to the base with the use of a blade. The slices were placed in 1% TTC-PBS buffer and then incubated in a thermostatic water bath at 37°C away from light for 20 mins. The heart tissue slices were then transferred into a 10% formalin solution. Photographs were taken, and the infarct size was determined.

Western blot

Proteins were denatured after boiling and loaded on 10% SDS-PAGE. The separated proteins were transferred onto NC membrane, which was blocked with a 5% skim milk-TBST solution at 4°C overnight. The membrane was then washed, and primary antibody was added, followed by incubation at 4°C overnight. HRP-labeled secondary antibody was added after the membrane was washed and incubated at room temperature for 1 hour. ECL luminescence reagents were applied to the membrane after washing; the film was then exposed, developed, and fixed. Semiquantitative analysis of the hybridization signal was carried out by using an image analysis system. Bands were normalized to the eukaryotic translation initiation factor 5 (eIF-5) expression.

Statistical analysis

Statistical analysis was done by using the SPSS version 20 software (SPSS Inc., Chicago, IL). The data were presented as mean ± standard error of the mean, and *t* test or one-way ANOVA with the Tukey post hoc test was used for comparison between groups. All the statistical tests were two-sided, and a *P* value of less than 0.05 was considered indicative of a statistically significant difference.

Results

Overview of sequencing data

After the sequencing, no low quality sequences were found in both Con Library and RIC Library and other types of contaminant reads, such as 3' adaptor null or insert null or 5' adaptor contaminants were removed from the original data. All the clean miRNA sequences for sequencing obtained after the removal of contaminant reads accounted for 97.12% in Con Library and 98.53% in RIC Library (**Table 1**). The data on the length distribution showed that the most frequently read length was 20-24 nt, the peak at 22 nt, indicating the high purity of the two libraries (**Figure 1**). **Figure 2** presents the analysis results for common and specific sequences. The expressions of the common sequences were found to be relatively concentrated, indicating good overall consistency in the sequencing of Con Library and RIC Library.

Differential microRNA expressions and qPCR verification

239 miRNAs were detected in the plasma samples from all subjects by high throughput

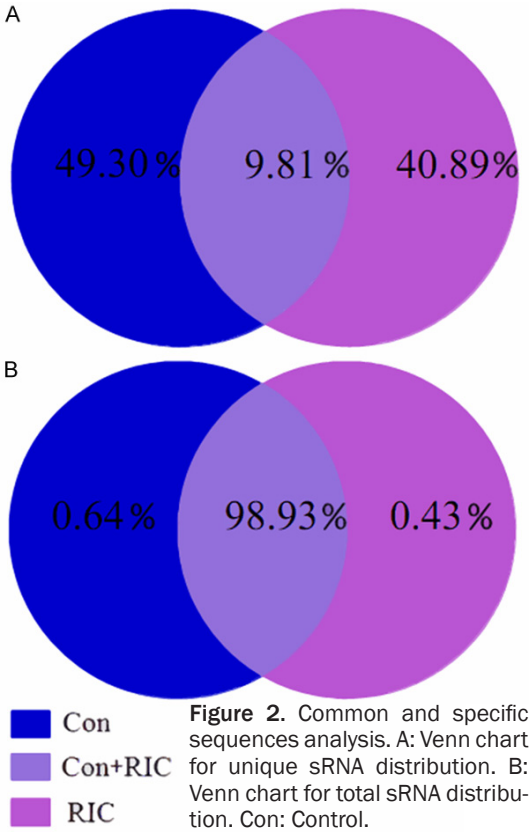

Figure 2. Common and specific sequences analysis. A: Venn chart for unique sRNA distribution. B: Venn chart for total sRNA distribution. Con: Control.

sequencing in this study. Among these, 37 miRNAs were significantly differentially expressed between the two libraries (P<0.05), which met the requirements of a fold-change $\log_2$ (RIC/Con) >1 or a fold-change $\log_2$ (RIC/Con) <-1. Of the 37 miRNAs, 4 were up-regulated, 33 were down-regulated (**Table 2**). To verify the results of the sequencing data, the expressions of hsa-miR-21-5p, hsa-miR-26b-5p and hsa-miR-92a-3p were further detected by using qPCR. The expression patterns of these 3 miRNAs were consistent with those in the sequencing study, which confirmed the accuracy of sequencing results (**Figure 3**).

GO enrichment analysis on the target genes of differentially expressed miRNAs

GO consists of three ontologies, including the biological process (P), molecular function (F), and cellular component (C). In this study, GO enrichment analysis was carried out in terms of the target genes of 37 differentially expressed miRNAs. The results showed that 28, 15, and 10 were significantly enriched in the P, F, and C ontology respectively. The predictive target genes of these differentially expressed miRNAs were mainly enriched in cell metabolism, cellular process regulation and so on (**Figure 4**).

Table 2. Differentially expressed plasma microRNAs after RIC treatment

microRNA	fold change (RIC/Con)	q-value	Style
hsa-miR-9-5p	20.59	0.000	Up
hsa-miR-192-5p	4.23	0.000	Up
hsa-miR-107	3.18	0.000	Up
hsa-miR-126-5p	2.04	0.000	Up
hsa-let-7b-5p	0.47	0.000	Down
hsa-let-7a-5p	0.47	0.000	Down
hsa-miR-340-5p	0.44	0.000	Down
hsa-miR-140-3p	0.43	0.000	Down
hsa-miR-221-3p	0.42	0.000	Down
hsa-let-7i-5p	0.40	0.000	Down
hsa-miR-30d-5p	0.37	0.000	Down
hsa-miR-151a-3p	0.36	0.000	Down
hsa-miR-30e-3p	0.36	0.000	Down
hsa-let-7e-5p	0.35	0.000	Down
hsa-miR-451a	0.32	0.000	Down
hsa-miR-22-3p	0.31	0.000	Down
hsa-miR-19b-3p	0.29	0.000	Down
hsa-miR-423-5p	0.28	0.000	Down
hsa-let-7c-5p	0.27	0.000	Down
hsa-let-7d-5p	0.26	0.000	Down
hsa-miR-30e-5p	0.25	0.000	Down
hsa-miR-92a-3p	0.24	0.000	Down
hsa-miR-98-5p	0.23	0.000	Down
hsa-miR-143-3p	0.23	0.000	Down
hsa-miR-148a-3p	0.21	0.000	Down
hsa-miR-16-5p	0.20	0.000	Down
hsa-let-7f-5p	0.18	0.000	Down
hsa-miR-19a-3p	0.17	0.000	Down
hsa-miR-21-5p	0.15	0.000	Down
hsa-miR-26b-5p	0.13	0.000	Down
hsa-let-7g-5p	0.13	0.000	Down
hsa-miR-30c-5p	0.10	0.000	Down
hsa-miR-30a-3p	0.10	0.000	Down
hsa-miR-146b-5p	0.10	0.000	Down
hsa-miR-93-5p	0.09	0.000	Down
hsa-miR-375	0.02	0.000	Down
hsa-miR-126-3p	<0.01	0.000	Down

Note: q-value: adjusted *P* value.

KEGG pathway enrichment analysis of miRNA candidate target genes

The KEGG pathway analysis was performed in terms of the target genes of these differen-

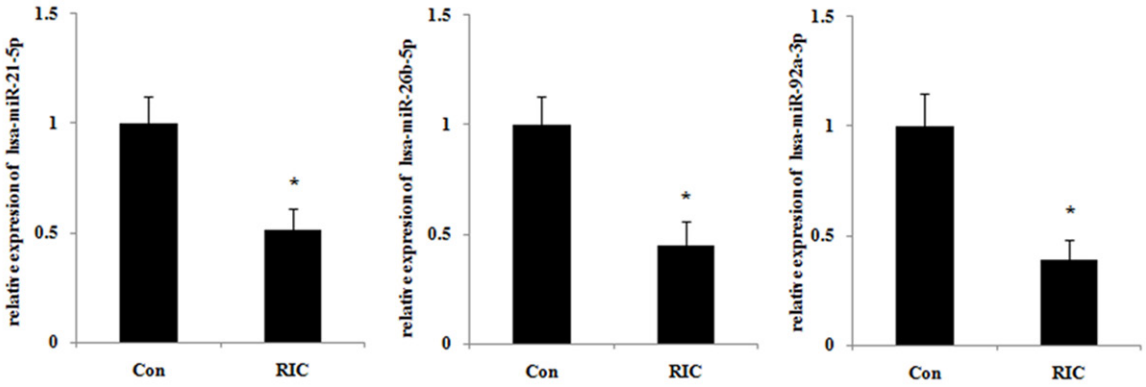

Figure 3. Validation of plasma miRNA expression with qRT-PCR. Con: Control. *P<0.05, compared to Con group, n=8 for each group.

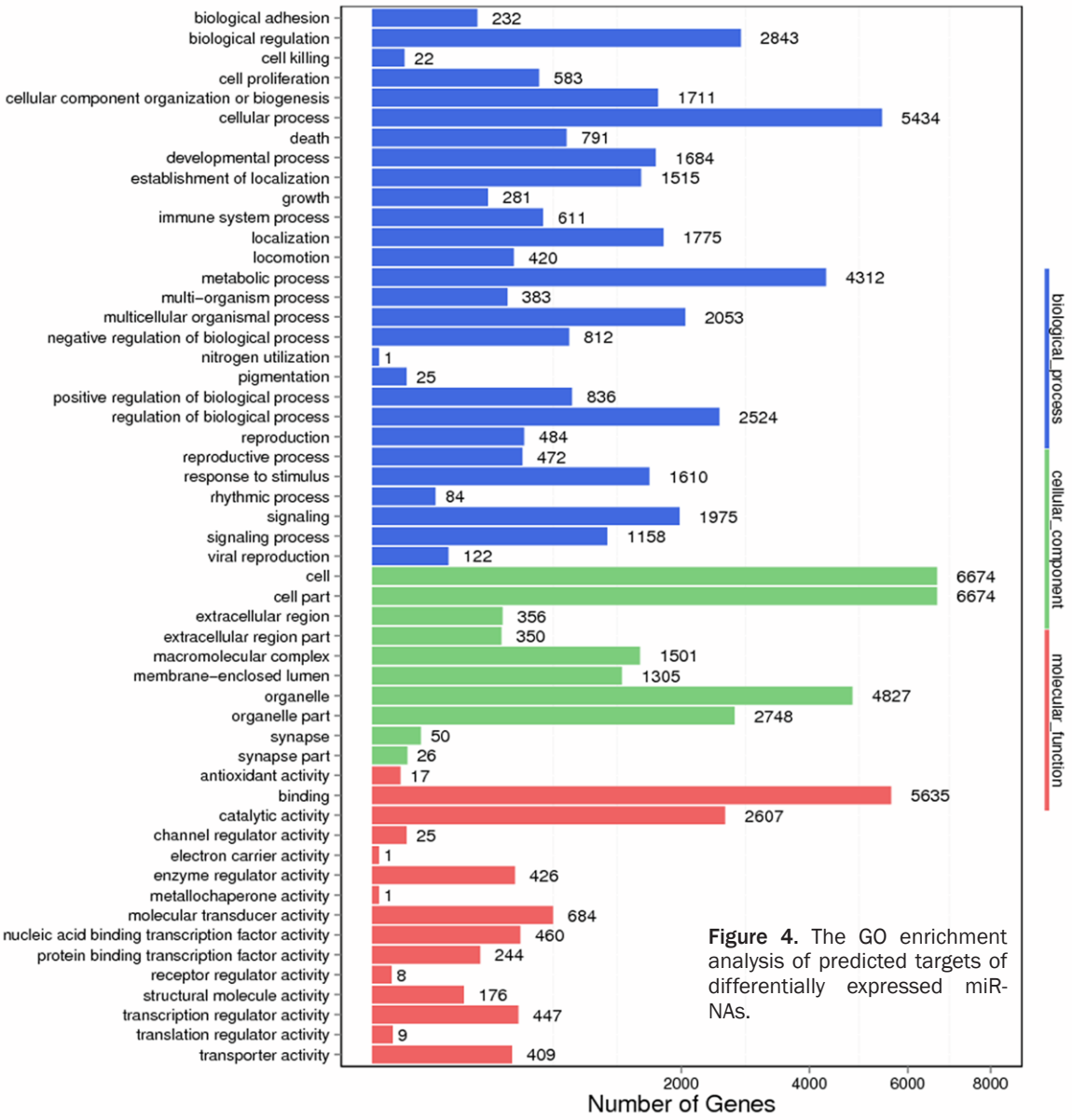

Figure 4. The GO enrichment analysis of predicted targets of differentially expressed miRNAs.

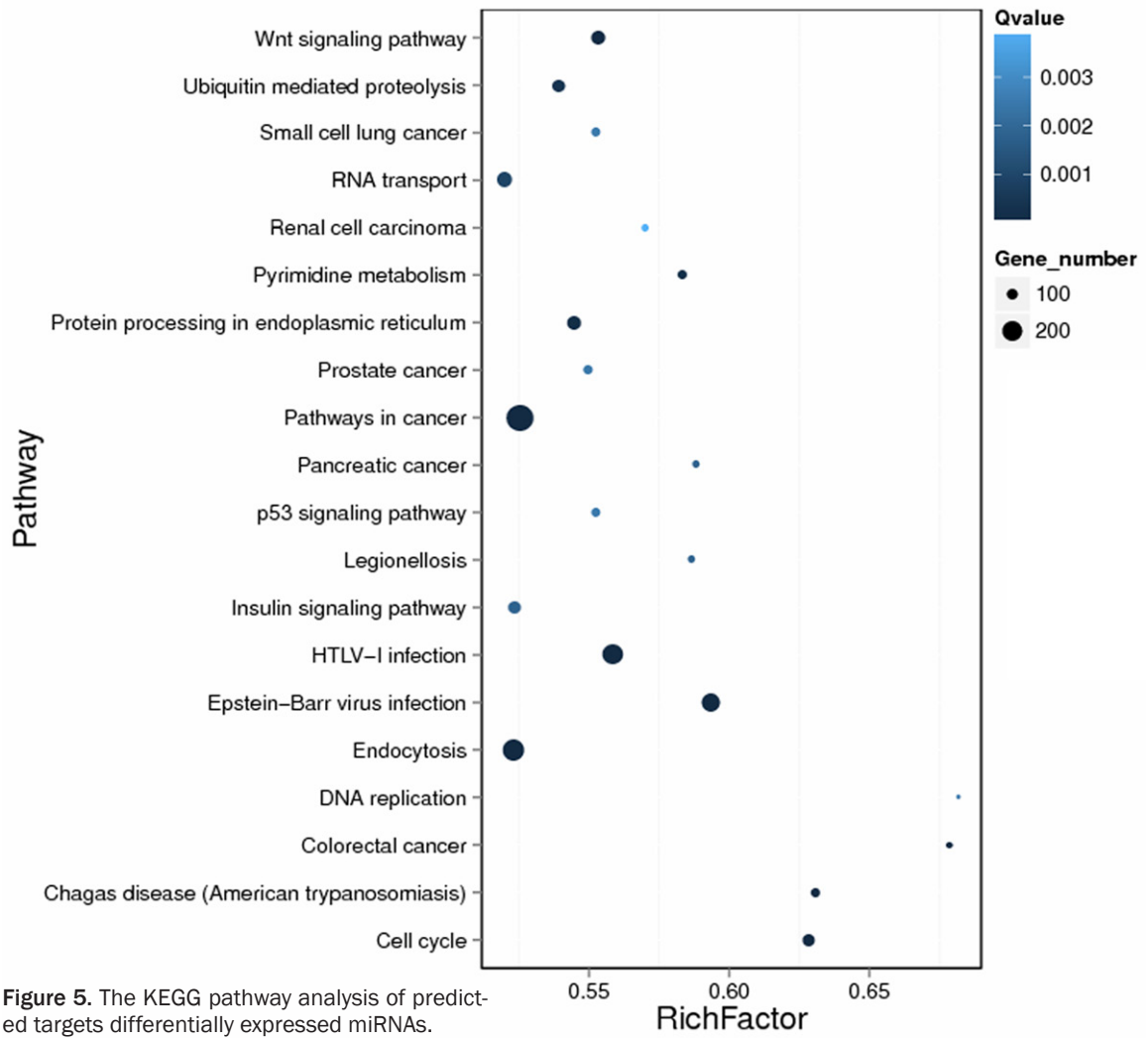

Figure 5. The KEGG pathway analysis of predicted targets differentially expressed miRNAs.

tially expressed 37 miRNAs. The results revealed that 20 metabolism pathways were enriched in the predictive target genes of these miRNAs (adjusted P<0.05) (**Figure 5**), such as the Wnt signaling pathway, RNA transport signaling pathway, endocytosis, the insulin signaling pathway, cell metabolism, and cell cycle signaling pathway.

RIPostC may activate the phosphorylation of GSK-3β in rat myocardium

A rat model of myocardial IR was established and RIPostC was carried out. RIPostC was found to significantly decrease the myocardial infarct size; the miR-21 levels in plasma and myocardium were both significantly decreased after RIPostC, compared with the IR group. The western blot results showed that after the application of RIPostC, the level of myocardial p-GSK-3β in rat increased. This finding was consistent with our KEGG analysis (**Figure 6**).

Discussion

In this study, the expression profile of plasma microRNA in five healthy subjects after RIC treatment was detected by the next generation high-throughput sequencing technique. 4 microRNAs were upregulated, and 33 were downregulated. The expressions of hsa-miR-21-5p, hsa-miR-26b-5p, and hsa-miR-92a-3p were verified through qRT-PCR. KEGG analysis showed that the Wnt, RNA transport, and endocytosis signaling pathways may be involved. Finally, a rat model of myocardial IR was created, and the myocardial protective effect of RIC, as well as the changes in miR-21 expression in plasma and myocardium, was verified. The results also confirmed that RIC could activate

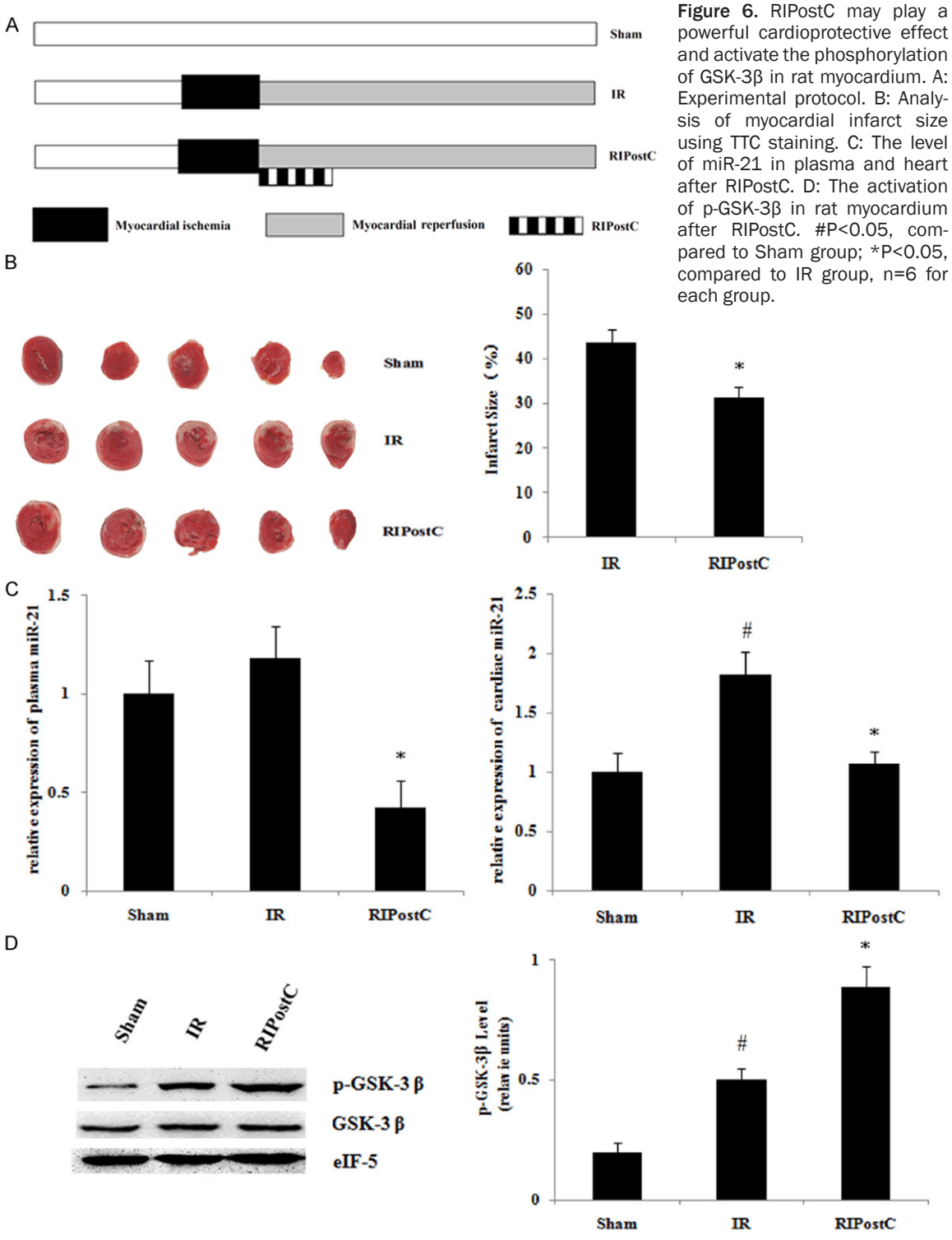

Figure 6. RIPostC may play a powerful cardioprotective effect and activate the phosphorylation of GSK-3β in rat myocardium. A: Experimental protocol. B: Analysis of myocardial infarct size using TTC staining. C: The level of miR-21 in plasma and heart after RIPostC. D: The activation of p-GSK-3β in rat myocardium after RIPostC. #P<0.05, compared to Sham group; *P<0.05, compared to IR group, n=6 for each group.

the phosphorylation of GSK-3β, the key molecule in the Wnt signaling pathway.

RIC is a promising technique with extensive clinical application because it is simple, noninvasive, safe, and effective. The mechanism involved in this procedure, however, remains unclear thus far. The literature indicates that after the application of RIC, a protection signal is sent from the extremities to the heart, exerting a myocardial protective effect. There are three main pathways, namely, the humoral,

neural, and immune pathways, among which the humoral pathway is the most notable [17]. In a study on five healthy volunteers in 2013, Hibert et al. found that RIC could cause a marked change in plasma proteome; similar results were found in a rat model [18, 19], indicating that the humoral pathway might play a key role in RIC. The main mechanism of the humoral pathway involves a marked change in circulating factors, which may eventually induce a powerful protective effect on the heart after RIC treatment of limbs [20]. The effect of RIC seems to extend beyond the above-mentioned pathways. RIPC has been reported to possibly modify the gene expression in mouse myocardium and human neutrophils [21, 22]. MicroRNAs have been shown to play a key role in gene expression. Besides, circulating microRNAs have been widely recognized to be potential markers for clinical diagnosis and to play important roles in the development of disease [23]. Thus, changes in microRNA expression and the role of microRNAs in RIC should be emphasized.

Studies have shown that the application of RIPC may induce the down-regulation of myocardial miR-1 and miR-21 [13]; Jing Li et al. [14] found that the expression of microRNA-144 was significantly increased in mouse plasma and myocardium after RIPC. Our omics study results showed that 37 microRNAs were differentially expressed in five healthy subjects after RIPC treatment. Of the 37 microRNAs, 4 were upregulated, and 33 were downregulated. In the present work, the expressions of hsa-miR-21-5p, hsa-miR-26b-5p, and hsa-miR-92a-3p were validated by using qPCR, and the results of qPCR verification were found to be consistent with that of the high-throughput sequencing, indicating the reliability of the latter. The above results showed that the expression profiles of plasma microRNAs in healthy subjects were significantly changed after RIC and indicated that various microRNAs might be involved in the regulation of the myocardial protective effect of RIC. In this study, of the 37 microRNAs, 33 microRNAs were down-regulation in plasma significantly after RIC. Cabrera-Fuentes HA et al. found that RNase1, which could confer powerful cardioprotective role in myocardial IR injury, increase significantly in plasma after RIPC, while the extracellular RNA reduce markedly. The down-regulation of plasma microRNAs after RIC might be due to the increase of plasma RNase1 [24, 25].

GO and KEGG pathway analysis are powerful tools in functional genomics; they can effectively predict the functions of gene products and thus successfully explore the potential bioinformatics information. GO analysis showed that the target genes of the differentially expressed microRNAs were closely related to cell metabolism, cellular process, and cell proliferation. KEGG pathway analysis indicated that the target genes of the differentially expressed microRNAs were associated with signaling pathways, such as RNA transport and endocytosis. RIC may be related to the transfer process of the protection signal from remote limb tissues to the heart, and microRNAs, as important functional small molecules, play an important role in remote information transmission. At present, the transport of microRNAs, especially mediated by exosome/microvesicle, is a hotspot in the study of trans-membrane transport. An exosome is a tiny vesicle less than 100 nm in diameter; a microvesicle is 100-1000 nm is diameter. They could mediate the trans-membrane information communication via transferring small functional molecule, including microRNA between different cells [26, 27]. The results indicate that microRNAs may be active in the transport process of RIC, which is consistent with the hypothesis that the RIC myocardial protection signal can be transported to the heart through the humoral pathway [28, 29].

KEGG analysis also showed that the target genes of the differentially expressed microRNAs were related to the Wnt signaling pathway, a classic signal pathway that plays an important role in multiple physiologic processes, including cell proliferation, apoptosis, growth, differentiation, and metabolism. The Wnt signaling pathway may induce the intracellular accumulation of β-catenin, and GSK-3β can add a phosphate group to the amino terminal of β-catenin, thus exerting a regulatory effect. Several studies have shown that the Wnt/GSK-3β/β-catenin signaling pathway has an important effect in the regulation in myocardial IR injury. The activated Wnt/GSK-3β/β-catenin signaling pathway may inhibit the apoptosis of myocardial cells and significantly reduce myocardial IR injury, thus protecting the myocardi-

um [30-32]. Our omics analysis showed that the target genes of differentially expressed microRNAs were closely related to the Wnt signaling pathway. The microRNAs in the expression profiles, such as miR-21, could regulate the phosphorylation of GSK-3β. We further constructed an IR rat model and carried out RIC treatment. The results showed an obvious phosphorylation of GSK-3β. Thus, the Wnt/GSK-3β/β-catenin signaling pathway may have an important regulatory effect on the myocardial protection of RIC.

The present study has some limitations. First, the sample size for the high-throughput detection was relatively small. Second, the objects of RIC were healthy subjects; in future research, patients with myocardial infarction will be recruited to determine the expressions of microRNAs. Finally, further data analysis is required, and more data have to be collected and organized. The focus of future research is to investigate the regulation effect and molecular mechanism of differentially expressed microRNAs in the myocardial protection of RIC.

By applying next generation high-throughput sequencing, this study found that 37 microRNAs were differentially expressed in plasma in five healthy subjects after remote ischemic conditioning. Further, the target genes of these miRNAs may be closely related to the RNA transport, endocytosis, and Wnt signaling pathways. MicroRNAs may play an important regulatory role in the mechanism of myocardial protection of RIC, providing a potential therapeutic target for the clinical treatment of myocardial IR injury in the future.

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Disclosure of conflict of interest

None.

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