Identification of Non-coding RNA Biomarkers in Stress-induced Depression via Comprehensive Analysis of Competing Endogenous RNA Network

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Abstract

Background

Depression is one of the most common psychiatric disease worldwide. Although the research about the pathogenesis of depression have achieved progress, the detailed effect of non-coding RNAs (ncRNAs) in depression are still not clearly elucidated. This study was aimed to identify non-coding RNA biomarkers in stress-induced depression via comprehensive analysis of competing endogenous RNA network

Methods

In this present study, we acquired RNA expression from RNA seq expression profile in three mice with depressive-like behaviors using chronic restraint stress paradigm and three C57BL/6J wild-type mice as control mice.

Results

A total of 41 differentially expressed circular RNAs (circRNAs) and 181 differentially expressed messenger RNAs (mRNAs) were up-regulated, and 65 differentially expressed circRNAs and 289 differentially expressed mRNAs were down-regulated, which were selected by a threshold of fold change ≥ 2 and a p -value < 0.05 . Gene Ontology was performed to analyze the biological functions, and we predicted potential signaling pathways based on Kyoto Encyclopedia of Genes and Genomes pathway database. In addition, we constructed a circRNA-microRNA (miRNA)-mRNA regulatory network to further identify non-coding RNAs biomarkers.

Conclusions

Our findings provide a promising perspective for further research into molecular mechanisms of depression, and targeting circRNA -mediated competing endogenous RNA (ceRNA) network is a useful strategy to early recognize the depression.

1. Background

Depression is one of the most common psychiatric disease, with increased incidence due to the continuous stress on life and work [1]. The lack of early detection would impact on inappropriate diagnose, inefficient treatment and therefore quality of life of the patients, which in worse cases can lead to suicide [2]. However, the severity of depression is due to multiple factors related with its etiopathology. Although the pharmacological treatments have achieved progress, the precise molecular mechanisms are still not clearly elucidated. For these reasons, it is imperative and essential to investigate the underlying mechanisms and identify the potential biomarker, which could facilitate the identification of new therapeutic target for depression.

Currently, accumulating evidences have been put into the functions of non-coding RNA (ncRNA) because it could regulate gene expression [3, 4]. NcRNA, including circular RNA (circRNA) and microRNA (miRNA), are involved in the regulation of molecular function [5]. CircRNA has covalently closed loop structures, which is not similar to linear RNA with a poly-adenylated tail and 5'-3' polarity [6, 7]. CircRNA is spatiotemporal specific ncRNA with the structure of a circle through covalent binding [8]. MiRNA is endogenous ncRNA with a phosphate group at the 5' end and a hydroxyl group at the 3' end. MiRNAs are widely found in eukaryotes, and degrade target mRNAs or block translation of target mRNAs by base complement pairing [9]. CircRNA has been shown to function as miRNA sponge to increase the expression level of target gene [10]. CircRNA play an important role in the regulation of gene expression at the post-transcriptional level via serving as sponge of RNA-binding protein [11]. In addition, circRNA functions as a layer of binding mRNA directly to mediate translation [12]. Previous report showed that circRNAs were abundant in the mammalian brain, especially highly expressed in synapses [13]. Synaptic dysfunction can exert an essential role in mental diseases [14, 15]. MiRNAs are involved in the process of protein synthesis, and changes in miRNA levels were associated with mental disorders [16]. Based on the modulation of miRNA on mRNA expression process involving in mental diseases in previous study [17], it was postulated that circRNA could contribute to neuropsychiatric disorders by serving as a sponge of miRNA. Recent study demonstrated that circRNA mediated miRNA function might affect the pathogenesis of schizophrenia [18]. Although ncRNAs have significant functions on neuropsychiatric disorders, the underlying mechanisms of ncRNAs have not been clarified in depression.

Increasing evidences showed that circRNA functioned as miRNA sponge to promote the activity of mRNA [19–21]. Recently, researchers have focused on circRNA-miRNA-mRNA, as a novel regulatory mechanism of competing endogenous RNA (ceRNA), aiming at understanding RNA interactions deeply [22]. CeRNA could modulate transcriptional activation and chromatin modification [23]. Accumulating evidences have demonstrated that circRNA mediated ceRNA might take crucial part in the process of human diseases, including cancer [24] and neurological disease [25]. Lukiw et al. [26] showed that circRNA-7 might act as a miR-7 sponge to serve as Alzheimer's disease promoter via regulating expression of ubiquitin-conjugating enzyme E2A(UBE2A) and the epidermal growth factor receptor (EGFR). Additionally, a group of circRNAs has also been identified to be crucial for neuropsychiatric disorders [27]. Mahmoudi and colleagues briefly investigated the capacity of circRNA for modulating miRNA function in schizophrenia disease [18]. Besides, Jiang et al. found that expression profiles of changed circRNAs can be used to clarify the pathogenesis of depression [28]. Therefore, circRNAs are involved in the development of depression. However, the potential role of circRNA-mediated ceRNA network in depression remain unclear.

It is important to note that circRNAs are enriched at synapses and synaptic function deficit contributes to the development of neuropsychiatric disorders [27]. To study the effect of circRNA in depression, we acquired RNA expression from RNA seq expression profile in hippocampus tissues enriched at synapses with abundant circRNAs from mice with depressive-like behaviors using chronic restraint stress paradigm and C57BL/6J wild-type mice. In the present study, we found differentially expressed circRNAs (DEcircRNAs) and mRNAs (DEmRNAs), and constructed novel circRNA-miRNA-mRNA regulatory networks. We hypothesize ncRNA may provide insight into further identification the potential role in the

pathogenesis of depression. Therefore, targeting circRNA mediated ceRNA network is a useful strategy to recognize the depression early.

2. Materials And Methods

2.1 Brain samples

We purchased four-week-old C57BL/6J male mice from Guangdong Medical Laboratory Animal Center, and adopted chronic restraint stress paradigm on C57BL/6J mice to induce depressive-like behaviors. C57BL/6J wild-type mice as the control group were placed in a mouse cage, and C57BL/6J mice with chronic restraint stress paradigm as the depression group were placed in another mouse cage. Mice of two groups were placed in a cyclic rhythm environment of 12 h light/12 h dark with food and water availability in the following 14 days. Apart from that, every mouse of depression group was placed in a tight-fitting embossing tape with only a small hole for breathing between 7 p.m. and 10 p.m. The mouse could not move at all after being restrained in this tight-fitting embossing tape, just like under the condition of stress. We repeated the above operation on depression group mice for 14 consecutive days to induce depressive-like behaviors. Next, we performed behavioral tests to validate depressive-like behaviors. Finally, we adopted the method of dislocation of cervical vertebra to make mice euthanize. Hippocampal tissue samples from three mice with depressive-like behaviors and three C57BL/6J wild-type mice were obtained, frozen in liquid nitrogen, and sent for RNA sequencing analysis.

2.2 RNA library construction and sequencing

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 6000 Nano Lab Chip Kit (Agilent, CA, USA) with RIN number > 7.0. Approximately 10 ug of total RNA representing a specific adipose type was used to deplete ribosomal RNA according to the manuscript of the Epicentre Ribo-Zero Gold Kit (Illumina, San Diego, USA). Following purification, the poly(A)-or poly(A) + RNA fractions was fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, USA), the average insert size for the paired-end libraries was 300 bp ($\pm$ 50 bp). And then we performed the paired-end sequencing on an Illumina Hiseq 4000 at the (LC Bio, China) following the vendor's recommended protocol.

2.3 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

GO analysis was performed to annotate genetic regulatory networks based on DEmRNAs in three categories, including biological processes, cellular component and molecular functions (www.geneontology.org). The $-\log_{10}$ (p -value) denoted enrichment scores, which mean the significance of GO enrichment among DEmRNAs. Potential signaling pathways were explored to predicted based on

DEmRNAs according to KEGG pathways database (<http://www.genome.jp/kegg/>). The $-\log_{10}$ (p -value) denoted an enrichment score for the significance of pathway correlations.

2.4 Prediction of circRNA-miRNA interactions and mRNA-miRNA interactions

We predicted circRNA-miRNA interactions and mRNA-miRNA interactions via using TargetScan's (www.targetscan.org) and miRanda's (www.microrna.org) miRNA target prediction software. Next, the circRNA-miRNA co-expression networks and mRNA-miRNA co-expression networks were constructed with Cytoscape software V 3.7.2 (San Diego, CA, USA).

2.5 Construction of circRNA-miRNA-mRNA regulatory network

We displayed two comprehensive circRNA-miRNA-mRNA regulatory networks based on circRNA-miRNA interactions and mRNA-miRNA interactions. The circRNA mediated ceRNA networks were constructed with Cytoscape software V 3.7.2 (San Diego, CA, USA).

2.6 Statistical analysis

Statistical analysis was conducted by Statistical Package for Social Sciences (IBM SPSS) version 22.0 (SPSS, Chicago, IL, USA). Student's t -tests were used for comparisons between two groups. DEcircRNAs and DEmRNAs were selected by a threshold of fold change ≥ 2 and a p -value < 0.05 . p -values were adjusted by using False Discovery Rate correction. p -values < 0.05 were considered statistically significant.

3. Results

3.1. Comparison of depressive-like behaviors between stress-induced depression (SD) and control groups mice.

3.2 Different expressed circRNAs and miRNAs in the three mice with depressive-like behaviors and three control mice

106 DEcircRNAs and 470 DEmRNAs in the hippocampus tissue samples were evaluated based on RNA seq expression profile, which were listed in volcano plots visualization (Figure 2). We found that 41 DEcircRNAs and 181 DEmRNAs were up-regulated, and the most up-regulated were circRNA10080 and Shox2 respectively. While 65 DEcircRNAs and 289 DEmRNAs were down-regulated, and the most down-regulated were circRNA8757 and Eomes respectively. The top 20 up-regulated and 20 down-regulated DEcircRNAs and DEmRNAs were showed in the heat maps (Figure 3).

3.3. Functional analysis of DEmRNAs

In order to further identify the functions of these DEmRNAs, GO analysis was performed to analyze the biological functions, and potential signaling pathways were predicted based on KEGG pathway database.

GO analysis indicated that the most enriched DEmRNAs correlated with protein phosphorylation of the biological process, correlated with membrane of cellular component, and correlated with protein binding of molecular function. The top biological functions, including 25 biological processes, 15 cellular components, and 10 molecular functions were shown in Figure 4.

Results from KEGG pathway database demonstrated that the most paramount pathway was MAPK signaling pathway. The top 20 KEGG pathways correlated with these DEmRNAs were mitogen-activated protein kinase (MAPK) signaling pathway, human cytomegalovirus infection, chemokine signaling pathway, endocrine resistance, rhoGTPase-activated protein 1 (rap1) signaling pathway, receptor tyrosine kinase (erbB) signaling pathway, insulin signaling pathway, human immunodeficiency virus 1 infection, proteoglycans in cancer, vascular endothelial growth factor (VEGF) signaling pathway, glucagon signaling pathway, neurotrophin signaling pathway, chronic myeloid leukemia, bacterial invasion of epithelial cells, fluid shear stress and atherosclerosis, Insulin secretion, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance, amoebiasis, small cell lung cancer, and fatty acid biosynthesis (Figure 5).

3.4. Prediction of circRNA-miRNA interactions

In order to understand the crosstalk of ncRNAs, circRNA-miRNA interactions were explored. We predicted 108 and 83 miRNAs targets that could potentially be regulated by 10 up-regulated and 10 down-regulated DEcircRNAs respectively. We construct the networks of circRNA-miRNA interactions as presented in Figure 6.

3.5. Prediction of mRNA-miRNA interactions.

In order to further identify potential miRNAs, we predicted interactions between DEmRNAs and their upstream potential miRNAs. In this study, there appears to be promising miRNA-mRNA regulatory networks, which were associated with depression. (Figure 7)

3.6. Construction of circRNA-miRNA-mRNA regulatory networks in depression

In view of ceRNA theory, combining the results of circRNA-miRNA and miRNA-mRNA co-expression networks, we constructed two circRNA-miRNA-mRNA regulatory networks to further explore the molecular mechanism of depression (Figure 8). The networks may be involved in the pathogenesis and development of depression.

4. Discussion

To the best of our knowledge, RNA seq expression profile is a requisite for revealing known and novel transcript, which could identify more gene expression than microarray analysis [29]. In this study, we found up-regulated 41 circRNAs and 181 mRNAs, and down-regulated 65 circRNAs and 289 mRNAs with significant differential expression between three mice with depressive-like behaviors and three control

mice. These results may be necessary to deeply investigate the effects and biomarkers of ncRNAs in the pathogenesis of depression.

CircRNA is an important ncRNA molecule, which regulates target gene by a post-transcriptional mechanism and takes part in critical function in gene expression [30]. Researchers have characterized gene expression as an essential factor in the development of disease [31]. However, there is a practical difficulty in obtaining human brain biopsy specimens for clinical exploration. It was reported that circRNAs existed abundantly and stably in synaptoneurosomes of mammals [7]. CircRNAs are suitable for serving as biomarkers to detect early and diagnosis disease because of the feature of circRNA stable structure [32]. Therefore, we can obtain DEcircRNAs from cerebrospinal fluid (CSF) or blood of patients to recognize diseases in clinical work. Among 106 DEcircRNAs in this study, we found that the most up-regulated DEcircRNA was circRNA10080 and the most down-regulated DEcircRNA was circRNA8757 between mice with depressive-like behaviors and control mice. These DEcircRNAs are viewed as special targets for recognize depression, and identified as noninvasive biomarkers for witnessing the therapeutic response with the treatment of depression in clinical work.

For further explore the biological functions of DEmRNAs in depression, we performed GO analysis, and predicted potential signaling pathways based on KEGG pathway database. Our study found that protein phosphorylation and MAPK signaling pathway were involved in depression. Protein phosphorylation is the process of transferring the phosphate group of ATP to the amino acid residues of the substrate protein (serine, threonine, tyrosine), and is a common mode of regulation in organisms. Previous study was consistent with that protein phosphorylation has been reported to play an important role in depression [33]. Argel et al. reported that eIF4E (eukaryotic initiation factor 4E) phosphorylation was particularly important for regulating depressive-like behaviors [34]. It was revealed that phosphorylated cAMP response element-binding protein (CREB) were altered in the hippocampus of depressive mice [35]. Notably, protein phosphorylation is recognized as an essential player in the development of depression. The mitogen-activated protein kinase (MAPK) is a group of serine-threonine protein kinases that can be activated by different extracellular stimuli, and the MAPK signaling pathway is an important transport pathway that carries signals from the cell surface to the inside of the cell nucleus [36]. The MAPK signaling pathway plays an important role in many important cellular physiological/pathological processes, such as environmental stress adaptation and inflammatory response [37]. As we known, depression is an affective disorder that occurs in response to prolonged external stressors and involves a range of pathophysiological processes including an inflammatory response [38]. Therefore, MAPK signaling pathway was thought to be associated with depression. Han et al. found that ghrelin control p38-MAPK signaling pathway took part in antidepressant effect in depressive mice [39]. It was reported that the NLRP3 inflammasome modulated MAPK signaling pathway in depressive mice [40]. In conclusion, we are consistent with the previous study that protein phosphorylation and MAPK signaling pathway were known to potential depression-related pathogenesis [41, 42].

Researches have emerged about the important effect of circRNAs on competing with pre-mRNA to interfere gene expression, and exerting its function as regulator to influence mRNA level [43, 44]. However,

there is still lack of evidences to explore the molecular etiology of depression. In fact, one circRNA can regulate other miRNAs based on similar binding sites [45]. Therefore, it is important for us to explore the effect of ncRNA in depression. In order to explore the interactions between circRNAs and miRNAs in depression, we selected 10 up-regulated DEcircRNAs and 10 down-regulated DEcircRNAs for further analysis. The 108 miRNAs and 83 miRNAs targets were predicted respectively based on miRNA target prediction software. As shown in Fig. 5 of this present study, miR-124 was the target of circRNA7256. It was reported that miR-124 was associated with the regulation of depressive-like behaviors [46]. Since miRNAs regulate target genes to control the levels of mRNAs, it is necessary for us to predict the interactions between miRNAs and mRNAs in depression. The upstream potential 447 miRNAs targets and 171 miRNAs targets were predicted based on 10 up-regulated DEmRNAs and 10 down-regulated DEmRNAs respectively through miRNA target prediction software. We found that miR-338-3p was able to interact with parvalbumin (pvalb) in Fig. 6. There were evidences that miR-338-3p and pvalb were identified to be abnormal expressed in depression, suggesting play a key role pathogenesis of depression [47, 48]. MiR-338-3p is a mature miRNA. Decreased level of miR-338-3p attenuates the inhibition of genic transcription and translation processes, and enhances the level of pvalb mRNA. Pvalb is the core molecule of different circuits in the brain in response to social defeat stress, and depression is associated with elevated pvalb-positive neuronal activity [49]. Our results are consistent with previous study, and offer promising insight into the crosstalk of ncRNAs. Following studies will be needed to confirm underlying circRNA-miRNA interactions and mRNA-miRNA interactions.

Previous researches have showed that ncRNA has a regulatory role in psychiatric diseases [50, 51]. According to circRNA share the miRNA response elements to abolish the suppressive effect on mRNA level [52], it is necessary to verify the circRNA-miRNA-mRNA regulatory network to further understand the effect of ncRNAs. For example, the circRNA *TLK1*/miR-335-3p/TIPARP signaling pathway was associated with neurological deficits [53]. Besides, circRNA *HDAC9*/miR-138 / Sirt1 was involved in synaptic dysfunction [54]. CeRNA analysis may offer promising insight into the mechanism of neuronal injury [55, 56]. However, the evaluation of circRNA mediated ceRNA in underlying effect of depression remains ambiguous. As shown in Fig. 7, two novel lncRNA-miRNA-mRNA regulatory networks involved in the development of depression were constructed. This supports the idea that 8 DEcircRNAs-28 miRNAs – 8 DEmRNAs and 7 DEcircRNAs-9 miRNAs – 2 DEmRNAs could serve as valid biomarkers for the early detection of depression. We can use reverse transcription-polymerase chain reaction to measure the levels of special DEcircRNAs- miRNAs -DEmRNAs biomarkers from blood or CSF of patients to recognize depression, and can overcome the limitations of conventional diagnostic technique. Our research could provide interactions of ncRNAs for further investigating the potential mechanisms of depression, and contribute to human healthy with potential therapeutic target of depression.

This is the early study to evaluate circRNA mediated ceRNA in the development of depression using RNA seq expression profile. Nevertheless, some potential limitations of the present study should be taken into consideration. Firstly, there is over- or under-estimation of numbers of DEcircRNAs and DEmRNAs because of limited sample size. The bigger data is required to be collected in future investigation. Secondly, one sample source in specific period might limit the results. More sample sources from diverse

periods using same molding technology are needed to reconfirm outcomes. Finally, the interactions of circRNAs and mRNAs still remain complex, our research might not reveal the detailed mechanism of circRNA in depression comprehensively. We are supposed to do further exploration based on excluding aforementioned factors.

5. Conclusion

To conclude, we found changed expressions of circRNAs and mRNAs according to RNA seq expression profile between mice with depressive-like behaviors and control mice. GO analysis was performed to analyze the biological functions, and potential signaling pathways were predicted based on KEGG pathway database. We constructed ceRNA networks by intergrading circRNA-miRNA interactions and mRNA-miRNA interactions to investigate the effect of ncRNAs in depression. These preliminary data suggest that circRNAs play a vital role in regulating their target gene, and may act as potential biomarkers to recognize depression. CircRNA-miRNA-mRNA regulatory network may provide a promising direction in the potential pathogenesis of depression, and even be targeted for therapeutic manipulation. However, our investigation was preliminary, further studies based on a bigger sample source should be explored to widen the knowledge of ncRNA in the pathophysiology of depression.

Abbreviations

non-coding RNAs, ncRNAs; circular RNAs, circRNAs; expressed messenger RNAs, mRNAs; competing endogenous RNA, ceRNA; ubiquitin-conjugating enzyme E2A, UBE2A; epidermal growth factor receptor, EGFR; differentially expressed circRNAs, DEcircRNAs; differentially expressed mRNAs, DEmRNAs; Gene Ontology, GO; Kyoto Encyclopedia of Genes and Genomes, KEGG; stress-induced depression, SD; mitogen-activated protein kinase, MAPK; rho GTPase-1, rap1; vascular endothelial growth factor, VEGF; epidermal growth factor receptor, EGFR.

Declarations

Ethics approval and consent to participate

Animal experimental protocols were approved by Ethics Committee of Experimental Animals of Jinan University (Guangzhou, China). We conducted an exploration on mice in accordance with the National Institutes of Health Guide for the Care and Committee Guidelines of Laboratory Animals.

Consent for publication

We agree with publication.

Availability of data and materials

We declare data and materials are available.

Competing interests

There are no conflicts of interest.

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Authors' contributions

Xuanjun Liu wrote the paper. LanYan, Chun Lin and Yiliang Zhang sorted out data, Haofei Miao, Shiyi Shen and Hui Zhao searched for documents. Shuming Zhong and Li Zhang modified paper. Yanbin Jia conceived and designed the study. The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

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Figures

Figure 1

(A) Crossing numbers in the open field test (OFT). (B) Rearing numbers from the open field test (OFT). (C) Grooming time in the open field test (OFT). (D) The sucrose preference ratio in the sucrose preference test (SPT). (E) The immobility in the forced swim test (FST). **P<0.01.

Figure 2

Volcano plots of DEcircRNAs (A) and DEmRNAs (B) between stress-induced depression (SD) and control samples. The horizontal axis represents the $-\log_{10}(\text{p-values})$, and the vertical axis represents the $\log_2(\text{fold change})$. The red dots represent the significantly up-regulated circRNAs or mRNAs, the green dots represent the significantly down-regulated circRNAs or mRNAs, and the black dots represent no significantly circRNAs or mRNAs.

Figure 3

Heat maps of the top 20 DEcircRNAs (A) and DEmRNAs (B) between stress-induced depression (SD) and control samples. Each column represents one sample, and each row represents one DEcircRNA (A) or DEmRNA (B). The left three columns named SD_HP1, SD_HP2 and SD_HP3 represent three samples of hippocampal tissues in the mice with depressive-like behaviors. The right three columns named Ctrl_HP1, Ctrl_HP2 and Ctrl_HP3 represent three samples of hippocampal tissues in the control mice. Red represents high relative expression, and blue represents low relative expression. The magnitude of deviation from the median is represented by color saturation.

Figure 4

Identification biological functions of DEmRNAs based on GO analysis. The horizontal lines represent the numbers of enriched DEmRNAs, and the vertical lines represent the top types of biological functions. Blue represents biological process, green represents cellular component, and orange represents molecular function.

Figure 5

Identification enriched pathways of DEmRNAs based on KEGG pathway database. Each row represents one enriched pathway, and circle size is depicted according to the enriched number of DEmRNAs. p-value

is represented by color saturation.

Figure 6

Prediction of circRNA-miRNA interactions. (A) 10 up-regulated DEcircRNAs and 108 miRNAs targets (B) 10 down-regulated DEcircRNAs and 83 miRNAs targets. The circRNA-miRNA co-expression networks were constructed with Cytoscape software V 3.7.2. Ellipse nodes represent DEcircRNAs and v-shaped nodes represent miRNAs. Red represents up-expression and blue represents down-expression.

Figure 7

Prediction of mRNA-miRNA interactions. (A) 10 up-regulated DEmRNAs and 447 miRNAs targets. (B) 10 down-regulated DEmRNAs and 171 miRNAs targets. The mRNA-miRNA co-expression networks were constructed with Cytoscape software V 3.7.2. Diamond nodes represent DEmRNAs and v-shaped nodes represent miRNAs. Red represents up-expression and blue represents down-expression.

Figure 8

Prediction of circRNA-miRNA-mRNA regulatory network. (A) 8 up-regulated-DEcircRNAs as decoy, 28 miRNAs as centre, and 8 up-regulated DEmRNAs as target. (B) 7 down-regulated DEcircRNAs as decoy, 9 miRNAs as centre, and 2 down-regulated DEmRNAs as target. The circRNA-miRNA-mRNA regulatory networks were constructed with Cytoscape software V 3.7.2. Ellipse nodes represent DEcircRNAs, v-shaped nodes represent miRNAs, and diamond nodes represent DEmRNAs. Red represents up-expression and blue represents down-expression.