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MicroRNA expression profiling and bioinformatics analysis of dysregulated microRNAs in obstructive sleep apnea patients

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Abstract

Obstructive sleep apnea (OSA) is a common chronic obstructive sleep disease in clinic. The purpose of our study was to use bioinformatics analysis to identify microRNAs (miRNAs) that are differentially expressed between OSA patients and healthy controls.

Serum samples were collected from OSA patients and healthy controls. To better reveal the sample specificity of differentially expressed microRNAs, supervised hierarchical clustering was conducted. We used the microT-CDS and TargetScan databases to predict target genes of the differentially expressed microRNAs and selected the common genes. The Search Tool for the Retrieval of Interacting Genes (STRING) was used to evaluate many coexpression relationships. Moreover, we used these potential microRNA-target pairs and coexpression relationships to construct a regulatory coexpression network using Cytoscape software. Functional analysis of microRNA target genes was conducted with FunRich.

A total of 104 microRNAs that were differentially expressed between OSA patients and healthy controls were identified. Supervised hierarchical clustering was conducted based on the expression of the 104 microRNAs in the OSA patients and healthy controls. Overall, 6621 potential target genes were predicted, and 119 target genes were screened based on coexpression coefficients in the STRING database. A regulatory coexpression network was constructed that included 23 differentially expressed microRNAs and 18 of the most related potential target genes. Metabolic signaling pathways were the most highly enriched category. Differentially expressed microRNAs, such as hsa-miR-485-5p, hsa-miR-107, hsa-miR-574-5p, and hsa-miR-199-3p, might participate in OSA. The target gene *CAD* might also be closely related to OSA.

Our results may provide a basis for the pathogenesis of OSA and the study of disease diagnosis, prevention, and treatment. However, more experiments are needed to verify these predictions.

Abbreviations: AHI = Apnea Hypopnea Index, CIH = chronic intermittent hypoxia, EPCs = endothelial progenitor cells, FDR = false discovery rate, HIF-1β = hypoxia-inducible factor-1β, OSA = obstructive sleep apnea, PSG = polysomnography, REDD1 = regulated in and DNA damage responses 1, STRING = The Search Tool for the Retrieval of Interacting Genes, STS = soft tissue sarcoma, TOR = target of rapamycin.

Keywords: bioinformatics analysis, microRNA, obstructive sleep apnea

1. Introduction

Obstructive sleep apnea (OSA) is a common chronic obstructive sleep disease in clinic. In recent decades, OSA has gradually been fully recognized due to media coverage and the improvement of people's health awareness. The pathogenesis, diagnosis, and treatment of OSA have also received significant academic attention. Epidemiological studies have confirmed that the prevalence of OSA has increased over the past 30 years[1,2] and suggest that OSA may be complicated by many serious diseases, for instance, cardiovascular diseases, diabetes, and metabolism syndrome. Studies have noted that OSA not only affects people's life and work seriously but also becomes a great financial burden for families.[3,4] Sleep structure is affected in OSA patients due to the complete or partial upper airway obstruction that occurs during sleep, leading to sleep fragmentation and daytime sleepiness because of repeated episodes of chronic intermittent hypoxia (CIH). The ineffective action of respiratory motion in the obstructive condition also results in increased pressure in the chest, intermittent asphyxia, and sleep-wake cycle disruption. CIH may also cause oxidative stress, inflammation, and metabolic disorders. Therefore, OSA can cause considerable damage to all body systems.

In 1993, Bartel first discovered microRNAs in *Caenorhabditis elegans*; however, people have not paid attention to the function of microRNAs in human disease until the last 10 years.[5] MicroRNAs are powerful regulators of gene expression. They are small single-stranded noncoding RNAs of approximately 18–24 nucleotides on average. MicroRNAs can regulate the expression of specific genes at the posttranscriptional level and can affect the

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level of protein expression by blocking the translation of mRNA.[6–9] Friedman believed that microRNAs can regulate the majority of human protein-coding genes.[10] MicroRNAs also have a very important role in the pathophysiology of the human body because of their regulatory effect on inflammation, oxidative stress, hypoxia, and metabolic disorders. Liu and his colleagues demonstrated that chronic intermittent hypopnea affected the expression of many microRNAs in a mouse model of intermittent hypoxia. In addition, CIH could induce endothelial cell damage and affect protein secretion in apoptosis and phagocytosis.[11] OSA can induce chronic intermittent hypopnea, leading to inflammation and oxidative stress. We have a new perspective on the mechanism of the occurrence and development of OSA and propose that the differential expression levels of microRNAs may serve as biomarkers for the diagnosis and prognosis of OSA.

To further study the mechanism of the role of microRNA in OSA and identify significant and novel diagnostic and therapeutic targets, we collected serum samples from OSA patients and healthy controls. Serum RNA was extracted, and libraries were constructed for RNA sequencing. We then performed an analysis to identify microRNAs that were differentially expressed between the 2 groups. Target genes of the differentially expressed microRNAs were confirmed, and the coexpression relationships among the target genes and microRNAs were analyzed via construction of a regulatory coexpression network. This study identified microRNAs and the corresponding potential target genes that may be involved in OSA, providing a basis for the study of the pathogenesis of OSA as well as the prevention, diagnosis and treatment of OSA.

2. Materials and methods

2.1. Research subjects

All participants were inpatients in the department of Otolaryngology Head and Neck Surgery of Beijing Anzhen Hospital affiliated with Capital Medical University. The participants enrolled in our study were divided into 2 groups: OSA patients and healthy controls. Each group included 3 participants. Blood samples were collected in tubes containing anticoagulant. Subsequently, separation of the serum was carried out, and was stored at −80°C. All participants underwent overnight polysomnography (PSG) in our department. The diagnostic standard of OSA is defined as Apnea Hypopnea Index (AHI) > 5 events/h, but the standard is further subdivided into mild ($5 < AHI \leq 15$ events/h), moderate ($15 < AHI \leq 30$ events/h), severe ($AHI > 30$ events/h). Our study population consisted of 3 OSA patients (mean age 48 ± 7.8 years; mean $AHI = 61.3 \pm 34.9$; and mean $BMI = 25.4 \pm 0.42$) and 3 health controls (mean age 45.7 ± 8.2 years; mean $AHI = 2.5 \pm 1.8$; and mean $BMI = 21.7 \pm 2.36$). A history of malignant tumor, ischemic heart disease, hypertension, diabetes mellitus, and chronic renal diseases was excluded in all participants. Our study was approved by the ethics committee of the hospital and was conducted in line with the Declaration of Helsinki. Informed consent was obtained for experimentation with human subjects.

2.2. MicroRNA expression profiles

Total RNA was extracted from the 6 samples, and small RNA libraries were established for deep sequencing based on the manufacturer's protocols. After adapters were ligated to the RNA, cDNAs were generated using SuperScript II Reverse Transcriptase (Life Technologies). The Illumina HiSeq 3000 (Illumina Inc) platform was used for sequencing after PCR amplification was performed. The clean reads acquired after trimming were used for mapping and further analysis. MicroRNA expression values were selected for the differential expression analysis using the edgeR tool from Bioconductor. The screening criteria used to identify differentially expressed microRNAs were as follows: normalization reads greater than 50, absolute value of fold-change (Log 2 [OSA/control]) greater than 2, and P value less than .05. The P value was then adjusted using the Benjamini-Hochberg method to generate the false discovery rate (FDR).[12]

2.3. Hierarchical clustering analysis of differentially expressed microRNAs

To better reveal the sample specificity of differentially expressed microRNAs, supervised hierarchical clustering[13] was conducted based on the Euclidean distance[14] of microRNAs in samples using the Pheatmap package[15] in R.

2.4. Target gene prediction for differentially expressed microRNAs

Identification of target genes is crucial for characterizing the functions of microRNAs. According to previous reports, microT-CDS is a credible tool for predicting target genes of microRNAs.[16] Based on comparative sequence analysis, TargetScan can also predict target genes of microRNAs in multiple genomes.[17] We used the microT-CDS and TargetScan databases to predict the target genes of the differentially expressed microRNAs. To increase the reliability of the predicted target genes, we selected the common target genes.

2.5. Construction of the regulatory coexpression network

Accurate understanding of the function of proteins is important. Because functional interactions in biological functions commonly require coexpression of proteins, coexpression analysis is important for identifying novel and significant genes and proteins. The Search Tool for the Retrieval of Interacting Genes (STRING)[18] is a reliable online tool that can be used to evaluate many coexpression relationships. In our study, we extracted the potential target genes with coexpression coefficients higher than 0.6 from STRING. Moreover, we used these potential microRNA-target pairs and coexpression relationships to construct a regulatory coexpression network using Cytoscape software.[19]

2.6. Pathway analysis of regulatory coexpression network

Functional analysis of microRNA target genes was conducted with FunRich, an open access standalone functional enrichment and interaction network analysis tool. After the microRNA target genes were imported into FunRich, significantly enriched pathways, defined as P value <.05, that involved the potential target genes were analyzed.

2.7. Isolation of miRNAs and real-time qRT-PCR analysis

The participants included in our research were divided into 2 groups: 12 healthy controls (Cont), 12 OSA patients. Total RNA was isolated from 600 μL of serum using the miRcute serum/plasma miRNA isolation kit (Tiangen: catalogue number DP503)

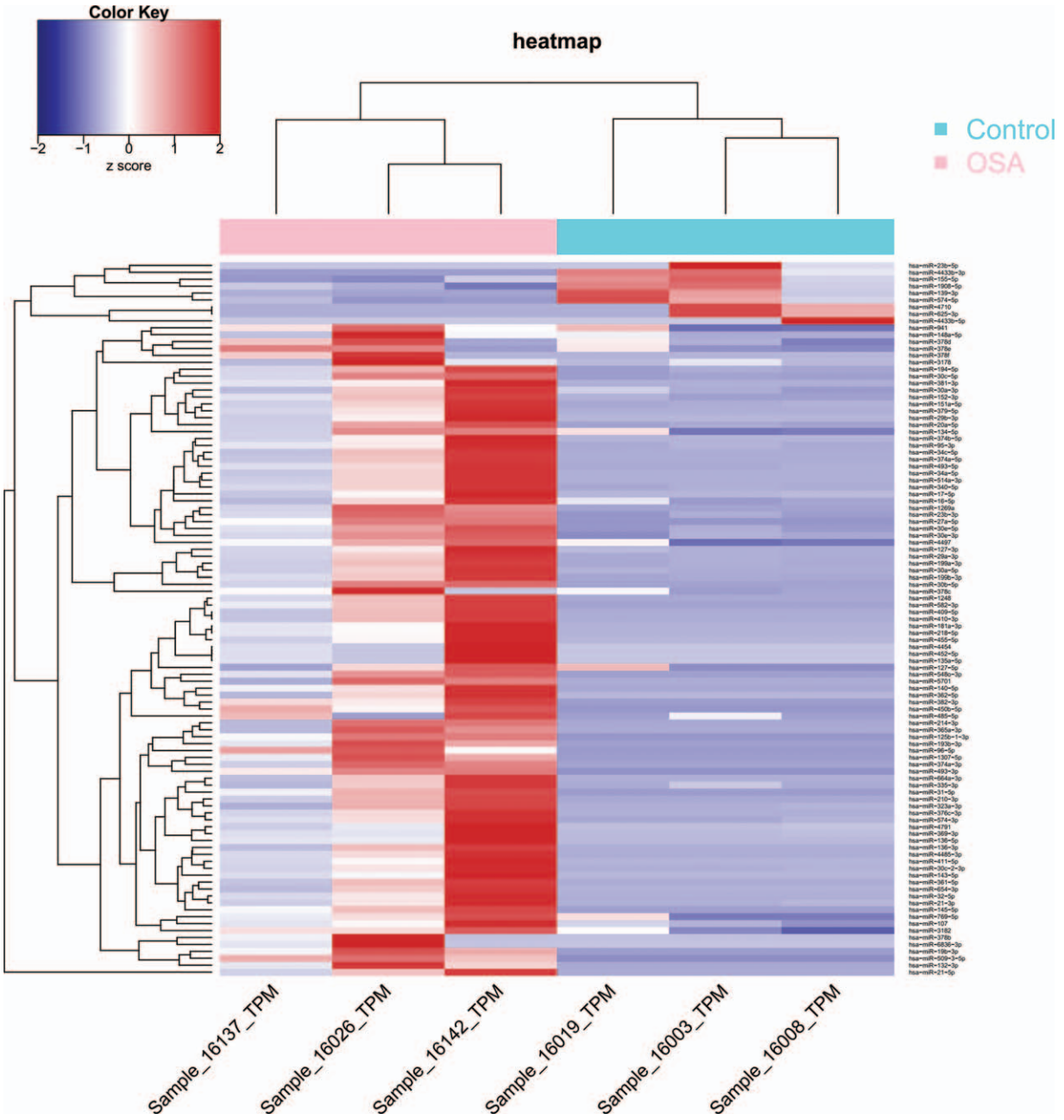

Figure 1. Hierarchical clustering analysis of differentially expressed microRNAs. Red represents a high expression value, whereas blue represents a low expression value. Control = healthy control sample, OSA = obstructive sleep apnea sample.

and dissolved in 65 μL of RNAase-free water. Because of the lack of a stable endogenous serum miRNA, samples were spiked with a synthetic *C. elegans* mir-39 miRNA mimic (cel-mir-39: Catalogue number 219610) as a control to monitor changes in RNA recovery. Previous experimental studies have confirmed that celmir-39 is suitable for cyclic miRNA formulation specification. Ten microliters of total RNA was reverse transcribed to cDNA with the M-MLV reverse transcriptase (Promega). Specific miRNA quantification was performed by SYBR Green-based real-time PCR using a miScript SYBR Green PCR kit (QIAGEN; catalogue number 218073).

3. Results

3.1. Identification of differentially expressed microRNAs and hierarchical clustering analysis

We obtained standard microRNA expression profiles after preprocessing the sequencing results and identified 104 differentially expressed microRNAs (FDR < 0.05 and |log2FC| ≥ 1), including 10 that were upregulated and 94 that were downregulated. Subsequently, we used hierarchical clustering analysis to illustrate the differential expression of microRNAs between the OSA and healthy samples (Fig. 1).

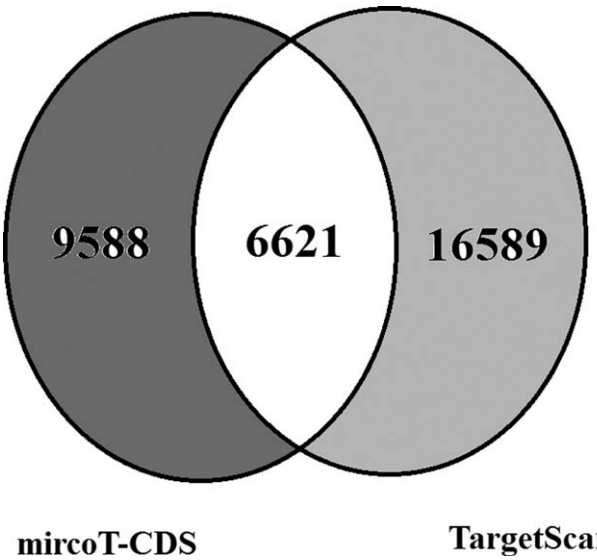

Figure 2. Target genes of the differentially expressed microRNAs. Target genes predicted based on the microT-CDS database and TargetScan database.

3.2. Target prediction of differentially expressed microRNAs and construction of the regulatory coexpression network

We used the microT-CDS and TargetScan databases to predict target genes of the differentially expressed microRNAs and selected the common genes; this procedure generated 6621 potential target genes (Fig. 2). Next, we identified 119 validated coexpressed gene pairs based on STRING (Fig. 3). By combining these target genes pairs and potential microRNA-target pairs, we constructed the regulatory coexpression network using Cytoscape (Fig. 4). This regulatory coexpression network included 41 nodes, with 23 differentially expressed microRNAs and 18 potential target genes. Several differentially expressed microRNAs had high degrees of differential expression, such as hsa-miR-485-5p, hsa-miR-107, hsa-miR-574-5p, hsa-miR-199-3p, hsa-miR-127-3p, and hsa-miR-139-3p.

3.3. Pathway analysis of regulatory coexpression network

FunRich was used to perform pathway analysis, and 100 pathways were identified that involved the potential target genes in the regulatory coexpression network. Notably, metabolic pathways was the most significant; it involved 9 genes, namely, *CAD*, *CPS1*, *ATP5L*, *COX6C*, *ATP5C1*, *COX5B*, *ATP5D*, *ATP5J*, and *CYP21A2*, targeted by hsa-miR-485-5p, has-miR-107 and so on. In addition, CYP21A2 may participate in the metabolism of lipids and lipoproteins pathway. CAD is also closely related to the insulin, PI3K-AKT, and mTOR signaling pathways.

3.4. MiRNAs have significantly different expression levels

The expression levels of miR-485-5p, 107, and 199-3p were downregulated in the OSA compared with Cont. The expression of miR-574-5p showed a significant upregulation in the OSA compared with Cont (Fig. 5).

4. Discussion

OSA is a very common disease and can cause many complications, such as cardiovascular disease, stroke, diabetes, and hypertension. These complications seriously endanger people's health, and although the diagnosis of OSA is very simple, PSG, which is the most important diagnostic measure of OSA, is cumbersome. By continually monitoring breathing, arterial oxygen saturation, EEG, ECG, heart rate, and other indicators, experts could understand the number of apnea-related pauses, the lowest blood oxygen value, and the impact on health. PSG is the internationally recognized gold standard for the diagnosis of sleep apnea hypopnea syndrome. PSG requires monitoring of patients while they are asleep, but the connecting wires of the equipment can easily fall off. Therefore, data loss and errors frequently occur. In addition, in many patients, the connecting lines can seriously affect sleep quality, which leads to inaccurate OSA diagnosis. At present, the treatment of OSA patients is divided into surgical treatment and CPAP therapy; the efficacy of surgical treatment is controversial, and patient compliance with CPAP therapy is poor. Therefore, the diagnosis and treatment of OSA needs further study.

In this study, bioinformatics analyses were performed to investigate the potential molecular mechanism of OSA and to identify molecular targets at the level of microRNA. A regulatory coexpression network was constructed that incorporated 23 differentially expressed microRNAs, including hsa-miR-485-5p, hsa-miR-107, hsa-miR-574-5p, hsa-miR-127-3p, hsa-miR-199-3p, and hsa-miR-139-3p, with a high degree of differential expression. To date, there has been no report on the relationship between OSA and microRNA. However, due to CIH, OSA can lead to metabolic disorders, inflammation, and oxidative stress, and considerable research has been conducted regarding the role of microRNAs in these processes. In our study, hsa-miR-485-5p, hsa-miR-107, hsa-miR-574-5p, hsa-miR-127-3p, hsa-miR-199-3p, and hsa-miR-139-3p have a high degree of differential expression; among these, the expression of hsa-miR-574-5p and hsa-miR-139-3p is upregulated, whereas that of hsa-miR-485-5p, hsa-miR-107, hsa-miR-127-3p, and hsa-miR-199-3p is downregulated. In addition, these microRNAs have been reported to play a role in hypoxia or metabolism.

The result of qRT-PCR analysis also demonstrated that the expression levels of miR-485-5p, 107, and 199-3p were downregulated in the OSA compared with Cont. The expression of miR-574-5p showed a significant upregulation in the OSA compared with Cont. Differentially expressed microRNAs might have a close relationship with OSA.

The main characteristic of OSA is recurrent hypopnea or apnea during sleep, a phenomenon that leads to intermittent hypoxia or frequent episodes of arousal. The ineffective action of respiratory motion in the obstructive condition also results in increased pressure in the chest, intermittent asphyxia, and sleep-wake cycle disruption. Hypoxia-inducible factor 1 (HIF-1) is mainly responsible for the adaptation of cells to hypoxia and is an important transcription factor in OSA. Some researchers have suggested that the expression of HIF-1α is regulated by many microRNAs. For example, Rane revealed that miR-199a could regulate the expression of HIF-1α in cardiomyocytes.[20] Reportedly, endothelial progenitor cells (EPCs) play an important role in tissue repair after ischemic heart disease, and Shu Meng and colleagues demonstrated that miR-107 was upregulated in hypoxia to prevent EPC differentiation via its target HIF-1β. To reveal the mechanism, they used an miR-107 inhibitor and

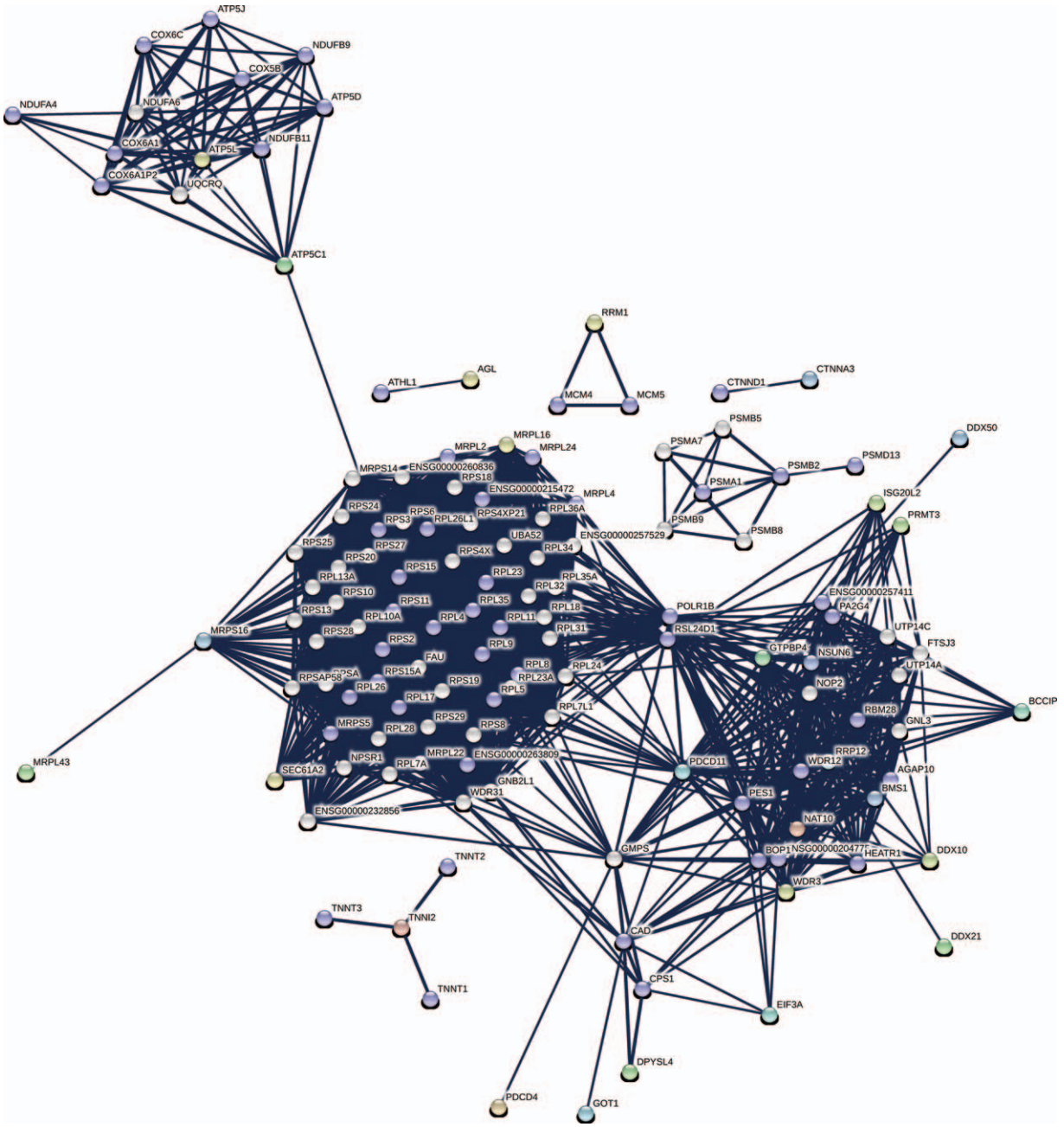

Figure 3. Coexpressed gene pairs. A total of 119 validated coexpressed gene pairs were identified based on STRING. Circles represent target genes. STRING = The Search Tool for the Retrieval of Interacting Genes

lentiviral vector expressing a short hairpin RNA (shRNA) that targets miR-107 and hypoxia-inducible factor-1β (HIF-1β) to alter miR-107 and HIF-1β expression. EPC miR-107 expression in hypoxic and normoxic conditions was measured by real-time qualitative PCR, and the results revealed that miR-107 expression in EPCs was increased under hypoxic conditions. Upregulation of miR-107 partly suppressed the EPC differentiation that was induced by hypoxia, whereas downregulation of miR-107 promoted EPC differentiation. HIF-1β was the target.[21] Recent research also demonstrated that miR-107 could mediate p53-mediated regulation of hypoxic signaling and tumor angiogenesis. miR-107 could decrease hypoxia signaling by suppressing expression of HIF-1β. In addition, the expression of HIF-1 in tumor cells could be significantly increased by knocking out miR-107. Conversely, overexpression of miR-107 inhibited HIF-1β expression and hypoxic signaling.[22] Our results showed that hsa-miR-107 and hsa-miR-199-3p were downregulated; moreover, hsa-miR-107 (|log2FC| = 2.47) and hsa-miR-199-3p (|log2FC| = 6.26) had a high degree of differential expression.

To gain better understanding of the mechanism of hypoxic responses in soft tissue sarcomas (STSs) and to study the prognostic factors of new therapies, a panel of 12 STS cell lines was exposed to hypoxic conditions. After verifying the conditions, microRNA expression profiles were assessed by LNA oligonucleotide microarrays and RT-PCR. Caroline

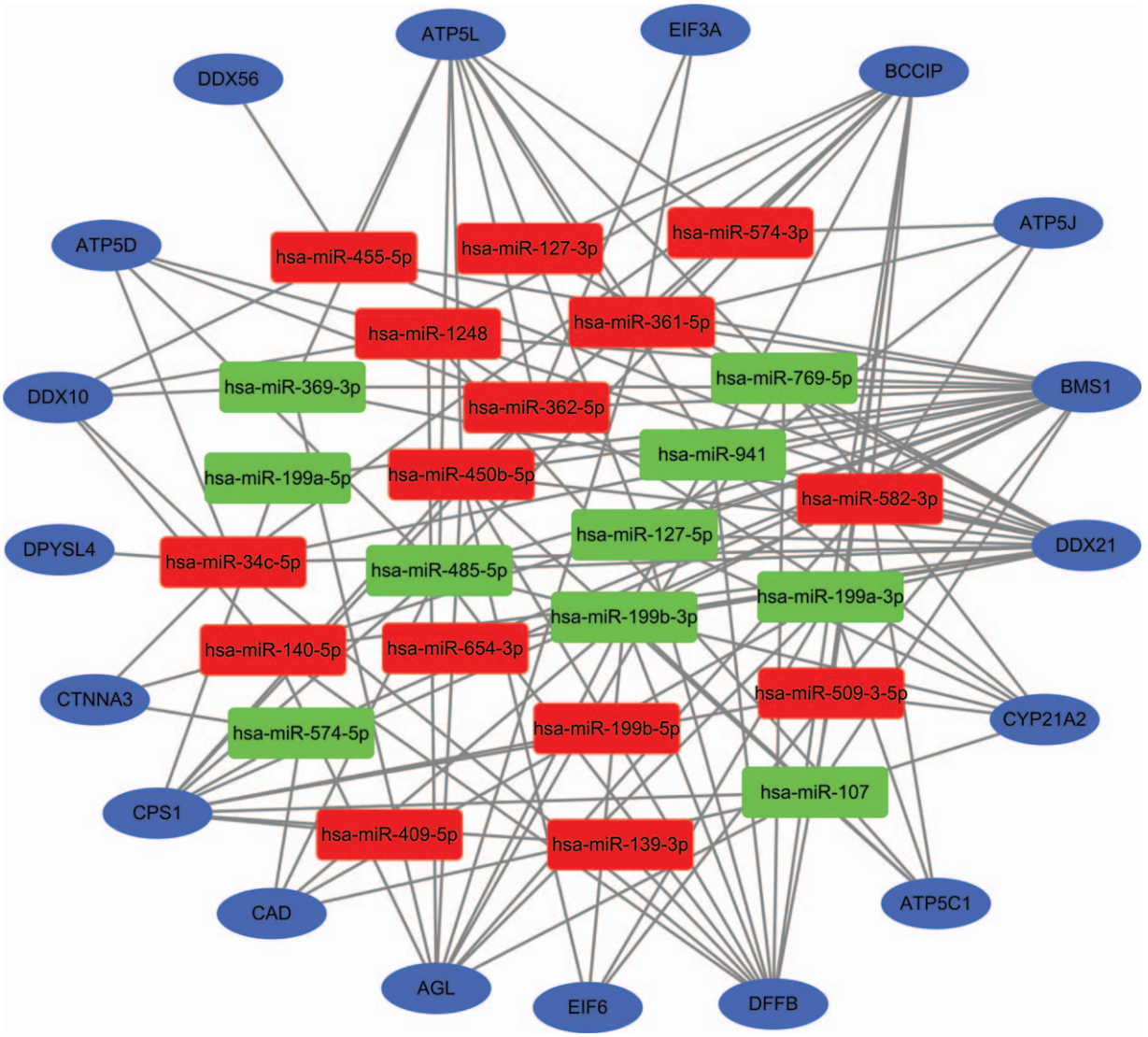

Figure 4. Regulatory coexpression network of differentially expressed microRNAs and potential target genes. Circles represent target genes. Red squares represent upregulated differentially expressed microRNAs. Green squares represent downregulated differentially expressed microRNAs.

provided evidence for miR-485-5p-mediated regulation of HIF-3α by hypoxia-responsive microRNAs in STSs.[23] Scientists also found that overexpression of miR-139-5-p could increase phosphorylation levels of p-AKT and p-GSK-3β in H9c2 cells treated with H_2O_2 and that miR-139-5-p could increase Bcl-2 expression and decrease Bax expression, which suggested that miR-139-5-p could inhibit apoptosis by activating the AKT/GSK-3β pathway. The oxidative stress injury could be inhibited by increasing the expression of miR-139-5-p, an effect that could be related to the elimination of intracellular oxidative stress products and resistance to apoptosis through the AKT/GSK-3β signaling pathway.[24] Xie and his colleagues demonstrated through experiments that the expression levels of hsa-mir-574-5p depend on HIF-1α. However, HIF-1α is an important regulator of hypoxia, thus confirming that hsa-mir-574-5p may be involved in the regulation of hypoxia.[25] HIF-1α interference could lead to downregulation of miR-127-3p as well as induction of its target gene *BCL6* in vivo.[26] Our results showed that these microRNAs all have a high degree of differential expression. Moreover, miR-139-5-p (|log2FC| = 2.93) and miR-574-5p (|log2FC| = 5.3) were upregulated, whereas miR-127-3p (|log2FC| = 4.53) and miR-485-5p (|log2FC| = 3.18) were downregulated.

As we all know, there are many metabolic pathways in the human body. OSA may affect metabolic disorders, and metabolic disorders may also affect the development and progression of OSA. So researchers have demonstrated that there may be a bidirectional relationship between OSA and metabolic disorders.[27–30]

Pathway analysis showed that the target genes in the network were significantly enriched in metabolic pathways; this category included 9 genes, namely, *CAD*, *CPS1*, *ATP5L*, *COX6C*, *ATP5C1*, *COX5B*, *ATP5D*, *ATP5J*, and *CYP21A2*, targeted by hsa-miR-485-5p, has-miR-107, and others. Moreover, CYP21A2 could participate in the metabolism of lipids and lipoproteins pathway. CAD is also closely related to the insulin, PI3K-AKT, and mTOR signaling pathways. These target genes, which were regulated by differentially expressed microRNAs, play a critical role in metabolic pathways. Therefore, microRNAs

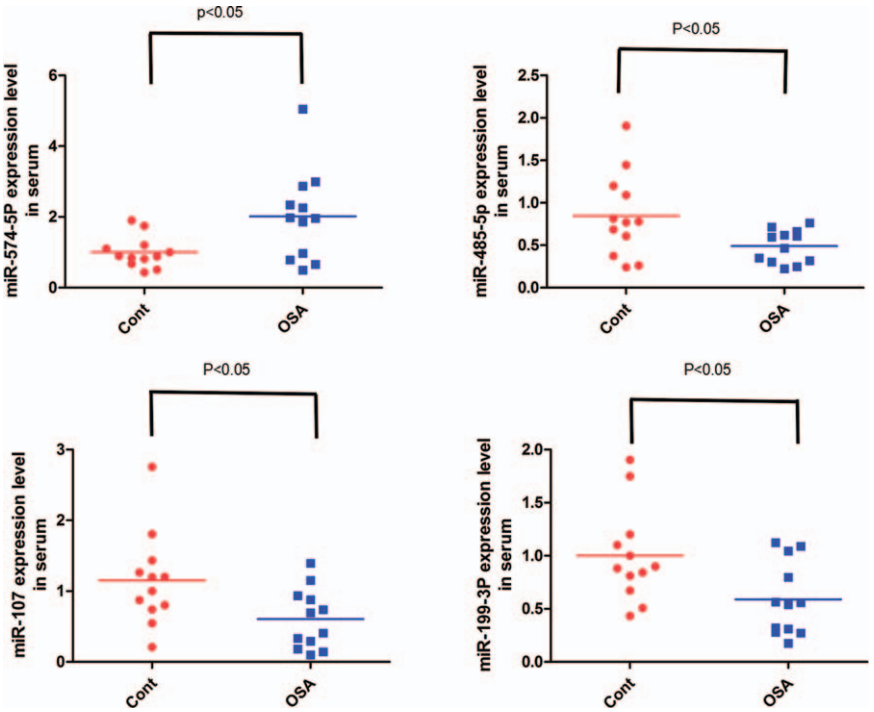

Figure 5. The relative expression levels of 4 miRNAs in the Cont and OSA groups. MiRNA expression level in serum: $\log_2$ (miRNA/miR-39).The horizontal lines indicate the mean. *P* values were generated by Mann-Whitney test. $P < .05$ was considered significant.

associated with OSA may be involved in metabolic disorders. OSA-induced metabolic perturbations include dyslipidemia, atherogenesis, liver dysfunction, and abnormal glucose metabolism.

Our results showed that miR-107 is downregulated in OSA samples compared with healthy control samples. Reportedly, miR-107 upregulation occurs in the livers of leptin-deficient mice and mice with diet-induced obesity.[31] Moreover, in these mouse models, antisense-mediated silencing of miR-107 could improve insulin sensitivity while also inducing the disorder of glucose homeostasis. The results revealed that miR-107 has a close relationship with the insulin pathway and might improve treatment of obese patients with insulin resistance.[32] Bioinformatics analysis predicts that miR-107 is a key regulator of lipid metabolism. miR-107 can regulate the *PANK* genes, so it plays a key role in metabolic pathway. The *PANK* genes have also been shown to play an important role in the regulation of intracellular coenzyme A.

CAD is not only related to the regulation of metabolism but also closely related to hypoxia and oxidative stress. CAD is regulated by hsa-miR-485-5p, hsa-miR-107, and hsa-miR-574-5p, and these different microRNAs also play a significant role in metabolism, hypoxia, and oxidative stress. CAD is also closely related to the PI3K-AKT and mTOR signaling pathways. Moreover, the PI3K-AKT and mTOR signaling pathways are significant for OSA.

Target of rapamycin (TOR) is an important serine/threonine protein kinase. TOR was originally discovered as a target of rapamycin in 1991. There is also a conserved sequence of the *TOR* gene in mammals called mTOR. The network of the mTOR kinase is increasingly recognized as a central regulator. mTOR regulates various signaling pathways that are involved in metabolism. Recent studies highlighted that mTOR is a crucial regulator of oxidative stress and metabolism by controlling several processes, such as the HIF-1α signaling pathway, insulin metabolism, glucose and lipid metabolism, and many other functions.[33] In mammals, mTOR forms 2 complexes: mTORC1 and mTORC2. Hypoxia selectively acts on mTORC1. In the case of severe hypoxia, protein translation is inhibited. The hypoxia-inducible gene *REDD1* (regulated in and DNA damage responses 1) inhibits mTOR activity.[34,35]

The PI3K/AKT signaling pathway is widely found in cells. This pathway acts as a link between extracellular signals and cellular responses by influencing downstream signaling molecules. It is also involved in cell growth, proliferation, and differentiation. More and more studies have shown that PI3K can inhibit oxidative-stress-induced apoptosis. Thus, it can protect against H_2O_2-induced oxidative damage.[36] PI3K plays a very significant role in cell proliferation, homeostasis, and oxidative stress.[37]

5. Conclusion

Differentially expressed microRNAs such as hsa-miR-485-5p, hsa-miR-107, hsa-miR-574-5p, and hsa-miR-199-3p might participate in OSA, and CAD might have a close relationship with OSA. Our results may provide a basis for the pathogenesis of OSA and the study of disease diagnosis, prevention, and treatment. However, more experiments are needed to verify these predictions.

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