Joint detection of miR-149-3p and hepcidin predicts the onset of obstructive sleep apnea syndrome in obese patients

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Abstract

Background: Obstructive sleep apnea syndrome (OSAS) is a potentially fatal sleep respiratory disorder, and hepcidin has been found to be related with OSAS onset and severity. This study aimed to examine the serum expression of microRNA-149-3p (miR-149-3p) and hepcidin in OSAS patients, and evaluate the predictive value of miR-149-3p and hepcidin for OSAS occurrence in obese population.

Methods: This study analyzed the data from 212 OSAS patients and 120 control individuals. OSAS severity was evaluated by apnea hypopnea index (AHI) from polysomnography. Serum miR-149-3p was examined using reverse transcription quantitative PCR, and hepcidin and inflammatory cytokines were measured using ELISA kits. Logistic regression analysis was used to evaluate the predictive value of miR-142-3p and hepcidin for OSAS in obese population, and ROC curve was plotted to assess the predictive accuracy.

Results: Serum miR-149-3p and hepcidin were increased in OSAS patients, especially in the severe cases, and had diagnostic potential to distinguish OSAS. High miR-149-3p and hepcidin were positively correlated with OSAS patients’ inflammatory cytokines. Obese OSAS patients had the highest miR-149-3p and hepcidin levels, and the two molecules had predictive value of OSAS present in obese population, and the combination of miR-149-3p and hepcidin showed the highest predictive accuracy.

Conclusion: Serum miR-149-3p and hepcidin levels were elevated in OSAS patients and correlated with disease severity and systemic inflammation. miR-149-3p and hepcidin levels have diagnostic value to distinguish OSAS, exhibited predictive value for OSAS in obese population, and the joint detection of the two molecules showed the highest predictive accuracy.

KEYWORDS
apnea hypopnea index, hepcidin, inflammation, miR-149-3p, obesity, obstructive sleep apnea syndrome, polysomnography
1 | INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a kind of sleep respiratory disease with unknown etiology. The clinical manifestations of OSAS include nocturnal sleep snoring accompanied by apnea and daytime sleepiness. OSAS-induced apnea leads to recurrent nocturnal hypoxia and hypercapnia, contributing to some complications, such as hypertension, coronary heart disease, diabetes and cerebrovascular diseases, and even sudden death at night. Thus, OSAS is considered as a potentially fatal sleep respiratory disorder. The direct pathogenesis of OSAS is the stenosis and obstruction of the upper airway. However, it is reported that the occurrence of OSAS is not only induced by simple airway obstruction, but also associated with upper airway collapse and disorders in respiratory central nervous regulatory factor. Activation of systemic inflammation is considered as one of the major mechanisms in the pathogenesis of OSAS, and increased inflammatory cytokines are observed in OSAS progression. It is noticed that obesity is also closely related with the presence of OSAS, and about 70% OSAS patients had obesity. Therefore, the early screening of obese population with high risk of OSAS would contribute to disease prevention.

Hepcidin serves as a regulator of transmembrane iron transport, and plays a pivotal role in iron homeostasis. Accumulated studies have demonstrated that hepcidin expression is significantly upregulated in response to inflammation and obesity. More important, we previously found that the elevated hepcidin was associated with OSAS onset and severity. Some iron-related genes have been investigated in colorectal carcinoma, and microRNA-149-3p (miR-149-3p) was identified as an iron-related miRNA-targeted divalent metal transporter (DMT1), which is either stored as ferritin or moved into serum by hepcidin, indicating the potential link between miR-149-3p and hepcidin. miR-149-3p is an inflammation-related molecular, and its reduction in obese mice has been found to restrain diet-induced obesity and metabolic dysfunctions. Importantly, a study by Yang et al. has reported that miR-149-3p was upregulated in OSAS patients compared with healthy controls, but the clinical significance of miR-149-3p in OSAS remains elusive.

To discover novel biomarkers to indicate the presence of OSAS, especially in obese population, this study aimed to analyze the serum expression of miR-149-3p and hepcidin, and evaluate the clinical significance of miR-149-3p and hepcidin in the diagnosis of OSAS. The study results would provide novel biomarkers and methods to diagnose OSAS, and might contribute to the screening of obese patients at high risk of OSAS.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment and serum collection

A total of 212 patients were investigated in this study, who received polysomnography and diagnosed as OSAS between June 2015 and October 2020 in Weifang People’s Hospital. All the analyzed patients met the following inclusion criteria: (1) all patients received overnight polysomnography, and were confirmed with OSAS based on the American Academy of Sleep Medicine (AASM) Guidelines, (2) age of >18 years. Patients were excluded if they met the following exclusion criteria: (1) cases had other sleep disorders [determined using the International Classification of Sleep Disorders (ICSD-II)]; (2) patients had received the treatment of sleep-related breathing disorders; (3) presence of family history of psychiatric disorders, abuse of alcohol or drug, any other severe diseases, such as diabetes, cardiopathy or malignant tumors; (4) pregnant or lactating women. One hundred and twenty control individuals, who had no clinical symptoms of OSAS, and matched with OSAS patients at age, gender, and body mass index (BMI). The OSAS patients were further categorized into three groups: normal weight group (BMI of 18.5–24.9 kg/m²; n = 26), overweight group (BMI of 25–29.9 kg/m²; n = 48) and obese group (BMI of ≥30 kg/m²; n = 138) based on BMI values.

Blood samples were collected from the study population after overnight fasting, and the OSAS patients had not received any therapy before the sampling. Serum samples were extracted from the blood using centrifugation at 4°C and stored at −80°C for further use. The protocols of this study were in accordance with the ethics guideline of the Helsinki Declaration and approved by the Ethics Committee of Weifang People’s Hospital. Each patient signed informed consent for the participation in this research.

2.2 | Study of sleep

OSAS patients received overnight polysomnography using standard techniques, and the apnea hypopnea index (AHI) was recorded and analyzed. The severity of OSAS was determined based on the range of AHI: cases with AHI < 15 were mild OSAS, cases with 15 ≤ AHI < 30 were moderate OSAS, and cases with AHI ≥ 30 were severe OSAS. Among the 212 OSAS patients, 32 (15.1%) were mild cases, 58 (27.4%) were moderate cases, and 122 (57.5%) were severe cases.

2.3 | Enzyme-linked immunosorbent assay

Serum levels of hepcidin and inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, were measured using enzyme-linked immunosorbent assay (ELISA) kit (Eliet Biotechnology, Wuhan, China) following the manufacturer’s protocols. The concentration results were quantified and calculated by reading the OD value at 450 nm.

2.4 | Total RNA extraction and reverse transcription quantitative PCR

Total RNA was extracted from serum samples using TRIzol reagent and mRNA pure mini kit (CWBiotech, Beijing, China)
following the manufacturer’s instructions. Then, the NanoDrop 2000 was adopted to evaluate the purity and concentration of RNA, and the OD ratio of A260/A280 closed to 2.0 meant the eligibility of RNA.

Reverse transcription was performed to synthesize cDNA from RNA using a miRNA cDNA synthesis kit (CWBiotech, Beijing, China), and the obtained cDNA was used as the template of qPCR. The qPCR was carried out using miRNA qPCR assay kit (CWBiotech, Beijing, China) on a 7500 real-time PCR system (Applied Biosystem, Thermo Fisher Scientific, MA), and the relative expression miR-149-3p was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to cel-miR-39-3p. The thermocycling conditions were 95°C for 10 min, 40 cycles of 95°C for

| Variables     | Controls (n = 120) | OSAS patients (n = 212) | p-value |
|---------------|--------------------|-------------------------|---------|
| Age (years)   | 56.23 ± 10.11      | 55.59 ± 10.88           | .596    |
| Gender (male, %) | 91 (75.8%)      | 161 (75.9%)             | .982    |
| BMI (kg/m²)   | 31.67 ± 5.67       | 31.68 ± 5.41            | .981    |
| Hypertension (n, %) | 42 (35.0%)    | 85 (40.1%)              | .359    |
| Diabetes (n, %) | 10 (8.3%)        | 22 (10.4%)              | .544    |
| TC (nM)       | 2.19 ± 1.35        | 5.96 ± 1.27             | <.001   |
| TG (nM)       | 1.98 ± 0.70        | 1.89 ± 0.58             | .207    |
| LDL-C (nM)    | 3.41 ± 1.04        | 3.93 ± 1.05             | <.001   |
| HDL-C (nM)    | 1.44 ± 0.36        | 1.35 ± 0.43             | .064    |
| AHI           | 1.99 ± 1.35        | 34.65 ± 17.58           | <.001   |

Abbreviations: AHI, apnea hypopnea index; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

**FIGURE 1**  Expression and diagnostic performance of serum miR-149-3p and hepcidin in OSAS patients. (A,B) Serum levels of miR-149-3p and hepcidin were both elevated in OSAS patients compared with control individuals. (C) Serum miR-149-3p was positively correlated with hepcidin in OSAS patients. (D) ROC curves based on serum miR-149-3p and hepcidin levels in OSAS patients (AUC, area under the curve). ***p < .001
30 s, 60°C for 20 s, and 72°C for 30 s. Following were the sequences of primers (5'-3'): miR-149-3p forward GCCGAGGGAGGGAGCG, miR-146-3p reverse CTCGTCGTCGTCGTA, cel-miR-39-3p forward GGTCACCGGTTGTAATC, and cel-miR-39-3p reverse GAGAGGAAGAGGAGA.

2.5 | Statistical analysis

The analysis data were expressed as mean ± SD and assessed using SPSS 26.0 (IBM Corp.) and GraphPad Prism 9.0 (GraphPad Software, Inc.). Differences between two groups were compared using t test, and one-way ANOVA with Tukey’s post hoc test was used to compare the differences between multiple groups. Chi-square test was used to analyze the differences in categorical variables between groups. Pearson correlation coefficient was applied to evaluate the correlation between miR-149-3p, hepcidin, AHI, and inflammatory cytokines. Receiver operating characteristic (ROC) curve was plotted based on serum miR-149-3p and hepcidin to evaluate their diagnostic performance to distinguish OSAS patients, and area under the curve (AUC), diagnostic cutoff value, sensitivity, and specificity were calculated from this analysis. Logistic regression analysis was used to analyze the predictive value of miR-149-3p and hepcidin for the onset of OSAS in obese patients. A p-value of less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Baseline clinical characteristics of the study population

The baseline characteristics of the enrolled participants were listed in Table 1, which showed that the control individuals matched with the OSAS patients at age, gender, BMI, and history of hypertension and diabetes (all \( p > .05 \)). The average age of OSAS

**FIGURE 2** Serum miR-149-3p and hepcidin levels were associated with OSAS severity. (A, B) Serum levels of miR-149-3p and hepcidin were positively correlated with AHI in OSAS patients. (C, D) Severe OSAS patients had highest levels of miR-149-3p and hepcidin levels, and the two molecule levels in moderate OSAS were higher than that in mild OSAS cases. *\( p < .05 \), **\( p < .01 \), ***\( p < .001 \)
patients was 55.59 ± 10.88 years old, and 161 (75.9%) males were included in the OSAS group, and the controls aged of 56.23 ± 10.11 years old and contained 91 (75.8%) males. Compared with the controls, OSAS patients had higher levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), and AHI (all \( p < .001 \)), whereas no differences were observed in triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) levels (both \( p > .05 \)).

3.2 | Expression and diagnostic performance of serum miR-149-3p and hepcidin levels in patients with OSAS

Expression of serum miR-149-3p and hepcidin levels were both elevated in OSAS patients compared with that in controls (both \( p < .001 \), Figure 1A, B). Moreover, the relative expression of miR-149-3p was positively correlated with serum hepcidin levels in OSAS patients \( (r = 0.762, p < 0.001; \text{Figure 1C}) \). Given the significant differential expression of miR-149-3p and hepcidin, the diagnostic potential of the two molecules to distinguish OSAS from normal controls was evaluated by constructing ROC curves. As shown in Figure 1D, miR-149-3p and hepcidin presented moderate diagnostic accuracy, and their synthetic role showed relatively high diagnostic accuracy for the screening of OSAS patients, and the diagnostic sensitivity and specificity for the joint role of miR-149 and hepcidin were 84.10% and 86.70%, respectively.

3.3 | Relationship of miR-149-3p and hepcidin with OSAS severity

OSAS severity was evaluated by the AHI, which was 34.65 ± 17.58 in OSAS patients. By the correlation analysis, this study found that serum miR-149-3p expression and hepcidin levels were both positively correlated with AHI in patients with OSAS \( (r = 0.751, p < .001 \text{ for miR-149-3p with AHI}; r = 0.611, p < .001 \text{ for hepcidin with AHI}; \text{Figure 2A,B}) \). Based on the definition of OSAS severity via AHI results, the 212 OSAS patients included 32 (15.1%) mild cases, 58 (27.4%) moderate cases, and 122 (57.5%) severe cases. Severe OSAS cases had the highest levels of miR-149-3p and hepcidin compared with mild and moderate OSAS patients (both \( p < .001 \)), and their levels in moderate OSAS patients were higher than mild cases \( (p < .01 \text{ for miR-149-3p}, p < .05 \text{ for hepcidin}; \text{Figure 2C,D}) \).

3.4 | Correlation between miR-149-3p, hepcidin and inflammatory cytokines in OSAS patients

miR-149-3p and hepcidin were both inflammation-related molecules, and inflammatory responses are activated during in the pathogenesis
of OSAS. The results showed in Figure 3 revealed that miR-149-3p expression levels were positively correlated with inflammatory cytokines, including IL-1β (r = 0.649), IL-6 (r = 0.561), and TNF-α (r = 0.533) (all p < .001, Figure 3A-C). Figure 3D–F also presented the correlation of hepcidin with IL-1β (r = 0.468), IL-6 (r = 0.391), and TNF-α (r = 0.332; all p < .001).

### 3.5 Deregulated levels of miR-149-3p and hepcidin in obese patients with OSAS

Most of OSAS patients combined obesity, the relationship between miR-149-3p and hepcidin with obesity in OSAS was evaluated. There were 138 (65.1%) obese patients in OSAS group and 79 (65.8%) obese cases in the control group. From the data of Figure 4, obese OSAS patients had the highest serum miR-149-3p and hepcidin levels compared with normal weight and overweight patients (both p < .001). In addition, overweight patients had increased miR-149-3p and hepcidin levels than normal weight patients (both p < .05).

### 3.6 High risk of OSAS in obese patients with high levels of miR-149-3p and hepcidin

This study assessed the data from obese OSAS patients and obese controls, aiming to find the factors associated with OSAS present in obese population. The univariate logistic regression analysis results showed that TC, LDL-C, miR-149-3p, and hepcidin were associated with OSAS (all p < .05), and further multivariate analysis results showed that miR-149-3p (OR = 2.201, 95% CI = 1.437–3.006, p = .002), and hepcidin (OR = 1.626, 95% CI = 1.213–2.076, p = .002).

### Table 2 Logistic regression to predict OSAS in obese patients

| Variables          | Univariate analysis | Multivariate analysis |
|--------------------|--------------------|-----------------------|
|                    | OR (95% CI)        | p-value               | OR (95% CI)        | p-value               |
| Age                | 1.022 (0.673–1.554)| .392                  | –                   | –                     |
| Gender             | 1.146 (0.892–1.605)| .081                  | –                   | –                     |
| BMI                | 1.104 (0.945–1.379)| .094                  | –                   | –                     |
| Hypertension       | 1.009 (0.647–1.495)| .423                  | –                   | –                     |
| Diabetes           | 1.342 (0.887–1.843)| .157                  | –                   | –                     |
| TC                 | 1.138 (1.045–1.439)| .039                  | 1.128 (0.921–1.457)| .134                 |
| TG                 | 1.072 (0.871–1.874)| .145                  | –                   | –                     |
| LDL-C              | 1.346 (1.092–1.743)| .036                  | 1.333 (0.965–1.477)| .121                 |
| HDL-C              | 1.106 (0.701–1.563)| .663                  | –                   | –                     |
| miR-149-3p         | 2.376 (1.543–3.229)| <.001                 | 2.201 (1.437–3.006)| .002                 |
| Hepcidin           | 1.669 (1.223–2.205)| .003                  | 1.626 (1.213–2.076)| .007                 |

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.
p = .007) were independent risk factors associated with the onset of OSAS (Table 2). To evaluate the predictive accuracy of miR-149-3p and hepcidin for OSAS risk in obese patients, we further performed ROC analysis. From the ROC curves in Figure 5, miR-149-3p and hepcidin showed highly predictive accuracy (AUC of 0.96 for miR-149-3p, AUC of 0.926 for hepcidin), and the best predictive performance was observed by joining miR-149-3p and hepcidin, which showed the improved AUC (0.992), sensitivity (97.10%), and specificity (97.50%).

4 | DISCUSSION

OSAS is a kind of sleep respiratory disease, and OSAS-induced apnea can even lead to sudden death at night. Obesity contributes to the present of OSAS, and about 70% OSAS cases had obesity. This study investigated two obesity-related molecules in OSAS, and found that serum miR-149-3p and hepcidin levels were both elevated in OSAS patients and associated with disease severity and inflammation. In obese OSAS patients, the expression of miR-149-3p and hepcidin were significantly higher compared with that in normal weight and overweight patients, and the two molecules were identified as indicators to predict OSAS present in obese population. Additionally, serum miR-149-3p and hepcidin had abilities to diagnose OSAS from normal controls, and these abilities were more significant in obese patients. The joint detection of miR-149-3p and hepcidin showed the highest diagnostic accuracy compared to the two molecules alone.

The current diagnosis of OSAS firstly dependent on the clinical symptoms, such as nocturnal sleep snoring accompanied by apnea and daytime sleepiness. Then, the sleep respiration monitoring using polysomnography is the standard technique to determine the disease present and severity. After the detection, AHI of individuals are records, and the cases with AHI equal to or more than 5 are diagnosed with OSAS. The 212 OSAS patients in this study had the average AHI of 34.65 ± 17.58, and 32 cases had mild OSAS, 58 with moderate OSAS, and 122 had severe OSAS. Accumulated studies indicated that obesity is one of the risk factors of OSAS, and more than half of OSAS are obese patients. The obese patients accounted for 65.1% of our study patients, which also supported the conclusion of the relationship between obesity and OSAS. Thus, obesity-related molecules might be involved in the development of OSAS, which might further contribute to the discovery of biomarkers to early indicate the risk of OSAS in obese population. Hepcidin has been found highly expressed...
We previously investigated the potential role of hepcidin in OSAS, and found that the increased expression of hepcidin was associated with OSAS severity and could indicate OSAS onset.\textsuperscript{11} In accordance with the previous findings, the present study found that hepcidin had diagnostic accuracy to distinguish OSAS patients from normal controls. Considering the relationship between hepcidin and obesity, we suspected that hepcidin might show more attractive clinical value in obese OSAS patients. By analyzing the data from obese patients and obese controls, it is found that hepcidin had higher diagnostic accuracy than that in the total OSAS population, suggesting that hepcidin as an obesity-related molecule had considerable clinical value to indicate OSAS in obese population.

Deregulated miRNAs have attracted increasing attentions for their potential clinical significance in disease diagnosis and prognosis.\textsuperscript{15,22} Some miRNAs have been reported to be related with OSAS development and progression. For instance, miR-126a has been found to alleviate OSAS-related hypertension, oxidase stress, and inflammation by targeting HIF-1\textalpha.\textsuperscript{23} Increased serum miR-92a in OSAS patients has been found to be related with disease severity.\textsuperscript{24} miR-21-5p was lowly expressed in OSAS patients, and miR-21-5p overexpression inhibited inflammation to adverse mononuclear cell apoptosis.\textsuperscript{25} Decreased expression of miR-664a-3p in OSAS patients has been determined as a marker of atherosclerosis.\textsuperscript{26} The current study analyzed miR-149-3p in OSAS. miR-149-3p has been reported as an iron-related miRNA by targeting DMT1, which is stored as ferritin and can be moved into serum by hepcidin.\textsuperscript{12,13} An important previous study reported that miR-149-3p expressed highly in OSAS patients compared with healthy controls,\textsuperscript{15} but did not investigate the clinical value of this dysregulation. In our study, elevated serum miR-149-3p was also found in OSAS patients, and its expression was positively correlated with serum hepcidin. Similar with hepcidin, serum miR-149-3p was positively correlated with OSAS severity, and also had ability to distinguish OSAS from normal controls. More importantly, the synthetic role of miR-149-3p and hepcidin showed relatively high accuracy in the diagnosis of OSAS. Thus, it is considered that the joint detection of miR-149-3p with hepcidin might be a novel method to diagnose OSAS.

Accumulated evidence showed the close relationship between obesity and OSAS, and OSAS progression also contributes to the development of obesity.\textsuperscript{21,27} Therefore, accurate prediction of OSAS risk in obese population is of great importance for disease prevention. Hepcidin and miR-149-3p are two obesity-related molecules,\textsuperscript{9,14} and thus were considered to exhibit specific associations with obese OSAS patients. By assessing the data from obese controls and OSAS patients, we found that serum miR-149-3p and hepcidin could independently predict the OSAS present in obese population, and the prediction accuracy was best when joining the two molecules. These findings indicated that the combined detection of miR-149-3p and hepcidin might facilitate the development of methods to predict OSAS in obese population.

In addition to the clinical value of miR-149-3p and hepcidin in OSAS, we also found the significantly positive correlation of miR-149-3p and hepcidin with systemic inflammation. It is well known that inflammatory responses play critical roles in the pathogenesis of OSAS.\textsuperscript{28} Thus, we deduced that miR-149-3p and hepcidin might be involved in OSAS progression through regulating inflammatory responses. In chronic obstructive pulmonary disease, miR-149-3p regulated inflammatory responses by targeting TLR-4 signaling pathway.\textsuperscript{29} miR-149-3p-mediated intestinal inflammation and malignancy induced by enterotoxigenic Bacteroides fragilis.\textsuperscript{30} Although our study evaluates the predictive value of miR-149-3p for OSAS onset especially in obese population, the mechanisms underlying miR-149-3p dysregulation were not explored in our study. Based on the relationship between miR-149-3p and inflammation in OSAS found in our study, we preliminarily predicted the potential targets of miR-149-3p using miRWalk (http://mirwalk.umm.uni-heidelberg.de/), and TargetSan, miRDB, and miRTarBase were used to screen the predicted targets (Figure 6). According to the reported studies regarding to the top 10 predicted targets, ZBTB7B, NR1H2, IKZF3, NFIX, LASP1, and TP53 were related with the development of inflammation.\textsuperscript{31–36} These predicted data may provide important reference materials and basis for future research, and we will further explore functional molecules that might be related with the development and progression of OSAS.

In conclusion, serum miR-149-3p and hepcidin levels were elevated in OSAS patients, especially in obese OSAS patients than normal controls, and were related with disease severity and systemic inflammation. High levels of miR-149-3p and hepcidin had abilities to diagnose OSAS, and exhibited predictive value for OSAS present in obese population. High predictive accuracy could be achieved by combining miR-149-3p and hepcidin for the prediction of OSAS, indicating the joint detection of miR-149-3p and hepcidin may provide a promising method for screening obese cases at high risk of OSAS.

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**CONFLICT OF INTEREST**

The authors declare that they have no competing interests.

**DATA AVAILABILITY STATEMENT**

The data used and analyzed can be obtained from the corresponding author under a reasonable request.

**ETHICS STATEMENT**

The experimental procedures were all in accordance with the guidelines of the Ethics Committee of Weifang People’s Hospital and had been approved by the Ethics Committee of Weifang People’s Hospital. This study complies with the declaration of Helsinki.

**CONSENT TO PARTICIPATE**

A signed written informed consent was obtained from each patient.

**CONSENT FOR PUBLICATION**

Written informed consent for publication was obtained from each participant.
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