Bioinformatics analysis of microRNA expression profiles in rat cortex following focal cerebral ischemia and reperfusion

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Abstract

Ischemic stroke is a serious disease of brain caused by the blood obstruction. Reperfusion is a necessary treatment for ischemic stroke, but itself also causes the secondary injury termed cerebral ischemia/ reperfusion (CIR) injury. In order to elucidate the molecular mechanism of CIR injury, We detected the miRNAs expression profile in rats cortex after focal cerebral ischemia and reperfusion by using miRNA microarray technique, and systematically analyzed the gene ontology function classifications as well as signaling pathways of genes targeted by these differentially expressing miRNAs with bioinformatics tools. The results showed that miRNAs expression profiles were significantly changed in the reperfusion phase of focal cerebral ischemia, and a total of 15 miRNAs were up-regulated and 44 miRNAs down-regulated. The target genes of these differentially expressed miRNAs were mainly involved in metabolic process and cellular process, which were identified as the hub node of miRNA-GO-network. The most correlated pathways included D-Glutamine and D-glutamate metabolism, renin-angiotensin system, peroxisome, PPAR signaling pathway, SNARE interactions in vesicular transport, and calcium signaling pathway. Our study suggested that miRNAs might play an important role in the pathological process of cerebral ischemia/ reperfusion injury. Understanding miRNAs expression and functions may shed a new light on illustrating the molecular mechanism of CIR injury.

Key Words: cerebral ischemia and reperfusion injury, molecular mechanism, miRNAs expression profiles, bioinformatics analysis

Introduction

Ischemic stroke, a serious disease of brain, is usually derived from the obstruction of blood supply artery. As one of the leading cause of death and disability worldwide, it brings about heavy medical burden. The main therapeutic methods was making recanalization to restore blood flow and obtain reperfusion [1]. But reperfusion itself could cause additional and unexpected brain damage termed as cerebral ischemia/ reperfusion (CIR) injury[2], which resulted in secondary energy failure and ensuing cell death in a delayed manner. There are some evidences supported this pathological process from animal model studies of gerbil[3] and rats[4], even from human patients trials[5]. And the pathophysiological mechanisms of CIR injury was very complex, including intracellular calcium overload, calcium sensitive proteinase activated, excitatory amino acid release, reactive oxygen species excessive generated, lipid peroxidation[6], inflammation, and mitochondrial respiratory function reduced[7], etc. And microRNA (miRNA) has been reported to implicate with the pathophysiological mechanisms of CIR injury[8] [9]. However, little was known about the bioinformatics of miRNAs genomics played in the molecular machenisms of CIR injury, and how they performed their effects.

MicroRNAs (miRNAs) are small noncoding single-stranded RNA molecules with mature transcripts of 18 to 25 nucleotides long that act as negative regulators of gene expression, which binds to the 3'-untranslated region (UTR) of complementary or partially complementary target messenger RNAs (mRNAs) and thereby downregulates the target mRNA via degradation or translational inhibition[10]. In CNS, miRNAs have been correlated with modulation of multiple diseases and pathological processes, such as neurodegenerative disorders[11], cancers[12], stroke[13], and cerebral ischemia reperfusion injury[14]. And the miRNA expressing profiles in CIR animal models are increasingly reported[15] [16] [17][18]. However, these studies merely provided the superficial changes of these miRNAs[19] [20] or focus on the role of single miRNA in cerebral ischemia[21][22], lacking of comprehensive analysis for their biological functions and downstream signaling transduction pathways.
In this study, we profiled the miRNAs expression following focal cerebral ischemia and reperfusion by using microarray technology, and systematically analyzed the gene ontology function classifications as well as signaling pathways of genes targeted by these differentially expressing miRNAs with bioinformatics tools. Our study suggested miRNAs might be as a novel promising therapeutic target, and will shed a new light on illustrating the molecular mechanism of CIR injury.

Materials and methods

Animal and Grouping

The total of 16 adult female Sprague-Dawley(SD) rats (weighing 200-240g) were used in these experiments. They were purchased from Chengdu dashuo experimental animal limited company (Chengdu dashuo Co., Ltd., Chengdu, China), and maintained in a constant 12-h light cycle with standard laboratorial chew and food-water available ad libitum. All animals were assigned randomly to two groups: sham group, and the cerebral ischemia/reperfusion (CIR) group (Table 1).

Focal cerebral ischemia/reperfusion rats model

The experimental group (CIR) rats were subjected to intraluminal occlusion of the right middle cerebral artery (MCAO) as described previously[23], using the MCAO monofilament suture with blunt tip (0.32±0.02mm in diameter, type 2432-A4, purchased from Beijing Cinontech Co.,Ltd.) for a period of 90min. Followed by 90min of occlusion, the suture was removed to restore blood flow reperfusion until 72h. While the sham group rats were only subjected to insert the suture into the origin of internal carotid artery but not the middle cerebral artery, and the other procedures were identity with the CIR group. Referring to Zea Longa scoring criteria[24] (Rating scales following operation of tMCAO/reperfusion), animal models were selected as follows: (1) Zea Longa scores≤2; (2) No concurrent subarachnoid hemorrhage or cerebral hemorrhage was found; (3) Surviving until the predetermined time point. During the surgery, the core temperature of rats were maintained at 37.0 ± 0.5°C by the means of heating lamp and animal body temperature maintenance instrument (type YLS-20A,ZS dichuang Beijing co., LTD). At day 3 postoperation (3dpo), all animals were sacrificed. The whole brain were rapidly removed and incubated with Triphenyl Tetrazolium Chloride (TTC) staining (n=5 in sham group, and n=5 in CIR group), and another ipsilateral cerebral cortex was harvested for miRNA microarray (n=3 in sham group, and n=3 in CIR group).

TTC staining and Quantitation of infarct volume

To confirm the infarction and evaluate the infarct volume, entire brain of rats were removed at 3dpo, were put into specific brain slices mould, and quickly frozen in -20°C refrigerator for 20min so as to slice easily. Be pay attention to keep the integrality of brains when removing. The whole brain was then sliced into 2.0 mm thick along with coronal level between -2.00 mm and +4.00 mm from Bregma. Brain slices were incubated in 2% TTC (Beijing Cinontech Co.,Ltd.) solution at 37°C for 30 min avoiding light with foil cover, as described before[25], and later photographed using a digital camera. Infarct volume was evaluated from digitized images using the Image J software package (Broken System Software Co.). The size of infarction as well as the total area of the contralateral hemisphere were measured in all slices. To compensate for the effect of brain edema after cerebral infarct, the corrected infarct volume was calculated as the sum of the infarct areas multiplied by the thickness of the slice (2.0 mm), and expressed as a percentage of the contralateral (non-occluded) hemisphere[26].

Isolation of total RNA

In CIR group, the tissue around the lesion from ipsilateral ischemic cortex of rats was obtained at 3d after tMCAO and reperfusion, and the correspondent cortex was harvested from sham group as well. Total RNA was extracted using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN) according to the manufacturer’s instructions, then dissolved into RNAase-free deionized water. The purity and integrity of total RNA was measured by using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and standard denaturing agarose gel electrophoresis.

RNA labeled and miRNA microarray test

After having passed RNA quality measurement, the samples of total RNA were labeled using miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) according to the manufacturer’s guideline. One microgram of each sample was 3'-end-labeled with Hy3TM fluorescent label, using T4 RNA ligase by the following procedure: RNA in 2.0 μL of water was combined with 1.0 μL of CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37°C, and was terminated by incubation for 5 min at 95°C. Then 3.0 μL of labeling buffer, 1.5 μL of fluorescent label (Hy3TM), 2.0 μL of DMSO, 2.0 μL of labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16°C, and terminated by incubation for 15 min at 65°C.

Following the labeling procedure, the Hy3TM-labeled samples were sequently hybridized on the miRCURYTM LNA Array (v.16.0) (Exiqon). The mixed total RNA labeled by Hy3TM of three samples in experimental group and control group performed the hybridizations on slide1 or slide2 respectively. According to array manual, the total 25 μL mixture from Hy3TM-labeled samples with 25 μL hybridization buffer were first denatured for 2 min at 95°C, incubated on ice for 2 min and then hybridized to the microarray for 16–20 h at 56°C in a 12-Bay Hybridization Systems (Hybridization System - Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signal. Following hybridization, the slides were washed several times using Wash buffer kit (Exiqon), and finally dried by centrifugation for 5 min at 400 rpm. The slides were then scanned using the Axon GenePix Pro 4000B microarray scanner (Axon Instruments, Foster City, CA).

Then, the Scanned images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data ex-
traction. All microarray signals were analyzed by subtracting the background and normalized using the Median normalization. After normalization, differentially expressed miRNAs were identified through Fold Change filtering. In all samples, only those normalized intensities ratio >2 or <0.5 (Fold Change>=2.0) were defined as significantly changed miRNAs and included in the calculation.

Bioinformatic analysis of the differentially expressed miRNA
According to both normalized intensities and fold changes of miRNAs in microarray arranged from large to small, we selected the top ten differentially expressed ones in up- and down-regulated miRNAs respectively, and analyzed the main functional categories of them using GO-Analysis, which based on Gene Ontology (http://www.geneontology.org/). GO-Analysis was regarded as a tool of illustrating biological process, cellular component, and molecular function that significant changed miRNAs enrolled in Gene Ontology, the major function classification in NCBI, provides a controlled vocabulary to describe gene and gene product that attributes in any organism (http://www.geneontology.org). GO-Analysis covers three domains: biological process (BP), cellular component (CC) and molecular function (MF). Two-side Fisher’s exact test was used to find whether overlap between the DE list and the GO annotation list more than expected by chance. The false discovery rate (FDR) of the GOID or PathwayID was used to correct the p-value. GOID refers to the ID of gene ontology term used in Gene Ontology Project, and similarly to the PathwayID. The p-value denotes the significance of GO terms enrichment in the DE genes. The lower the p-value, the more significant the GO Term (p-value<=0.05 is recommended). Enrichment Score, abbreviation of enrichment score value of the GOID or PathwayID, equals (-log10(Pvalue)). Fold Enrichment, abbreviation of the fold enrichment value of the GOID or PathwayID, equals (Count/Pop.Hits) / (List.Total/Pop.Total). These all indicators applied for GO category and pathway analysis.

Then, miRNA-GO-Network was used to elucidate the core gene function centralized by multiple miRNAs. Firstly, we determined the target genes of miRNA from prediction database miRecords, Targetscan, MicroCosm, Miranda, and Pictar, and selected the intersection part of retrieval results from these databases. Next, through topo analysis for target genes, we obtained the enrichment results of significant biological process (BP) in GO-analysis. Finally, we connected the miRNAs with the enrichment results of BP via gene-sets association, and constructed the miRNA-GO-Network mapping. The count of overlap >10 and Overlap Coefficient (>0.4) were used as the strength index of correlation. The red square type Node in the map represents miRNAs, blue black circular Node represents function terms. The Node size reflects how many Edges correlated with.

Furthermore, we also analyzed predicted target genes of these differentially expressed miRNAs by using pathway-analysis, a functional analysis method mapping genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The p-value (EASE-score, Fisher-Pvalue or Hypergeometric-Pvalue) indicates the significance of the correlated Pathways. The lower is p-value, the more significant is the pathway (The recommend p-value cut-off is 0.05).

Results
The cerebral ischemia/reperfusion rats model was established successfully
The whole brain tissue was harvested after 90min ischemic injury followed by 72h reperfusion, and sliced for TTC staining to determine the ischemic lesion. TTC is somewhat heat and light unstable dye, it can be enzymatically reduced by dehydrogenases rich in living tissue, which is red in color, while remains white color in necrotic tissue due to lack of such enzymatic activity. So the infarct region appears as white color, while non-ischemic region is red. Compared with sham group, the significant white infarct area in both cortex and striatum by TTC staining were seen in CIR group(Figure 1a), and the percentage of corrected infarct volume was calculated as 29.08 ± 1.39 and demonstrated in the histogram (Figure 1b). These results confirmed the animal model induced successfully by intraluminal obstruction and reperfusion.

Assessment of total RNA quality
The ratio of absorbance 260 nm to 280 nm (OD260/280 Ratio) in all samples were between 1.8 and 2.1, and the ratio of absorbance 260 nm to 230 nm (OD260/230 Ratio) in all samples were more than 1.8. There was no obvious evidence that the total RNA contaminated with protein or organic compounds (Table 2). The image of agarose gel electrophoresis demonstrated that the 28S and 18S ribosomal RNA bands were intense and clear. There were no obvious degradation had been found(Figure 2). These results indicated that a high purity and integrity of obtained total RNA, which was accordant with the standard one and could meet the requirements for the subsequent miRNA microarray experiment.

miRNA express profile in rats cortex following cerebral ischemia/reperfusion
In order to identify the expressing profile of miRNAs in rats cortex after focal cerebral ischemia/reperfusion, the 6th generation of miRCURYTM LNA Array (v.16.0) (Exiqon), which contains more than 1891 capture probes, was used to detect the differentially expressed miRNAs. And the signal strength (Normalized intensity) ratio >2 or <0.5 was considered as the differentially expressed miRNA, using the Fold Change method. The results showed that there were total 15 miRNAs up-regulated and 44 miRNAs down-regulated dramatically more than twice in the cortex of cerebral ischemia/reperfusion rats, compared with that of the sham rats (table 2). Then the top ten differentially expressed miRNAs from increased and decreased miRNAs were selected respectively for subsequent bioinformatics analysis, based on the largest quantitative value of both Fold Change and Normalized Intensity from our miRNA microarray data. They
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Table 1 Animal groups for microRNA array analysis and TTC staining

<table>
<thead>
<tr>
<th>Group</th>
<th>Model</th>
<th>Experimental procedure</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>sham</td>
<td>At 3dpo, correspondent cortex taken from 3 rats for microRNA array analysis, and taken from 5 rats for TTC staining</td>
<td>n = 3</td>
</tr>
<tr>
<td>II</td>
<td>CIR</td>
<td>At 3dpo, tissue around the lesion in isdemia ipsilateral cortex taken from 3 rats for microRNA array analysis, and the whole brain taken from 5 rats for TTC staining</td>
<td>n = 5</td>
</tr>
</tbody>
</table>

Table 2 Assessment of total RNA purity by Nanodrop ND-1000. There was no obvious evidence that the total RNA contaminated with protein or organic compounds. It indicated that the high purity of total RNA was.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>OD260/280 Ratio</th>
<th>OD260/230 Ratio</th>
<th>Conc. (ng/μl)</th>
<th>Volume (μl)</th>
<th>Quantity (ng)</th>
<th>QC result Pass or Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIR</td>
<td>2.00</td>
<td>2.24</td>
<td>1508.39</td>
<td>10</td>
<td>15083.90</td>
<td>pass</td>
</tr>
<tr>
<td>Sham</td>
<td>2.00</td>
<td>2.26</td>
<td>878.04</td>
<td>20</td>
<td>17560.80</td>
<td>pass</td>
</tr>
</tbody>
</table>

There was no obvious evidence that the total RNA contaminated with protein or organic compounds. It indicated that the high purity of total RNA was.

Included rno-miR-224*, -183*, -331*, -758*, -7a-2*, -1193-3p, -377*, -103-1*, -742, -3568 of up-regulated miRNAs, and rno-miR-338, -29b, -let-7a, -219-5p, -451, -3597-5p, -129-2*, -126*, -let-7i, -142-3p of down-regulated miRNAs (Figure 3).

GO analysis of differentially expressed miRNAs

Using GO-Analysis which based on the Gene Ontology, the functional categories associated with top ten up- and down-regulated miRNAs were determined. This covers three domains: biological process (BP), cellular component (CC) and molecular function (MF). The results were described as follows:

**Biological Process analysis of differentially expressed miRNAs**

For up-regulated miRNAs, the cascade relationship of each biological process could be seen from the Pvalue tree structure (Figure 4a), the most correlated biological processes were metalolic process, cellular component organization or biogenesis at cellular level, and long-chain fatty acid biosynthetic process, according to Count (Figure 4b), enrichment score (Figure 4c), and fold enrichment (Figure 4d) of target genes GO terms. Similarly, as for down-regulated miRNAs, the cascade relationship of each biological process displayed in the Pvalue tree structure (Figure 5a). The most correlated biological processes were cellular process, response to organic substance, and cell proliferation involved in metanephros development, according to Count (Figure 5b), enrichment score (Figure 5c), and fold enrichment (Figure 5d) of target genes GO terms.

**Cellular Component analysis of differentially expressed miRNAs**

For up-regulated miRNAs, the cascade relationship of each biological process could be seen from the Pvalue tree structure (Figure 6a), the most correlated cellular component categories were metabolic process, cellular component organization or biogenesis at cellular level, and long-chain fatty acid biosynthetic process, according to Count (Figure 6b), enrichment score (Figure 6c), and fold enrichment (Figure 6d) of target genes GO terms. Similarly, as for down-regulated miRNAs, the cascade relationship of each cellular component displayed in the Pvalue tree structure (Figure 7a). The most correlated cellular component categories were cellular process, response to organic substance, and cell proliferation involved in metanephros development, according to Count (Figure 7b), enrichment score (Figure 7c), and fold enrichment (Figure 7d) of target genes GO terms.

**Molecular Function analysis of differentially expressed miRNAs**

For up-regulated miRNAs, the cascade relationship of each molecular function could be seen from the Pvalue tree structure (Figure 8a), the most correlated molecular function categories were metalolic process, cellular component organization or biogenesis at cellular level, and long-chain fatty acid biosynthetic process, according to Count (Figure 8b), enrichment score (Figure 8c), and fold enrichment (Figure 8d) of target genes GO terms. Similarly, as for down-regulated miRNAs, the cascade relationship of each molecular function displayed in the Pvalue tree structure (Figure 9a). The most correlated molecular function categories were cellular process, response to organic substance, and cell proliferation involved in metanephros development, according to Count (Figure 9b), enrichment score (Figure 9c), and fold enrichment (Figure 9d) of target genes GO terms.
Figure 3: Differentially expressed miRNAs detected by microarray following cerebral ischemia and reperfusion injury.
(a) The diagram showed the significantly changed miRNAs selected by Fold Change and Normalized Intensity. Bars above the abscissa represented the top ten up-regulated miRNAs, while the bottom bars of the abscissa represented the top ten down-regulated miRNAs. (b) Heat map of the top ten up-regulated miRNAs. The heat map was constructed by signal log ratio values, up-regulated miRNAs were shown in red while down-regulated in green, and the color column from green to red depicted the altered miRNA expression from the lowest to highest.

Figure 4: Biological Process classification in GO analysis of top ten up-regulated miRNAs.
(a) The P-value tree structure illustrated the cascade relationship of each biological process regulated by these up-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the biological process. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten biological process based on Enrichment Score. (d) The bar plot ranked the top ten biological process based on Fold Enrichment.

Molecular Function analysis of differentially expressed miRNAs
For up-regulated miRNAs, the cascade relationship of each molecular function could be seen from the P-value tree structure (Figure 8a), the most related molecular function were binding, channel inhibitor activity, according to Count (Figure 8b), enrichment score (Figure 8c), and fold enrichment (Figure 8d) of target genes GO terms. Similarly, in down-regulated miRNAs, the cascade relationship of each cellular component displayed in the P-value tree structure (Figure 7a). The most correlated cellular component were cell, cell part, and compact myelin, according to Count (Figure 7b), enrichment score (Figure 7c), and fold enrichment (Figure 7d) of target genes GO terms.

Figure 5: Biological Process classification in GO analysis of top ten down-regulated miRNAs.
(a) The P-value tree structure illustrated the cascade relationship of each biological process regulated by these down-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the biological process. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten biological process based on Enrichment Score. (d) The bar plot ranked the top ten biological process based on Fold Enrichment.
Figure 6 Cellular Component classification in GO analysis of top ten up-regulated miRNAs.
(a) The Pvalue tree structure illustrated the cascade relationship of each cellular component located by these up-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the cellular component. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten cellular component based on Enrichment Score. (d) The bar plot ranked the top ten cellular component based on Fold Enrichment. (1: cellular_component; 2: cell; 3: cell part; 4: intracellular; 5: intracellular part; 6: organelle; 7: cytoplasm; 8: intracellular organelle; 9: organelle part; 10: membrane-bounded organelle; 11: intracellular organelle part; 12: intracellular membrane-bounded organelle).

Figure 7 Cellular Component classification in GO analysis of top ten down-regulated miRNAs.
(a) The Pvalue tree structure illustrated the cascade relationship of each cellular component located by these down-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the cellular component. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten cellular component based on Enrichment Score. (d) The bar plot ranked the top ten cellular component based on Fold Enrichment. (1: cellular_component; 2: cell; 3: organelle; 4: membrane; 5: cell part; 6: membrane-bounded organelle; 7: organelle part; 8: cell fraction; 9: intracellular; 10: organelle membrane; 11: intracellular part; 12: cytoplasm; 13: intracellular organelle; 14: cytoplasmic part; 15: intracellular membrane-bounded organelle).

miRNA-GO-network analysis
In order to reveal the core gene function that controlled intensively by multiple miRNAs, we constructed the miRNA-GO-network via gene-sets between miRNAs and the significant biological process in GO-analysis. The results showed that the most concentrated biological process were metabolic process, which was modulated by up-regulated miRNAs (Figure 8a). And cellular process was centralized by down-regulated miRNAs (Figure 9a). These suggested that multiple miRNAs co-participated in regulating a same biological process, and played key roles in this biological process.

Pathway analysis of differentially expressed miRNAs
Following GO analysis, KEGG database was employed to analyze the signaling pathways of specified miRNA target genes, to further study the functions and underlying mechanisms of these differentially expressed miRNAs under cerebral ischemia reperfusion conditions. The results found that up-regulated miRNAs involved in 30 pathways in CIR group, compared with that of sham group. According to the Enrichment Score, the main signaling pathway associated with up-regulated miRNAs were D-Glutamine and D-glutamate metabolism, and Renin-angiotensin system signaling pathways (Figure 12a). And the most correlated pathways of down-regulated miRNAs were peroxisome, PPAR signaling pathway, SNARE interactions in vesicular transport, and Calcium signaling pathway (Figure 12b).

Discussion
Cerebral ischemia/reperfusion (CIR) injury was a complex pathophysiological process, which was regulated by multiple factors. Recently, miRNA has been reported to be act as an important regulator in the neuronal death induced by CIR injury [27]. This indicated that miRNA might be responsible for CIR injury. In this study, the distinct patterns of miRNAs expression following focal cerebral ischemia and reperfusion were detected by microarray analysis. A total of 15 miRNAs have been found to be up-regulated while 44 miRNAs down-regulated in ipsilateral ischemic cortex of ex-
Figure 8 Molecular Function classification in GO analysis of top ten up-regulated miRNAs.
(a) The Pvalue tree structure illustrated the cascade relationship of each molecular function regulated by these up-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the molecular function. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten molecular function based on Enrichment Score. (d) The bar plot ranked the top ten molecular function based on Fold Enrichment. (1: molecular_function; 2: catalytic activity; 3: binding; 4: ligase activity; 5: nucleic acid binding; 6: protein binding; 7: ligase activity, forming carbon-nitrogen bounds; 8: G-protein alpha-subunit binding; 9: protein domain specific binding; 10: SMAD binding; 11: acid–amino acid ligase activity; 12: small conjugating protein ligase activity; 13: ubiquitin–protein ligase activity).

Figure 9 Molecular Function classification in GO analysis of top ten down-regulated miRNAs.
(a) The Pvalue tree structure illustrated the cascade relationship of each molecular function regulated by these down-regulated miRNAs. (b) The pie chart showed the top ten counts of the significant enrichment terms which represented the molecular function. The terms with pane marks are significant enrichment, more large more significantly. (c) The bar plot ranked the top ten molecular function based on Enrichment Score. (d) The bar plot ranked the top ten molecular function based on Fold Enrichment. (1: molecular_function; 2: catalytic activity; 3: transporter activity; 4: binding; 5: substrate-specific transporter activity; 6: substrate-specific transmembrane transporter activity; 7: protein binding; 8: substrate-specific transmembrane transporter activity; 9: enzyme binding; 10: protein dimerization activity; 11: ion transmembrane transporter activity).

Figure 10 miRNA-GO-network analysis via gene-sets for the top ten up-regulated miRNAs.
This network map revealed the core gene function that controlled intensively by multiple miRNAs. Green marked nodes were associated with GO Biological Process, red marked nodes were associated with up-regulated miRNAs.

Experimental rats. Among these, miR-29c has been reported to target both Birc2 and Bak1 genes to aggravate the CIR injury in rat models[28]. MiR-21, miR-29b/c, miR-145, miR-181, miR-200 family, miR-338, and miR let-7 family were found to change their expressing levels in the response to CIR injury in vivo[29]. Another study has also found miR-21 and miR-29b were up-regulated both in neurons and astrocytes exposed to oxygen and glucose deprived (OGD), which mimicked ischemic conditions and reperfusion in vitro[21]. All of these miRNAs that showed aberrant expression in cerebral ischemia/reperfusion have also been detected in our microarray data. The rest of differentially expressed miRNAs from our microarray data have not been reported yet, which need to further study and validation. Until recently, most of studies focused on the alternation of miRNA expression in the early stage of CIR injury or stage when the most serious injury occurred, but miRNAs expressing profiles and their roles during the middle and late stage of reperfusion have not been fully elucidated. Therefore, we performed our study at the late phase of the most serious injury. MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules that act as important regulators of their target genes. In CNS, these miRNAs have been correlated with pathological processes of neurological diseases, including ce-
rebral ischemia reperfusion injury[30]. Currently, increasing microarray studies of these miRNAs are available[31], but lacking of systematic analysis for their biological functions and downstream signal transduction pathways. Therefore, in this study, we selected the most dramatically changed miRNAs to perform further comprehensive bioinformatics analyses, including Gene Ontology classification, miRNA-GO-network analysis, and KEGG pathway analysis. The results indicated that up-regulated miRNAs were mainly participated in the regulation of metabolic process (fig 10), while down-regulated miRNAs modulated cellular process (fig 11). The pathways that associated with these functions were renin-angiotensin system, D-Glutamine and D-glutamate metabolism(fig 12a), peroxisome, PPAR signaling pathway, SNARE interactions in vesicular transport, and calcium signaling pathway(fig 12b).

In the group of up-regulated miRNAs, the center of miRNA-GO-network was metabolic process (fig 10). This suggested that majority of up-regulated miRNAs co-participate in the same biological process and play a key role in this process. As it is well known that brain exclusively depends on energy and the energy supply-consumption balance must be maintained. Energy metabolic disorder is the basis of ischemic injury. In this study, KEGG pathway analysis suggested that D-Glutamine and D-glutamate metabolism signaling pathway was potentially implicated with energy metabolic process. Glutamine is the main source of glutamate through its enzymatic hydrolysis. And glutamate is the most abundant free amino acid in brain[32], as an important transmitter of CNS. During cerebral ischemia reperfusion, disturbance of mitochondrial energy metabolism resulted in ATP synthesis decreasing, triggered the superfluous release of glutamate, increased additional influx of calcium, and thereby aggravated the cytotoxic edema and cellular injury[33]. To maintain glutamine and glutamate metabolism balance would be a good strategy to eliminate the cell damages from redundant glutamate. Moreover, pathway analysis results indicated that Renin-angiotensin system (RAS) was also implicated in metabolic process. As we know that RAS is an important body fluid regulation system, involved in the regulation of water-sodium metabolism to maintain the fluid balance. Angiotensin II (Ang II) is the main active medium of RAS, whose function implemented by the receptor AT1R and AT2R. Following cerebral ischemia and reperfusion, the mRNA expression of AT1R and AT2R were significantly increased within brain tissues[34], and Ang II persistent increasing could exacerbate cerebral edema and inflammatory damage[35]. Research has shown that RAS in the brain was involved in the pathological process of CIR injury. Activation of proinflammatory cytokine NF-kB by Ang II could enhance oxidative stress reaction, and then aggravate CIR injury[36]. These suggested that up-regulated miRNAs involved in metabolic process, associated with renin-angiotensin system, D-Glutamine and D-glutamate metabolism signaling pathways, and then played an significant role in CIR injury.

Majority of down-regulated miRNAs co-participated in
the regulation of cellular process (Fig 11). From our pathway analysis results, peroxisome, PPAR signaling pathway, SNARE interactions in vesicular transport, and calcium signaling pathway might correlated with regulation of this biological function by down-regulated miRNAs. The observations under electron micrographs[37] confirmed that there were three types of cell death induced by cerebral ischemia and/or reperfusion: necrosis, apoptosis and autophagy. Necrosis dominated the ischemic core, while apoptosis and autophagy mainly occupied the penumbra surrounding ischemic core. And Rami et al[38] found that autophagy and apoptosis might be interacted with each other in the peri-infarct area after cerebral ischemia. Following cerebral ischemia and reperfusion, disorder of energy metabolism let to depolarization of mitochondrial membrane potentials, thereby generated excessive reactive oxygen species (ROS). When the excess generation of ROS overwhelmed the capacity of endogenous free radicals removal systems such as peroxisome, the peroxidation damage of lipids, proteins, nucleic acids and polysaccharides subsequently occurred[39]. PPAR is peroxisome proliferator-activated receptor, activation of PPARγ could inhibit the oxidative stress and inflammatory response related to the up regulation of catalase[40]. While in PPAR gene knockout mouse, the expression of superoxide dismutase(SOD) and glutathione(GSH) were significantly decreased, while IFNγ significantly increased[41]. In addition to cause peroxidation damage, the depolarization of mitochondrial membrane potentials, triggered an additional influx of calcium[42]. After that, the mitochondrial permeability transition pore (MPTP) opened [43] and cytochrome c (CytC) released[44], and promoted the pro-apoptotic proteins[45] release thereby initiated the cell death cascade. Moreover, SNARE protein can regulate all events of cell membrane fusion due to it is a key component of cell membrane fusion protein complex[46]. It played an important role in the vesicular transport. In the nervous system, SNARE complex mediated the membrane fusion process between neurotransmitter vesicles and presynaptic membrane then releasing neurotransmitter, which was induced by influx of calcium[47]. Upon reperfusion, disorders of energy metabolism resulted in ATP synthesis decreasing while ROS generation increasing, triggered oxidative stress including endoplasmic reticulum stress-autophagy response[48]. Autophagy is a hydrolysis process mediated by lysosome. Activation of autophagy after cerebral ischemia might be as protection mechanism due to clear the cytotoxic substance, but it also induced cells eventually to be collapse and death, if the autophagy activated persistently[49]. In the late stage of autophagy, autophagosome and lysosome fusion are typical vesicle fusion events, which mediated by SNARE protein. These suggested that in CIR injury, peroxisome, PPAR signaling pathway, SNARE interactions in vesicular transport, and calcium signaling pathway were involved in the cellular processes such as necrosis, apoptosis and autophagy, and these processes were potentially modulated by down-regulated miRNAs from our microarray data.

Conclusion

From what has been discussed above, we can preliminarily conclude that miRNAs expression profiles were significantly changed in the reperfusion phrase of focal cerebral ischemia. To our knowledge, our study for the first time systematically analyzed the biological functions and downstream signaling pathways associated with target genes of miRNAs using by bioinformatics prediction methods. These differentially expressed miRNAs were mainly involved in metabolic process, cellular process, and played an important role in the infarction related brain injury. But it was required further study to validate them.

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