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Comprehensive analysis of differentially expressed miRNAs in mice with kidney injury induced by chronic intermittent hypoxia

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Background: miRNAs have been reported to participate in various diseases. Nevertheless, the expression patterns of miRNA in obstructive sleep apnea (OSA)-induced kidney injury remain poorly characterized. In the current study, miRNA sequencing (miRNA-seq) was conducted to investigate miRNA expression profiles in a chronic intermittent hypoxia (CIH)-induced renal injury mouse model.

Methods: The mouse model of chronic intermittent hypoxia was established. Differentially expressed miRNAs (DEmiRs) were detected using miRNA-seq technology. The sequencing data were subjected to Gene Ontology (GO) functional enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using a bioinformatics approach. RT-qPCR was further used to evaluate the sequencing results. Finally, we created a network for clarifying the relationship between the miRNAs and target genes.

Results: In total, nine miRNAs were identified to be upregulated and nine to be downregulated in a mouse model of renal injury induced by chronic intermittent hypoxia. The Kyoto Encyclopedia of Genes and Genomes analyses revealed that the Wnt signaling pathway was involved in the development of chronic intermittent hypoxia-induced renal injury. Subsequently, eight DEmiRs, namely, mmu-miR-486b-3p, mmu-miR-215-5p, mmu-miR-212-3p, mmu-miR-344-3p, mmu-miR-181b-1-3p, mmu-miR-467a-3p, mmu-miR-467 d-3p, and mmu-miR-96-5p, showed a similar trend of expression when verified using RT-qPCR. Finally, five selected DEmiRs were used to construct a miRNA-mRNA network.

Conclusion: In conclusion, a total of 18 DEmiRs were identified in the mouse model of chronic intermittent hypoxia-induced renal injury. These findings advance our understanding of the molecular regulatory mechanisms underlying the pathophysiology of obstructive sleep apnea-associated chronic kidney disease.

KEYWORDS

miRNA, microRNA, chronic intermittent hypoxia (CIH), kidney injury, miRNA sequencing, bioinformatics analysis

Introduction

Obstructive sleep apnea (OSA), a breathing disorder, is characterized by chronic intermittent hypoxia (CIH). The prevalence of OSA is about 17% and 34% in middle-aged women and men, respectively (Yeghiazarians et al., 2021). OSA has been linked to numerous comorbidities, including metabolic disease (Light et al., 2018), nonalcoholic fatty liver disease (Mesarwi et al., 2019), cardiovascular diseases (Schütz et al., 2021), and Alzheimer's disease (Liguori et al., 2021). Although efforts have been undertaken to comprehend the consequences of OSA, there may be more comorbidities or problems associated with OSA than initially expected. Mounting evidence demonstrates that chronic kidney disease (CKD) is a highly prevalent comorbidity of OSA (Nowinski et al., 2020; Badran et al., 2021). Moreover, the prevalence of OSA in CKD patients is significantly higher than that in the general population (Lin et al., 2020). Patients with severe OSA exhibit a relatively high prevalence among people with CKD. Meanwhile, it has further been found that OSA could accelerate the loss of kidney function. To date, the molecular mechanisms of OSA-associated CKD have remained poorly understood.

miRNAs are small noncoding RNAs that have acted as critical post-transcriptional regulators of gene expression by binding to targeted mRNAs for translational inhibition or destabilization. Hundreds of miRNAs have received substantial attention over the last decades. They play a crucial role in multiple biological processes and pathological events (Pan et al., 2013; Torres et al., 2018; Matsuyama and Suzuki, 2020). For liver diseases, dysregulation of numerous miRNAs is reported to be associated with liver fibrosis, liver injury, and liver metabolism dysregulation (Wang et al., 2021a). In addition, ectopic miR-155-5p expression inhibits radiation-induced epithelium-to-mesenchymal transition *via* the GSK-3 β /NF- κ B pathway in the pathogenesis of radiation-induced pulmonary fibrosis (Wang et al., 2021b). A recent study demonstrates that miRNAs play an essential role in regulating vascular smooth muscle cells in cardiovascular diseases (Nguyen et al., 2021). However, a comprehensive analysis of the miRNA profile and functions in OSA-induced kidney injury remains to be thoroughly investigated.

In our research, miRNA sequencing (miRNA-seq) was implemented to identify miRNA expression profiles in a mouse model of renal injury induced by OSA. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to explore the functions of the mRNAs targeted by differentially expressed miRNAs (DEmiRs). In addition, RT-qPCR was further used to verify the sequencing

results. Finally, a miRNA-mRNA regulatory network was established. The present study aimed to uncover the potential mechanisms underlying kidney injury in the pathological processes of OSA-related CKD.

Materials and methods

Animals

Male balb/c mice were acquired from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. All experiments were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Fujian Medical University.

Mouse model of chronic intermittent hypoxia

The mice were randomly divided into two groups: the CIH group and the control group. The CIH mouse model was established as described in our previous study (Lai et al., 2021). In brief, the mice were placed in cages with a gas control system, which regulated nitrogen, air, and oxygen flow into the chambers. During the whole period of CIH, the fractional inspired O₂ was decreased from normal levels (21%) to about 6% within 60 s, followed by reoxygenation to normoxia levels within the next 60 s. The duration of CIH treatment was 8 weeks. The oxygen level was detected with an O₂ concentration monitor.

Histopathological examinations

After being euthanized, we collected kidney tissues and fixed them in 4% paraformaldehyde for 24 h. Then, the renal tissues were embedded in paraffin. Subsequently, we performed hematoxylin-eosin (HE) staining on 4 μ m thick sections to detect the changes of general cell morphology in kidney tissues. Finally, to make a precise pathological diagnosis, we carefully observed the sections under a light microscope.

miRNA-seq and bioinformatics analysis

Total RNA was extracted from the injured kidney tissue of mice following CIH exposure. The small RNA libraries were generated with the help of the Illumina TruSeq Rapid SR Cluster Kit. The purified cDNA libraries were utilized for cluster

TABLE 1 Primers used for RT-qPCR.

Gene	Sequence (5'→3')	Length (bp)
U6	F:5'GCTTCGGCAGCACATATACTAAAAT3' R: 5'CGCTTCACGAATTTGCGTGCAT3'	89
mmu-miR-467a-3p	GSP: 5'GGGGGGCATATACATACACACA3' R: 5'GTGCGTGTCGTGGAGTCG3'	66
mmu-miR-486b-3p	GSP: 5'GAAACGGGGCAGCTCAGT3' R: 5'GTGCGTGTCGTGGAGTCG3'	62
mmu-miR-215-5p	GSP: 5'GGGGGATGACCTATGATTG3' R: 5'GTGCGTGTCGTGGAGTCG3'	64
mmu-miR-344-3p	GSP: 5'GGGTGATCTAGCCAAAGCCT3' R: 5'GTGCGTGTCGTGGAGTCG3'	64
mmu-miR-181b-1-3p	GSP: 5'GGGGGCTCACTGAACAATG3' R: 5'GTGCGTGTCGTGGAGTCG3'	64
mmu-miR-467days-3p	GSP: 5'GGGGGGGATACATACACACAC3' R: 5'GTGCGTGTCGTGGAGTCG3'	65
mmu-miR-96-5p	GSP: 5'GGTTGGCACTAGCACAT3' R: 5'CAGTGGTGTCGTGGAGT3'	66
mmu-miR-212-3p	GSP:5'GGGGGATAACAGTCTCCAGTCA3' R:5'GTGCGTGTCGTGGAGTCG3'	66

generation on an Illumina's flow cell and then sequenced on Illumina NextSeq 500 according to the manufacturer's instructions.

The miRNA deep sequencing data were analyzed by miRDB and miRBase. Volcano plot analysis was conducted using R software. Hierarchical clustering was performed using Cluster 3.0. GO analysis was used to categorize the function of DE miRs. The KEGG pathway analysis was used to identify the main signaling pathways. The miRNA-seq experiment was employed in triplicate.

Validation of reverse transcription-quantitative PCR (RT-qPCR)

The expression of DE miRs was measured by RT-qPCR using an ABI 7500 System. Primer sequences are presented in Table 1. The relative amounts were determined using the $2^{-\Delta\Delta C_t}$ method, with U6 siRNA as the internal control. All RT-qPCR reactions were performed three times.

Prediction of miRNA targets

To reveal the potential functions of these DE miRs, their predicted target genes were filtered out according to the intersection of the miRDB and TargetScan databases. The miRNA-mRNA interaction network was created and visualized with the use of Cytoscape software.

Statistical analysis

Data were analyzed using SPSS (version 19.0). They were presented as the mean \pm Sd. Comparisons between the two groups were performed using an unpaired Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Chronic intermittent hypoxia-induced renal histological damage

With HE staining, we found that the glomerular and tubular structures were normal in the control group. Compared to the control groups, the CIH groups had significant changes such as glomerular atrophy, glomerular swelling, and renal tubular epithelial cell swelling (Figure 1).

Identification of DE miRs in the renal injury mouse model induced by chronic intermittent hypoxia

miRNA-seq was carried out to validate the differential expression of miRNAs in the renal injury mouse model caused by CIH. In total, 18 DE miRs were identified in the mouse model of CIH-induced kidney injury, including nine upregulated and nine downregulated DE miRs. Hierarchical

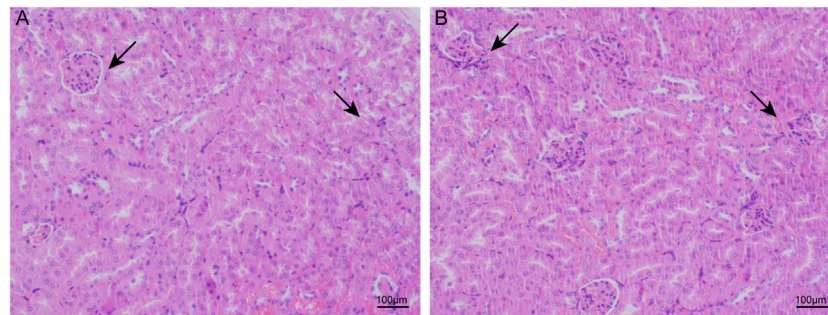


FIGURE 1

CIH caused renal structure damage in mice. In light microscopic examination, glomerular and renal tubular epithelial cell swelling and glomerular atrophy were more remarkable in the renal tissues of the CIH groups (B).

clustering analysis and volcano plot analysis of all differentially expressed miRNAs are displayed in Figures 2A,B, respectively.

Gene Ontology enrichment analysis

To better reveal the potential roles of these DE miRNAs in the etiopathogenesis of CIH-induced kidney injury, the targeted genes were then submitted to the GO database for functional annotation. That is, all the predicted genes were analyzed with their GO annotation, which included cellular components (CCs), molecular functions (MFs), and biological processes (BPs). The results demonstrated that the targeted genes of all DE miRNAs were mostly responses to the terms “cellular process” (BP), “intracellular anatomical structure” (CC), and “protein binding” (MF) (Figures 3A,B). This finding indicated that the expression of these DE miRNAs was crucial during the occurrence of renal injury caused by CIH.

Kyoto Encyclopedia of Genes and Genomes enrichment analysis

There were 141 KEGG pathways for all DE miRNAs. The top 10 pathways of up- and down-regulated DE miRNAs are listed in Figures 4A,B, including the MAPK signaling pathway, Wnt signaling pathway, and the FoxO signaling pathway. The most significantly enriched pathway of down-regulated DE miRNAs was the Wnt signaling pathway. These results suggested that the annotated pathways were important for the DE miRNAs involved in the development of CIH-induced renal injury. In addition, our findings pointed to 61 KEGG pathways for the up-regulated and 80 KEGG pathways for the down-regulated miRNAs.

Verification of DE miRNAs using RT-qPCR

RT-qPCR was used to determine the expression levels of the eight selected DE miRNAs. The results demonstrated that five up-regulated miRNAs (mmu-miR-486b-3p, mmu-miR-215-5p, mmu-miR-212-3p, mmu-miR-344-3p, and mmu-miR-181b-1-3p) and three down-regulated miRNAs (mmu-miR-467a-3p, mmu-miR-467 d-3p, and mmu-miR-96-5p) identified through miRNA-seq showed a similar trend of expression when verified using RT-qPCR (Figure 5). This finding suggested that our miRNA-seq data were reliable.

Construction of the miRNA–mRNA network

In total, five selected miRNAs, namely, mmu-miR-486b-3p, mmu-miR-344-3p, mmu-miR-215-5p, mmu-miR-212-3p, and mmu-miR-467a-3p, and their targeted genes were assembled in the integrated miRNA–mRNA network. The miRNA–mRNA network generated with the use of the five aforementioned DE miRNAs was created using Cytoscape (Figure 6). Our results provided a novel research strategy to explore the underlying mechanism of these DE miRNAs by revealing their targeted mRNAs.

Discussion

To the best of our knowledge, our current study is the first to report a comprehensive analysis of the differential expression profile of miRNAs in kidney tissue of a CIH mouse model. The possible functions and enriched signaling pathways of DE miRNAs in CIH-induced renal injury were then systematically analyzed. Specific miRNAs were selected to validate the sequencing data

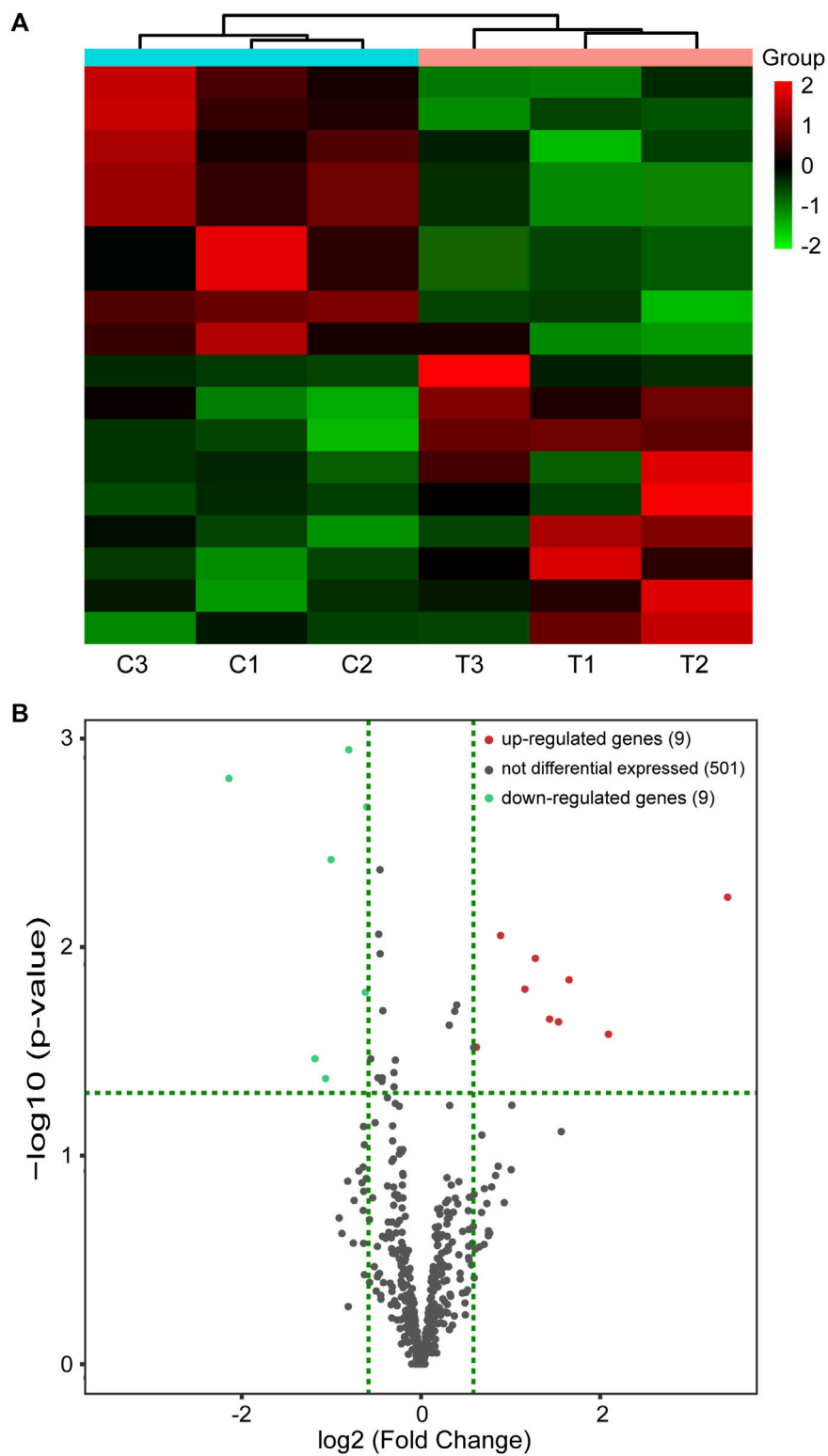
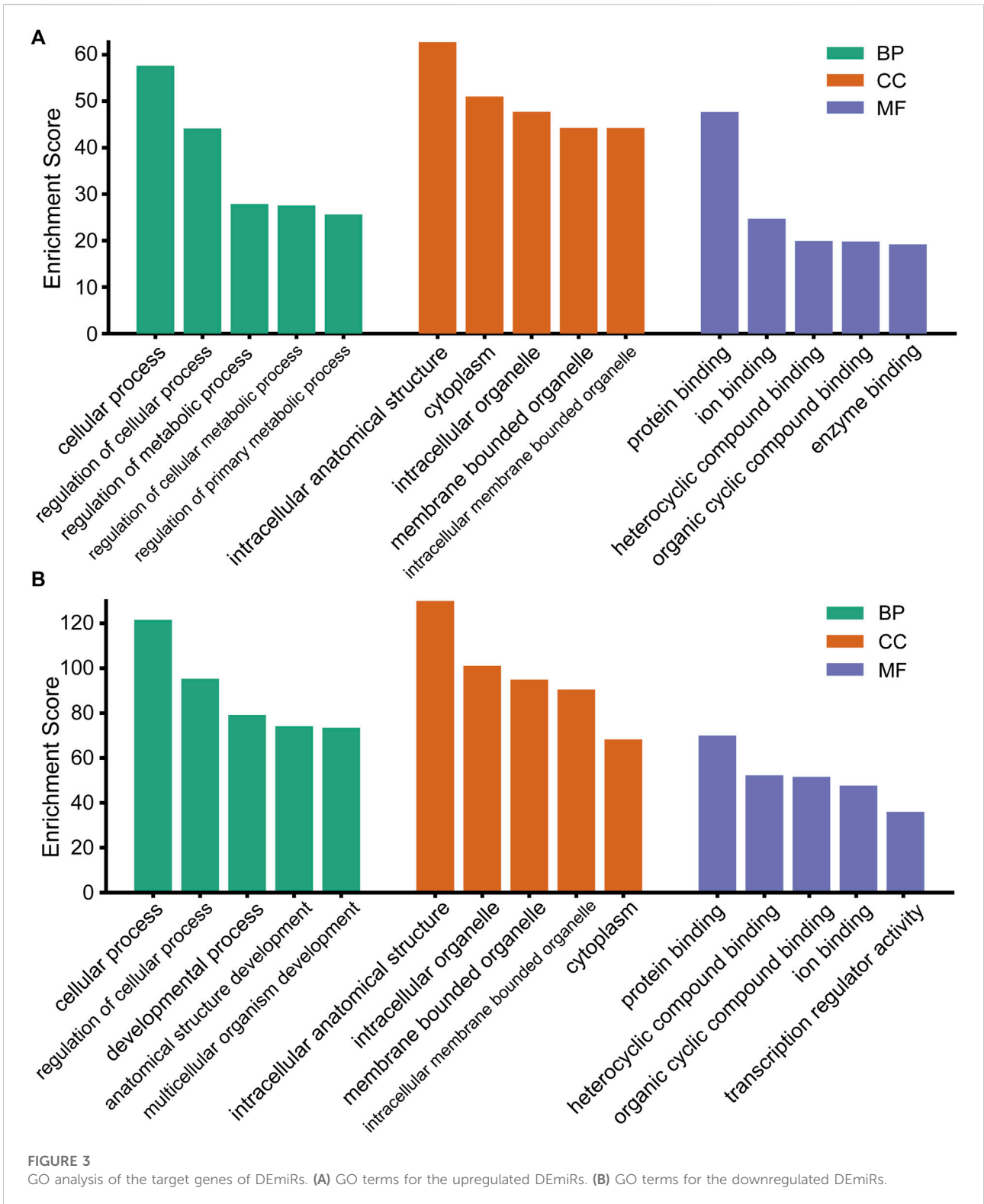
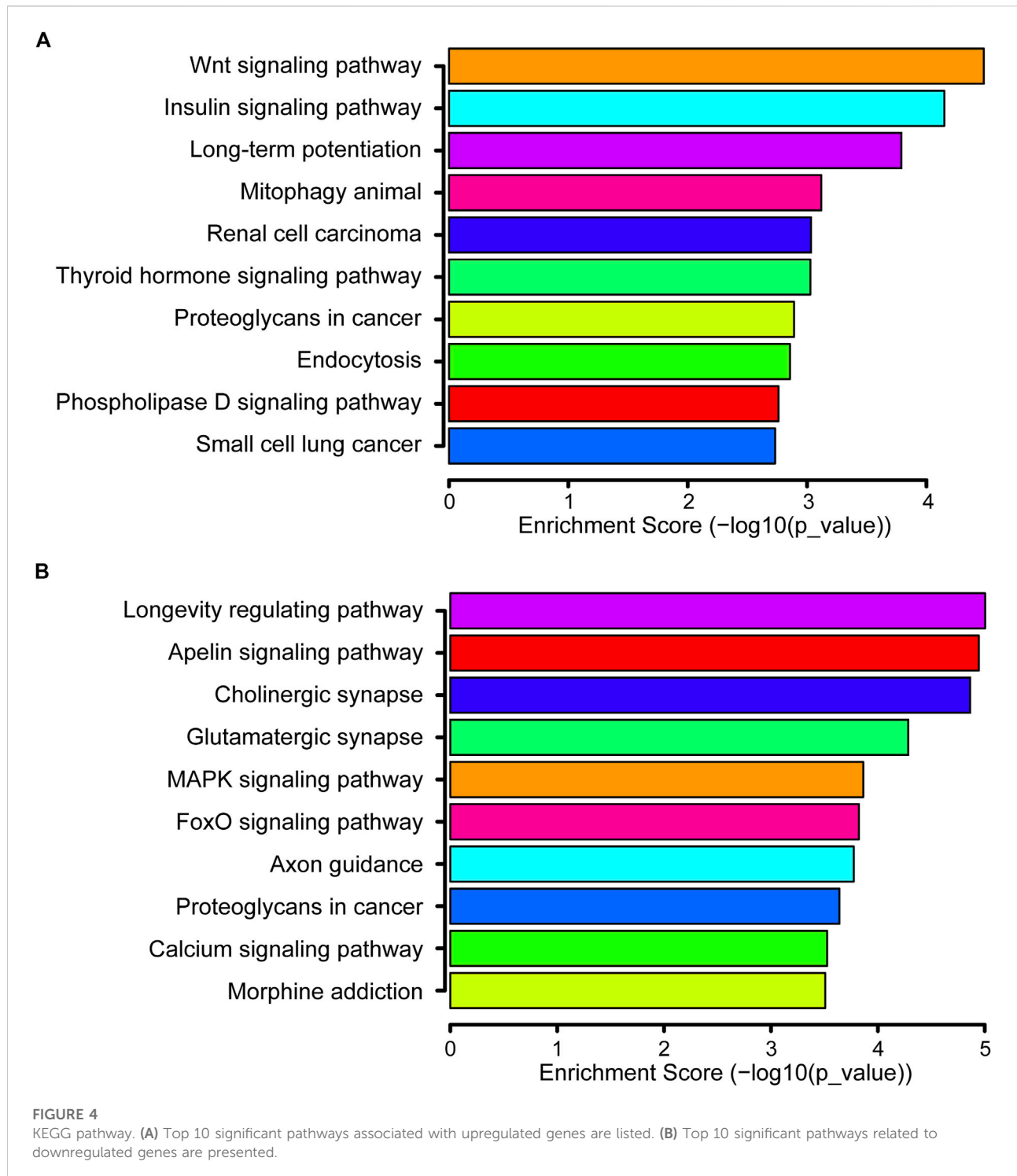


FIGURE 2 miRNA-seq data corresponding to the DEmiRs. **(A)** Hierarchical cluster of DEmiRs. Green represents downregulation and red represents upregulation. **(B)** Volcano plots. Red and blue dots represent DEmiRs. miRNA-seq, miRNA sequencing and DEmiRs, differentially expressed microRNAs.



using RT-qPCR. Our findings deepen our understanding of how changes in miRNA expression were involved in the underlying mechanisms of OSA-associated CKD.

OSA has been deemed as one of the leading causes of kidney impairment. OSA-associated CKD is mainly triggered by CIH-induced renal damage (Lin et al., 2018; Beaudin et al., 2022). OSA



triggers a subthreshold wake-up reaction because of a temporary shortage of oxygen to the various organs. Accumulating evidence has demonstrated that CKD is a relatively frequent complication in subjects with OSA. Recent studies suggested that the prevalence of OSA was significantly increased as renal

function decreased, and the treatment of CPAP may be beneficial for kidney function among individuals with OSA (Voulgaris et al., 2019). CIH, a key characteristic of OSA, might result in renal disease *via* its potential association with inflammation, increased sympathetic nervous system activity,

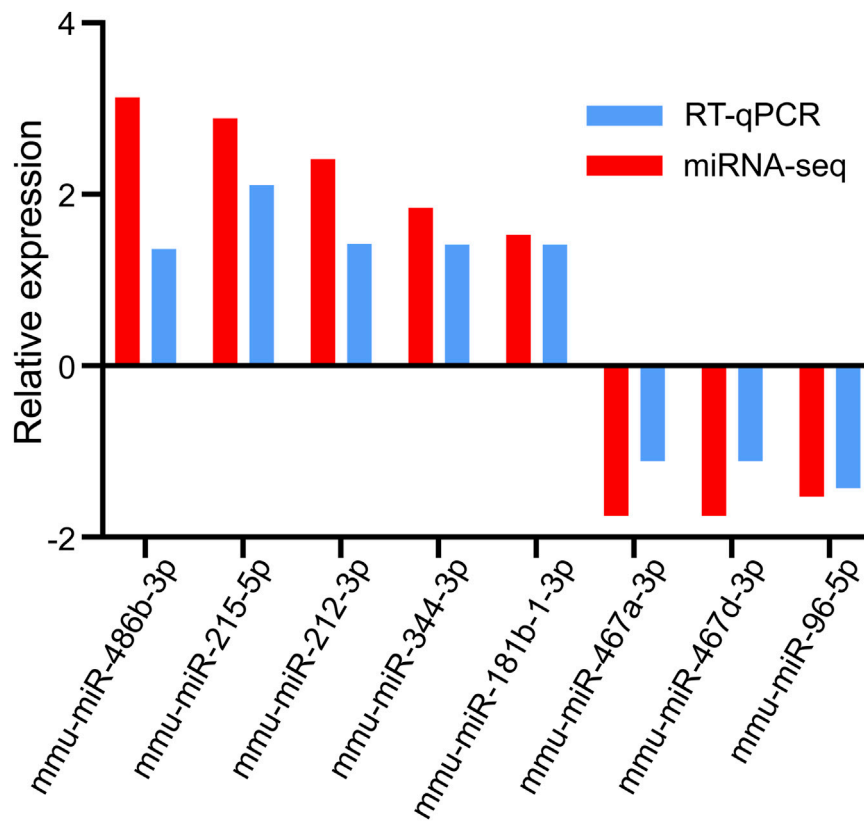


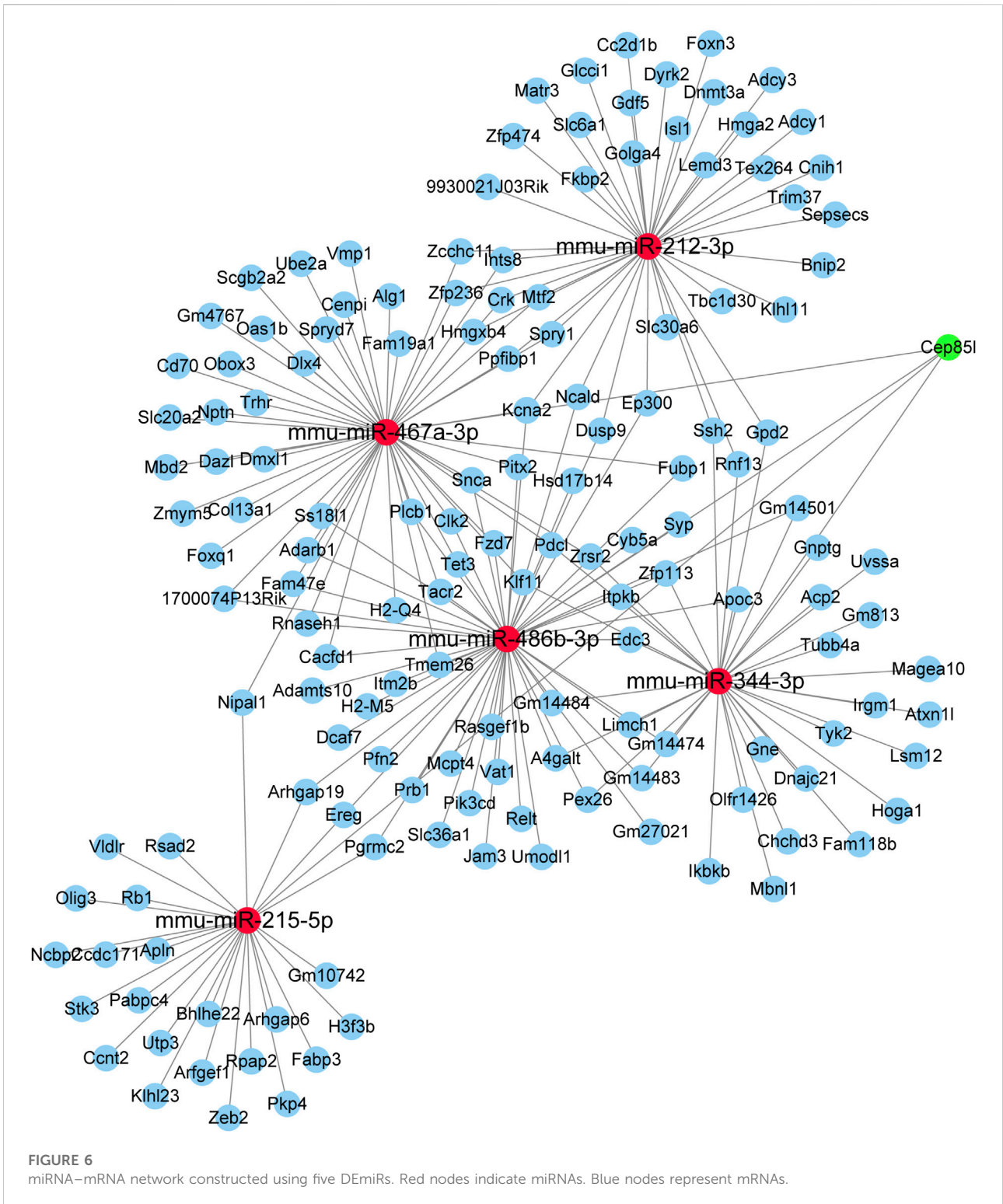
FIGURE 5
Validation of candidate DE miRNAs by RT-qPCR. miRNA-seq, miRNA sequencing.

activation of the renin-angiotensin-aldosterone system, intrarenal hypoxia, and oxidative stress (Turek et al., 2012; Hanly and Ahmed, 2014; Lu et al., 2017). However, the exact underlying mechanism of OSA-related renal injury remains unclear.

miRNAs have attracted increasing attention because of their universality in gene regulation and their stability among different samples. They are small noncoding RNA molecules less than 22 nucleotides in length. Moreover, miRNAs are important mediators of several pathological and physiological processes and have been found to affect various diseases. A previous study suggested that miRNAs may represent one of the promising therapeutic targets to treat inflammatory diseases in clinical practice (Lu et al., 2019). Liu et al. found that miR-132-3p could regulate osteoblast differentiation by repressing Smad5 in MC3T3-E1 osteoblastic cells in response to cyclic tensile stress (Liu et al., 2019). Additionally, it was reported that miR-374b could promote neural stem cell differentiation and proliferation *via* binding to Hes1 (Wu et al., 2018). However, the role of miRNAs in the pathophysiology of

OSA-related CKD has not yet been described. Therefore, we applied our previously described CIH mouse model to reveal the underlying mechanisms of miRNAs under the renal injury induced by CIH.

In this study, 18 miRNAs were found to be differentially expressed in the process of CIH-induced kidney damage. Of these DE miRNAs, nine miRNAs were upregulated and nine were downregulated. The eight selected DE miRNAs were assessed using RT-qPCR, and the results of RT-qPCR were in accordance with the miRNA-seq data. These identified miRNAs were further found to be involved in a variety of biological and pathological processes. For instance, miR-212-3p in HGSOV inhibited cell invasion, proliferation, and migration by targeting MAP3K3 in high-grade serous ovarian cancer (Zhang et al., 2020). Additionally, miR-215-5p inhibition attenuated doxorubicin-induced HL-1 cell apoptosis and death by upregulating ZEB2 expression (Xiong et al., 2021). Furthermore, miR-96-5p could promote cell migration in breast cancer by activation of the MEK/ERK signaling pathway (Qin et al., 2020). A previous study also revealed that miR-96-5p downregulation might be



used as a novel therapeutic strategy for treating allergic rhinitis (Zhan et al., 2021). However, the roles of these DE miRNAs in the development of CIH-induced renal injury remain unclear.

To further reveal the biological function of these screened miRNAs in the progress of renal damage caused by CIH, GO and KEGG enrichment analyses were carried out. Our findings showed that 141 pathways might be regulated by these

DEmiRs. For the upregulated DEmiRs, the Wnt signaling pathway was the most enriched one. Xiong et al. (2020) reported that the Wnt/ β -catenin pathway was related with hypoxia-related factors (HIFs). Meanwhile, it has been demonstrated that HIFs are very relevant in patients with OSA (Prabhakar et al., 2020). In addition, it was suggested that the Wnt/ β -catenin signaling pathway was implicated in the development of CIH-induced cognitive impairment (Pan et al., 2016). Previous studies have also found that hypoxic conditions might activate Wnt/ β -catenin pathways through DKK1 inhibition (Wang et al., 2022) and GSK3 β inactivation (Mayes et al., 2011). One latest study explored that CIH could aggravate the process of skeletal muscle aging by inhibiting KLC1/GRX1 expression through the Wnt/ β -catenin pathway (Guo et al., 2021). Thus, even though it was demonstrated that Wnt/ β -catenin was a key signaling pathway concerning OSA, the exact mechanism in CIH-induced kidney injury was not well understood.

Since the prominent biological function of miRNAs is to negatively regulate downstream mRNAs by targeting their 3'-untranslated region, we then predicted putative targets of these DEmiRs using two databases: miRWalk and miRDB. Through the complicated miRNA-mRNA network analysis, we discovered that one miRNA controlled a series of target genes, and numerous target genes were modulated by more than one miRNA. Elucidating the function of these targeted genes (e.g., Cep85L and EREG) was useful to enrich the current understanding of the complex molecular basis of renal damage induced by CIH. Meanwhile, erythropoietin (EPO), a small signaling molecule, was produced by the kidneys. Andrade et al. (2018) found that EPO was very relevant for patients affected by OSA. Thus, up/down-regulation of gene encoding EPO may be involved in the process of CIH-induced renal injury. Further research is urgently needed to clarify the functions and molecular mechanisms of all these differentially expressed miRNAs.

However, limitations were present in the study. First, no cell-specific identification of these miRNAs was conducted, and how these miRNAs function in CIH-associated renal injury remained unclear. This should be the subject of further investigations. Second, our investigation is only limited to male mice. Whether gender plays any significant role in altered miRNA expression of CIH-induced kidney injury remains to be studied. Third, our research falls short of exposing mice to acute hypoxia for 4 h and measuring the expression profile of miRNAs. This will reduce the persuasiveness of the study. Fourth, we only use HE staining to verify the pathological damage of CIH. It is better to report the oxygen concentration in a cellular environment, the partial pressure of oxygen and partial pressure of carbon dioxide in blood, and the pH of blood and the Handerson-Hasselbalch ratio together. Fifth, we did not observe the effects of hypoxia in the

different phases of sleep. Sixth, it lacks relevant details for a clinical perspective, which is very important for a common disease like OSA. Last but not least, the sample size was too small making the generalizability of the present results difficult to achieve.

Conclusion

In summary, the present study first demonstrated the profiling of DEmiRs in a mouse model of kidney injury induced by CIH using miRNA-seq and revealed their functional interaction network. These findings expanded the current understanding of the molecular etiology of OSA-associated CKD, which may contribute to providing new therapeutic strategies for this disease.

Data availability statement

The data presented in the study are deposited in the GEO repository, accession number GSE202480.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Fujian Medical University.

Author contributions

LY and QS designed the study. YN, CW, and WF established the mouse models and conducted the statistical analysis. YN and YB wrote the manuscript. QS revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2022.918728/full#supplementary-material>

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