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MiR-664a-3p expression in patients with obstructive sleep apnea

A potential marker of atherosclerosis

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Abstract

The early prediction of atherosclerosis (AS) is important in the management of obstructive sleep apnea patients (OSA). MicroRNA (miRNA) plays a vital role in the evolution of OSA and AS. Its differential expression may therefore serve as a diagnostic and prognostic biomarker of AS in OSA. The aim of this study was to identify specific serum miRNAs that could serve as a novel screening signature of AS in OSA patients. The specificity and sensitivity of these miRNAs in the early diagnosis of AS in OSA patients were then determined.

The 128 participants in this study underwent maximum carotid intima-media thickness (CIMT) measurements and polysomnography and were divided into 4 groups: 27 healthy volunteers with normal max-CIMT, 31 healthy volunteers with increased max-CIMT, 35 OSA patients with normal max-CIMT, and 35 OSA patients with iCIMT. MiRNA was extracted from the 12 participants' serum (3 participants each groups) and used to establish miRNA libraries for deep sequencing. A total of 116 participants were quantified by qRT- PCR. Correlations between differential expression of miRNAs and CIMT were assessed using the Spearman correlation coefficient. Our study was approved by the Ethics Committee of our hospital and was conducted in line with the Helsinki Declaration.

MiR-664a-3p expression was quantified by qRT-PCR. Correlations between miR-664a-3p expression and CIMT were assessed using the Spearman correlation coefficient. The results showed that the miR-664a-3p was downregulated in the OSA, OSA with iCMIT, and nCIMT groups compared with the control group.

The demonstrated potential of circulating miR-664a-3p as a noninvasive marker of AS in essential OSA patients should be confirmed in further studies.

Abbreviations: AHI = Apnea Hyponea Index, AS = atherosclerosis, CIH = chronic intermittent hypoxia, HIF-1β = hypoxia-inducible factor-1β, iCIMT = increased max-CIMT, LOS = lowest oxygen saturation, miRNA = MicroRNA, OSA = obstructive sleep apnea, PSG = polysomnography, SD = standard deviation.

Keywords: atherosclerosis, microRNA, miR-664a-3p, obstructive sleep apnea

1. Introduction

Obstructive sleep apnea (OSA) is a common chronic sleep disorder[1] whose pathogenesis and complications have been extensively investigated. It is characterized by recurrent episodes of complete or partial airway obstruction, which can lead to apnea or hypopnea. OSA is associated with several serious diseases, including cardiovascular disease, hypertension, diabetes, and metabolic syndrome.[2–6] These have been attributed to the additional characteristics of the disease, which include systemic inflammation, oxidative stress, and vascular endothelial dysfunction.[7,8] All of these features have also been identified in the pathogenesis and pathophysiology of atherosclerosis (AS). Consequently, OSA may be an independent risk factor for AS.[9,10] AS is not only a lipid deposition disease but is also associated with broader disorders of lipid metabolism.[11,12] In patients with OSA, the incidence of cardiovascular events increases significantly with increasing OSA severity and progression.[13] Thus, OSA seems to play an important role in the development and progression of AS.

MicroRNA (miRNA) regulates inflammation, oxidative stress, hypoxia, and lipid metabolic disorders. In a mouse model of intermittent hypoxia, chronic intermittent hypopnea was shown to alter miRNA expression and the endothelium.[14] Thus, miRNA may play an important role in the development of OSA and AS. Its differential expression in OSA may therefore be a useful biomarker to diagnose the risk of AS in OSA patients. The present study investigated the differential expression of miRNA in OSA as a biomarker of AS development. It also presents a novel view of the mechanism underlying the association of AS and OSA.

2. Materials and methods

2.1. Study participants

All participants were from Beijing AnZhen Hospital, affiliated with Capital Medical University. The 116 participants assessed in the study included 24 healthy controls with normal maximum carotid intima-media thickness (CIMT) (Con), 28 participants with an increased max-CIMT (iCIMT), 32 patients with OSA (OSA), and

Editor: Giuseppe Insalaco.

This work was supported by The Public Development and Reform Pilot Project of Beijing Municipal medical research Institute [grant numbers: 2016-4], Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support [grant numbers: ZYLX201605], and National Natural Science Fundation of China [grant numbers: 81470567].

The authors have no conflicts of interest to disclose.

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Medicine (2018) 97:6(e9813)

Received: 30 October 2017 / Received in final form: 2 January 2018 / Accepted: 17 January 2018

http://dx.doi.org/10.1097/MD.0000000000009813

32 with OSA and an increased max-CIMT (OSA with iCIMT). Polysomnography (PSG) was used to measure the severity of OSA and hypoxemia. OSA is diagnosed based on the Apnea Hyponea Index (AHI) index, with OSA defined as >5 events/h. The standard is further subdivided into mild ($5 < AHI \leq 15$ events/h), moderate ($15 < AHI \leq 30$ events/h), and severe (AHI > 30 events/h). The diagnostic standard for hypoxemia is based on the lowest oxygen saturation (LOS), which is further subdivided into mild ($0.85 \leq LOS < 0.9$), moderate ($0.65 \leq LOS < 0.85$), and severe ($LOS < 0.65$). CIMT was measured in all participants using diagnostic Color Doppler ultrasound (Toshiba Aplio 500 ultrasound scanner). CIMT was defined as the distance between the blood-intimae and media-adventitia interfaces of the arterial wall. The study protocol and criteria were based on current sonographic guidelines. A max-CIMT < 0.9 mm was defined as normal, a max-CIMT > 0.9 mm as intimal thickening, and a max-CIMT > 1.3 mm as an atherosclerotic plaque.[15,16] Participants with a history of malignant tumor, ischemic heart disease, hypertension, diabetes mellitus, or chronic renal diseases were excluded. Our study was approved by the Ethics Committee of our hospital and was conducted in line with the Helsinki Declaration.

2.2. MicroRNA expression profiles

Total RNA was extracted from the 12 participants' serum and used to establish miRNA libraries for deep sequencing according to the manufacturer's protocol. After adapters were ligated to the RNA, cDNAs were generated using SuperScript II reverse transcriptase (Life Technologies). PCR amplification was followed by sequencing using the Illumina HiSeq 3000 (Illumina) platform. Clean reads acquired after trimming were used for mapping and further analysis. MicroRNA expression values were determined 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 > 50, absolute value of the fold-change > 2, and a $P < .05$. The P value was then adjusted using the Benjamini–Hochberg method to generate the false discovery rate (FDR).

2.3. Hierarchical clustering analysis of differentially expressed microRNAs

To determine the sample specificity of differentially expressed microRNAs, supervised hierarchical clustering was conducted based on the Euclidean distance of microRNAs in samples using the Pheatmap package.

2.4. RT-qPCR assay

A total of 116 participants (24 Con, 28 iCIMT, 32 OSA, 32 OSA +iCIMT) were quantified by qRT-PCR. From each fasting participant, 2 mL of whole blood was collected in anticoagulant tubes. Total RNA was extracted using Trizol as previously described. The miRNAs were reverse-transcribed with the TaqMan microRNA reverse transcription kit (Invitrogen, CA) according to the manufacturer's instructions. The miRNA-specifc TaqMan microRNA assay (Invitrogen) was used to measure serum miRNA expression. The data were processed using the $2^{-\Delta\Delta CT}$ method as previously described. For the analysis of miRNA expression levels, both an internal reference (U6) and an external normalization using cel-miR-39 were applied.

2.5. Statistical analysis

Continuous variables were expressed as the mean and standard deviation (SD). Categorical variables were expressed as a number. The Mann–Whitney U test was used to compare differences in serum miRNA expression, determined from the scatter plots of the log-transformed relative expression levels. Hierarchical cluster analysis (average linkage) was performed using Cluster software and Treeview. The associations between miR-664a-3p expression levels and AHI, LOS, and CIMT were analyzed using the Spearman correlation analysis. All statistical analyses were performed using STATA 9.2, and visualized with GraphPad Prism 5.0 software. A P value <.05 was considered to indicate statistical significance.

3. Results

3.1. Study participants

The 116 participants in this study included 24 healthy controls (Con), 28 patients with an increased maximum carotid intima-media thickness (iCIMT), 32 with OSA (OSA), and 32 with OSA and iCIMT. In all study participants, the carotid artery was evaluated ultrasonographically and PSG was performed. The baseline data of the study participants are presented in Table 1. From the results of Table 1, we could learn that the level of blood lipids in OSA group is significantly more than that in patients of Con group, especially in the level of TC and TG. However, the level of HDL-c expression in OSA patients is not significantly different from that in patients of Con group. The expression level of Hcy was not significant in the OSA patients compared with the patients of Con group. The expression level of CRP is significant in OSA patients. Therefore, OSA might cause disorders of blood lipid levels and the inflammatory response of the body.

3.2. Identification of differentially expressed microRNAs and hierarchical clustering analysis

Differentially expressed microRNAs were identified after pre-processing of the sequencing results and an evaluation of the microRNA expression profiles (FDR < 0.05 and $|\log2FC| \geq 1$). Hierarchical clustering analysis was used to illustrate the differential expression of microRNAs between the 4 groups (Fig. 1). A comparison of the OSA and control groups revealed

Table 1

Characteristics of the study participants.

	Con (n = 24)	OSA (n = 32)	OSA+ iCIMT (n = 32)	iCIMT (n = 28)
Gender (M/F)	11/13	27/3	26/4	17/7
Age, y	43.17 ± 14.83	42.07 ± 9.84	50.28 ± 11.83*,†	53.54 ± 14.16*,†
BMI, kg/m^2	22.33 ± 3.49	24.10 ± 4.46	26.82 ± 2.95	24.50 ± 3.27
SBP, mm Hg	114.13 ± 15.41	126.44 ± 9.96	125.65 ± 11.89	121.75 ± 10.57
DBP, mm Hg	72.25 ± 9.62	80.00 ± 7.23*	80.24 ± 8.16*	77.4 ± 10.58
TC, mmol/L	4.26 ± 0.88†	4.57 ± 0.67*	5.21 ± 1.22*,†	5.0 ± 1.12*,†
TG, mmol/L	1.11 ± 0.73†	1.62 ± 0.67*	1.84 ± 1.03*,†	1.94 ± 1.92*,†
HDL-c, mmol/L	1.26 ± 0.33	1.3 ± 1.13	1.17 ± 0.25†	1.94 ± 1.92*,†
LDL-c, mmol/L	2.56 ± 0.81†	2.89 ± 0.58*	3.32 ± 1.00*,†	1.19 ± 0.32*,†
AHI, n/h	2.58 ± 1.36†	47.44 ± 24.64*	48.41 ± 28.44*	2.59 ± 1.30*,†
LSL, %	87.93 ± 16.95†	75.20 ± 12.17*	73.14 ± 13.30*	91.92 ± 1.91*,†
Max-IMT, mm	0.88 ± 0.05	0.86 ± 0.07	1.27 ± 0.15*,†	1.24 ± 0.14*,†
Hcy, μmol/L	11.81 ± 4.62	11.66 ± 9.19	13.64 ± 12.29*,†	11.71 ± 5.32
CRP, μmol/L	2.50 ± 1.16†	3.93 ± 4.60*	6.00 ± 10.58*,†	2.99 ± 4.07†

Continuous and categorical variables are expressed as the mean ± SEM or the number of participants, respectively.

DBP = diastolic blood pressure; SBP = systolic blood pressure.

* Different from Con ($P < .05$).

† Different from OSA group ($P < .05$).

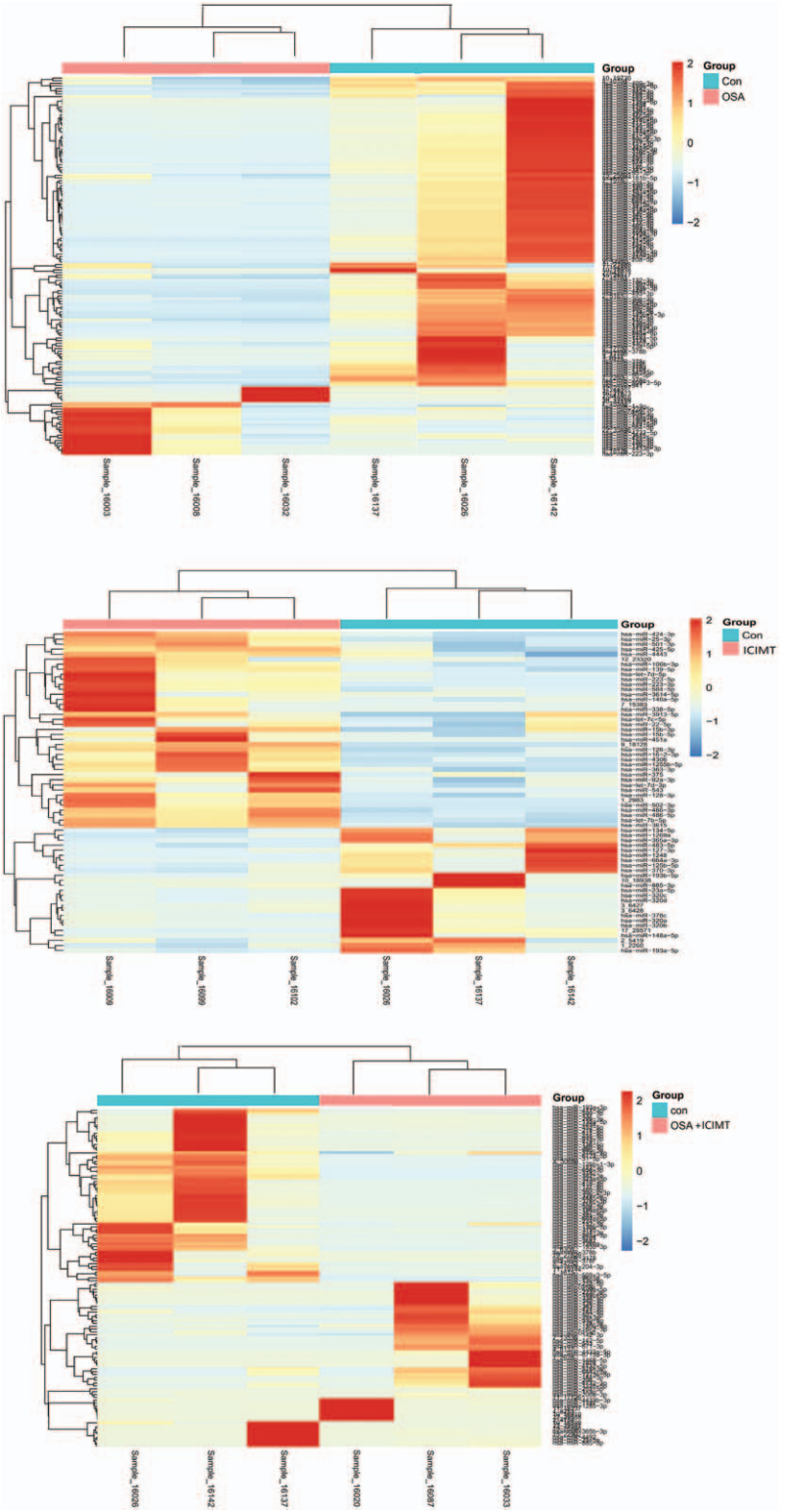

Figure 1. Hierarchical clustering analysis of differentially expressed microRNAs (miRNAs). High-level miRNA expression is shown in red, and low-level miRNA expression in blue. Con = healthy individuals, iCIMT = healthy individuals with an increased maximum carotid intima-media thickness, OSA = obstructive sleep apnea sample.

104 differentially expressed miRNAs (OSA/Con) potentially related to OSA. In a comparison of the iCIMT and Con (iCIMT/Con) groups, 54 differentially expressed miRNAs potentially related to AS were identified, and in a comparison of the OSA +iCIMT and Con (OSA+iCIMT /Con) groups, 100 differentially expressed miRNAs potentially related to OSA or AS. The intersection graph showed the miRNAs that might serve as screening signature of AS in OSA patients (Fig. 2).

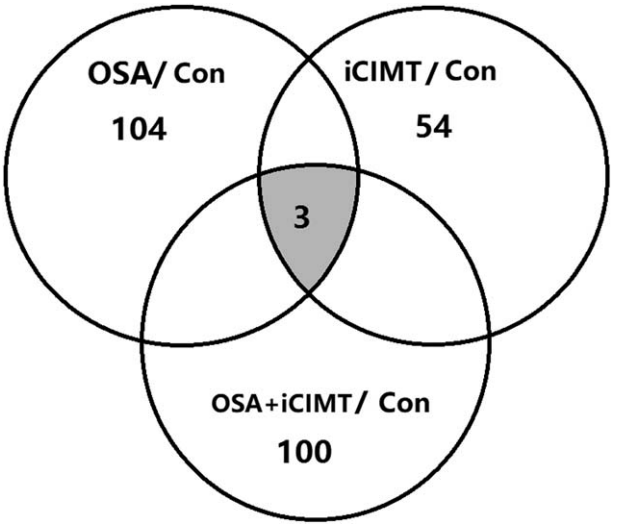

Figure 2. Intersection graph shows candidate screening signature miRNAs of atherosclerosis in patients with obstructive sleep apnea (OSA).

3.3. Significant differential expression of miRNAs

The candidate miRNAs were further examined by RT-qPCR in a training sample and a validation set (24 Con, 28 iCIMT, 32 OSA, and 32 OSA with iCIMT). As shown in Figure 2, 3 miRNAs, miR-664a-3p, miR-365a-3p, and miR-1269a, downregulated in the serum samples of Cont versus OSA, iCIMT, and OSA+iCIMT groups were identified. Of these, only the expression of miR-664a-3p was consistent with the results of earlier experiments (Fig. 3). The other 2 miRNAs were ruled out based on the inconsistent results with previous experiments (miR-365a-3p) or their low-level expression (miR-1269a).

3.4. Association of miR-664a-3p expression levels with the AHI, LOS, and CIMT

Our study showed that the expression level of miR-664a-3p correlated significantly with AHI ($r=-0.314$, $P<.01$), LOS ($r=0.318$, $P<.01$), max-CIMT ($r=-0.235$, $P<.05$; Table 2). In addition, the AHI correlated positively with LOS ($r=-0.865$, $P<.01$), and the total cholesterol (TC) level with OSA and AS: AHI ($r=0.204$, $P<.05$), LOS ($r=-0.236$, $P<.05$), and max-CIMT ($r=0.250$, $P<.01$). The TG level correlated significantly with OSA, AHI ($r=0.345$, $P<.01$), and LOS ($r=-0.312$, $P<.01$; Table 2).

4. Discussion

This study investigated the well-established close association of OSA with AS, by examining the differences in candidate miRNA expression. After identifying miR-664a-3p as a candidate biomarker of AS in OSA, we showed its expression was significantly higher in patients in the OSA, iCIMT, and OSA with iCIMT groups than in healthy controls (Fig. 1). We further demonstrated that miR-664a-3p expression is associated with AHI, LOS, and max-CIMT (Table 2).

AS is one of the most important and serious complications of OSA. In OSA and non-OSA patients, it can lead to coronary heart disease, stroke, and peripheral vascular disease. These serious complications are not only life-threatening, they also impose large familial, societal, and economic burdens. The early ability to predict the risk of AS in OSA patients would avoid many of the serious complications of this disease. Echocardiography is used in the clinical and differential diagnosis of AS, and PSG to measure the severity of OSA and related hypoxemia. Additional tools are a determination of CIMT, to assess the severity of AS, and measurements of AHI and LOS to evaluate the severity of OSA. We identified a significant correlation between serum mir-664a-3p expression and AHI ($r=-0.314$, $P<0.01$), LOS ($r=0.318$, $P<0.01$), and max-CIMT ($r=-0.235$, $P<.05$) (Table 2). In addition, AHI correlated positively with LOS ($r=-0.865$, $P<.01$). The TC level correlated significantly with OSA and AS, based on the AHI ($r=0.204$, $P<.05$), LOS ($r=-0.236$, $P<.05$), and max-CIMT ($r=0.250$, $P<.01$), and the TG level with OSA, based on the AHI ($r=0.345$, $P<.01$) and LOS ($r=-0.312$, $P<.01$). Consistent with previous studies, our results suggest that OSA influences lipid metabolism.

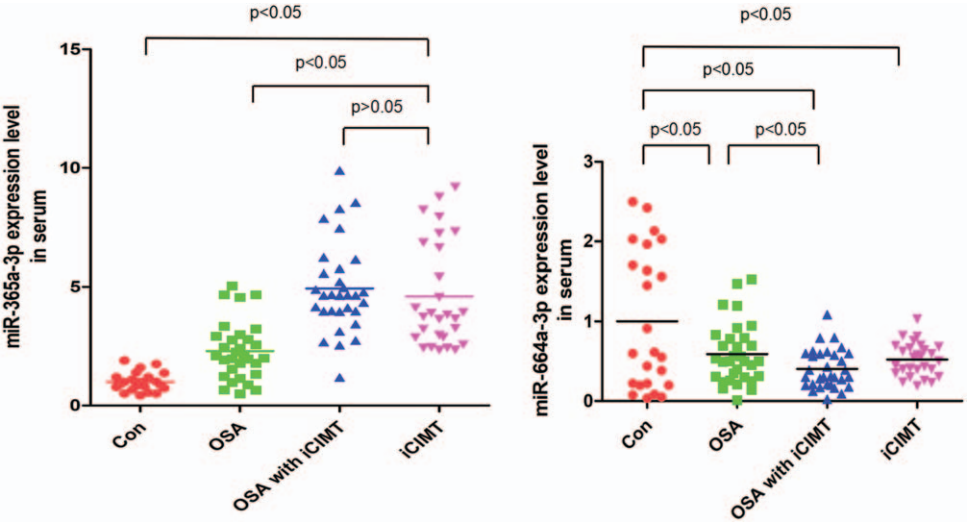

Figure 3. The relative expression levels of miRNAs in 4 groups. Serum miRNA expression: log2 (miRNA/miR-39). The horizontal lines indicate the mean. *P* values were generated in a Mann–Whitney *U* test; $P<.05$ was considered to indicate statistical significance.

Table 2

Correlations between circulating microRNAs critical to atherosclerosis and clinical parameters.

Correlations	AHI	LSL	MaxIMT	miR664a3p
Spearman's rho				
AHI correlation coefficient	1.000	−.865†	0.154	−0.314†
Sig. (2-tailed)		0.000	0.099	0.001
LSL correlation coefficient	−0.865†	1.000	−0.107	0.318†
Sig. (2-tailed)	0.000		0.254	0.001
MaxIMT correlation coefficient	0.154	−0.107	1.000	−0.235*
Sig. (2-tailed)	0.099	0.254		0.011
TC correlation coefficient	0.204*	−0.236*	0.250†	−0.057
Sig. (2-tailed)	0.028	0.011	0.007	0.546
TG correlation coefficient	0.354†	−0.312†	0.130	−0.076
Sig. (2-tailed)	0.000	0.001	0.163	0.419
HDLc correlation coefficient	−0.169	0.152	0.105	0.006
Sig. (2-tailed)	0.070	0.103	0.260	0.953
LDLc correlation coefficient	0.313†	−0.342†	0.128	−0.081
Sig. (2-tailed)	0.001	0.000	0.169	0.389
CRP correlation coefficient	−0.092	0.082	0.081	0.012
Sig. (2-tailed)	0.327	0.381	0.390	0.899
HCY correlation coefficient	0.282†	−0.240†	0.125	−0.026
Sig. (2-tailed)	0.002	0.010	0.182	0.784
miR664a3p correlation coefficient	−0.314†	0.318†	−0.235*	1.000
Sig. (2-tailed)	0.001	0.001	0.011	

NA = not applicable, *P* = sig. (2-tailed), *R* = correlation coefficient.

* Correlation is significant at the .05 level (2-tailed).

† Correlation is significant at the .01 level (2-tailed).

Human plasma or serum may contain specific miRNAs that can serve as biomarkers of disease, and therefore an early warning of, for example, cardiovascular disease or target organ damage.[17–24] Our study demonstrated that patients in the OSA and OSA with iCIMT groups had significantly higher levels of miR-664a-3p expression than healthy controls. This result suggested the utility of miR-664a-3p as a marker of AS in OSA patients. In fact, both the role and mechanism of miR-664 in disease have been investigated in several studies.[25–28]

Our study also demonstrated the negative correlation of miR-664a-3p expression with AHI and CIMT, and the positive correlation with LOS. This indirectly suggests a close relationship between miR-664a-3p and OSA and AS as well as the increased severity of both diseases, evidenced by the decreasing expression of the miRNA. A previous study reported the downregulation of miR-664 expression in human cervical cancer tissues compared with the corresponding noncancerous tissues. Moreover, the downregulated expression of miR-664 was significantly linked with lymphatic invasion and distant metastasis. The authors thus proposed miR-664 as a noninvasive prognostic biomarker in cervical cancer.[29] Zhang et al found that miR-664 inhibited the expression of the proinflammatory cytokines interleukin 6, tumor necrosis factor, and mitogen-activated protein kinase 1.[30] OSA may induce the high-level expression of systemic inflammation markers, which links this disease to AS. The common element in the pathogenesis of AS in OSA patients may thus include miR-664 downregulation.

There were several limitations to our study. First, the sample size used in the previous sequencing and QPCR validation was relatively small, which might have compromised the accuracy of the results. Second, the choice of statistical methods might have led to the selection of a smaller number of miRNAs than the actual possible number, thus affecting the postvalidation experiments. Third, neither intracellular nor extracellular experiments were performed to further validate the difference in miRNA expression levels. Further experimental studies are needed to explore the functions of miR-664a-3p in OSA patients and its relation to AS.

5. Conclusions

In summary, our study demonstrated the positive association of miR-664a-3p levels with AHI, LOS, and CIMT and thus its possible role in the pathogenesis of AS in OSA patients and as a noninvasive marker of these related conditions.

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