

# The Relationship Between HIF1 $\alpha$ and Clock Gene Expression in Patients with Obstructive Sleep Apnea

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**Purpose:** In this study, we aimed to investigate the precise relationship between hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ), circadian clock genes, and OSA.

**Methods:** We recruited 21 patients with OSA and 22 age-matched controls who underwent polysomnography and had their peripheral blood collected on the evening before and the morning after sleep. OSA was defined as an apnea hypopnea index (AHI)  $\geq 15$  events/h. Patients in which T90  $> 0$  were defined as having nocturnal hypoxemia (NH) and were referred to as the NH group. The mRNA levels of HIF1 $\alpha$ , HIF1 $\beta$  and several clock genes (*Timeless*, *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Ckl1 $\delta$* , *Rora*, *NR1D1*, and *NPAS2*) were determined by RT-qPCR. The percentage difference in gene expression levels when compared between the morning and evening was then determined as referred to as morning-evening variation (MEV).

**Results:** The MEV for HIF1 $\alpha$  mRNA expression in OSA patients increased significantly by 23% ( $P = 0.008$ ) when compared to patients without OSA. The gene expression levels of *Timeless* ( $P = 0.038$ ) and *Cry2* ( $P = 0.012$ ) decreased with AHI. The MEV of *Bmal1*, *Rora*, and HIF1 $\alpha$  mRNA levels were upregulated by 16% ( $P = 0.006$ ), 14% ( $P = 0.027$ ), and 25% ( $P = 0.005$ ), respectively, in participants with NH when compared to those without NH. Furthermore, the MEV for HIF1 $\alpha$  mRNA levels was positively correlated with the MEV of *Bmal1*, *Cry1*, and *CK1 $\delta$*  mRNA levels ( $R = 0.638$ ,  $P < 0.001$ ;  $R = 0.327$ ,  $P = 0.002$ ;  $R = 0.332$ ,  $P = 0.001$ , respectively) and negatively correlated with LSpO<sub>2</sub> ( $R = -0.464$ ,  $P = 0.009$ ) and Mean SpO<sub>2</sub> ( $R = -0.500$ ,  $P = 0.003$ ).

**Conclusion:** Our data suggest that patients with OSA or NH tend to develop circadian rhythm disorders that may be induced by the hypoxia-mediated augmentation of HIF1 $\alpha$  gene expression in OSA.

**Keywords:** obstructive sleep apnea, hypoxia-inducible factor, circadian rhythm, apnea hypopnea index

## Introduction

Obstructive sleep apnea (OSA) syndrome is a partial or complete block of the airways that can cause hypopnea or apnea accompanied by a variety of clinical manifestations, including night snoring, daytime sleepiness, and a reduction in cognitive performance.<sup>1</sup> OSA is estimated to affect 34% and 17% of middle-aged men and women in the general population;<sup>2</sup> the prevalence of this condition is increasing coincident with the epidemic of obesity.<sup>3</sup> Emerging data also suggest that the presence of OSA and nocturnal hypoxemia (NH) are constantly accompanied by an increased risk of hypertension,<sup>4</sup> diabetes,<sup>5</sup> coronary artery disease,<sup>6</sup> and stroke.<sup>7</sup> In OSA, the upper airway collapses repeatedly during sleep, thus causing cycles of apnea, arousal, and compensatory hyperventilation. Collectively, these events lead to alterations in day/night physiological and circadian gene expression and associated biological processes.<sup>8–10</sup> In addition, hypoxic signals regulate the clock by slowing the circadian cycle and dampening the amplitude of oscillations in a dose-dependent manner.<sup>11</sup> It is speculated that the disrupted expression of circadian genes induced by OSA through the hypoxia-inducible factor (HIF) pathway could result in an increased risk of a variety

of metabolic diseases and cardiovascular diseases.<sup>12</sup> Investigating the association between HIF and the expression of circadian genes could enhance our understanding of the pathophysiology of OSA and help reduce complications associated with this condition.

The internal 24-h circadian clocks are achieved in humans and most mammals by the ocular photoreception of light from the environment and the relay of signals to the suprachiasmatic nucleus (SCN) in the hypothalamus.<sup>13</sup> Previous studies suggested that the expression of clock genes in human monocytes isolated from peripheral whole blood could be used as indicators of changes in the central circadian rhythm.<sup>14,15</sup> A genetic network of transcriptional and translational negative feedback loops generates rhythmic 24-h oscillations in the expression levels of core clock components. The major transcription factor, *Bmal1*, interacts with *Clock* and *NPAS2* (the Clock homolog) to activate the circadian gene expression of their target genes including *PER* (*PER1*, *PER2*, and *PER3*), *CRY* (*CRY1* and *CRY2*), *NR1D1*, and *ROR $\alpha$* .<sup>16,17</sup> Although several studies have reported changes in the expression of canonical clock genes in patients with OSA,<sup>18–21</sup> the results derived from these studies were not consistent.

HIF1 is a heterodimer consisting of an unstable HIF1 $\alpha$  subunit and a constitutively expressed HIF1 $\beta$  subunit that is ubiquitously expressed in all tissues and functions as a primary regulator of oxygen homeostasis in humans.<sup>22</sup> Gene expression array and chromatin immunoprecipitation assays have demonstrated that more than 1000 genes are directly trans-activated by HIF1 in response to hypoxia.<sup>23,24</sup> Several studies have reported an increase in HIF1 $\alpha$  in patients with OSA when compared with control subjects.<sup>25,26</sup> Furthermore, HIF1 $\alpha$  has also been proven to exert functionality as the regulatory node that connects hypoxia signaling with the circadian clock.<sup>11</sup> A body of evidence now suggests that HIF1 and circadian clock genes (eg, *Bmal1* and *Per2*) undergo bidirectional interactions.<sup>27,28</sup> According to these previous studies, we know little about the specific interactions between *HIF1 $\alpha$*  and clock genes in patients with OSA; furthermore, existing information is not consistent.<sup>29</sup>

In this study, we investigated the disturbance in circadian clock gene expression in subjects experiencing different severities of OSA without any other complications. Secondly, we aimed to investigate the association between the expression of *HIF1 $\alpha$*  and clock genes in patients with OSA to gain a further understanding of the effect of intermittent hypoxia induced by OSA on genes responsible for circadian rhythm.

## Materials and Methods

### Participants

Polysomnography (PSG) was carried out in accordance with the American Academy of Sleep Medicine (AASM) manual 2.5 in order to score sleep and associated events.<sup>30</sup> PSG results were then used to consecutively recruit 21 males with an apnea hypopnea index (AHI)  $\geq 15$  events/h and 22 male controls (mean AHI: 1.6 events/h). Patients were all recruited between August 2019 and December 2019 from the Sleep Disordered Breathing Laboratory of Peking Union Medical College Hospital (PUMCH). The present study was carried out as described previously.<sup>31</sup> The exclusion criteria were as follows: (1) patients who had been treated for sleep apnea by continuous positive airway pressure (CPAP); (2) patients with self-reported chronic diseases including uncontrolled hypertension, chronic liver diseases, chronic kidney diseases, chronic lung diseases, perivascular diseases, heart diseases, diabetes, thromboembolism diseases, tumor, nerve system diseases and mental diseases; (3) patients with acute infectious over the previous two weeks; and (4) patients who performed shift work and slept for less than 6 h. Written and informed consent was provided by all participants. The study was approved by the PUMCH ethics committee (protocol number: JS-2013) and conducted in accordance with the Declaration of Helsinki.

### Polysomnography and the Definition of Key Sleep Variables

Overnight PSG was performed using an Embla N7000 system (Natus Medical Incorporated, USA) from 10 PM to 6 AM in the sleep-disordered breathing laboratory of our hospital. The overall process and manual scoring were performed by skilled technicians who were supervised by experienced sleep physicians in accordance with the AASM manual 2.5.<sup>30</sup> The AHI was defined as the total number of apnea and hypopnea events per hour of sleep. LSpO<sub>2</sub> was defined as the lowest pulse oxygen saturation during sleep. The oxygen desaturation index (ODI) was defined as the frequency of

instances when the blood oxygen levels decreased (desaturation) by 3% or more. T90 was defined as the percentage of time when the oxygen saturation was < 90% during the period of monitoring. An AHI  $\geq 15$  events/h was used as a cutoff to divide the patients into two groups: the control group featuring patients with an AHI < 15 events/h and an OSA group of patients with an AHI  $\geq 15$  events/h based on the International Classification of Sleep Disorders (3rd Edition).<sup>31</sup> In addition, we classified patients with a T90 > 0 into a nocturnal hypoxemia (NH) group and those with a T90 = 0 as a no NH group.

## Clock Gene Expression and Biological Measurements

Samples of whole peripheral blood were collected on the evening (9–10 PM) before and early morning (6–7 AM) after PSG using PAXGene Blood RNA tubes (2.5 mL) in the sleep-disordered breathing laboratory and then stored at  $-80^{\circ}\text{C}$ . Samples were processed within 6 months of acquisition. RNA was isolated with a PAXGene Blood RNA kit in accordance with the manufacturer's instructions (QIAGEN, Germany). Total RNA was converted to cDNA using a Prime Script RT Master Mix kit (TAKARA, Japan). Relative gene expression was then quantified by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) performed on an Applied Biosystems StepOnePlus Real-time PCR system (Applied Biosystems, USA), using Fast SYBR Green Master Mix (Applied Biosystems, USA). Relative gene expression was analyzed by the  $2^{-\Delta\Delta\text{Ct}}$  method<sup>32</sup> and normalized relative to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

All reactions were performed in duplicate, and mean expression levels for each transcript were used for analyses. The target genes were as follows: *Timeless*, *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Ckl $\delta$* , *Rora*, *NR1D1*, *NPAS2*, *HIF1 $\alpha$* , and *HIF1 $\beta$* . Forward and reverse primers specific for each gene of interest are listed in Table 1.

The levels of key biochemical parameters were determined in fasting peripheral blood within 1 h using standard techniques. Plasma was obtained after centrifugation for 30 min at 3000 g at  $4^{\circ}\text{C}$  for 15 min, stored at  $-20^{\circ}\text{C}$  for less than 1 week and then transferred to  $-80^{\circ}\text{C}$ . Plasma levels of HIF1 $\alpha$  were measured in batches within 6 months of acquisition using a Human/Mouse Total HIF1 $\alpha$  ELISA kit (R&D Systems, USA) in accordance with the manufacturer's protocol.

## Statistical Analysis

All calculations were performed using SPSS version 22.0 (IBM, USA) and GraphPad Prism 5.0 (GraphPad, USA). The normality of variables was assessed by the Kolmogorov–Smirnov normality test. We used t-tests or Chi-squared tests to compare between groups for normally distributed variables and categorical variables, respectively. The non-parametric Mann–Whitney *U*-test was used when data were not normally distributed. An ANOVA or Kruskal–Wallis test was used for non-parametric multi-group comparisons. For each sample of whole blood, CT data were examined, and samples in which the expression level of GAPDH was  $\geq 23$ , thus indicating unreliable; these samples were excluded from our analyses. Gene expression data were analyzed by comparing the mean fold change of transcript levels between groups. The percentage difference between morning and evening ( $\frac{\text{morning} - \text{evening}}{\text{evening}}$ ) was used to describe morning-evening variation (MEV). Pearson's or Spearman's methods were used to analyze the correlation between gene expression and PSG results.  $P < 0.05$  was considered statistically significant.

## Results

### Characteristics of Participants

Demographic variables, clinical characteristics, PSG parameters, and laboratory data are shown in Table 2. The mean age of the cohort was 39 years (range, 26–56 years); there was no significant difference between the control and OSA groups in terms of age. The mean body mass index (BMI) was  $26.6 \text{ kg/m}^2$  and was significantly higher in OSA ( $P = 0.001$ ) or NH ( $P < 0.001$ ) subjects than those in the control group. In addition, 49% of the study cohort were diagnosed as NH because T90 (percent of time spent with SpO<sub>2</sub> below 90%) was > 0. After adjustment for age and BMI, the serum HbA1c (hemoglobin A1C) levels in OSA patients were significantly higher than those of the control participants ( $P = 0.03$ ). Moreover, the levels of glucose, HbA1c, and ALT (Alanine aminotransferase) in patients with NH were significantly

**Table 1** Primer Sequence Details of the Analyzed Gene (F: Forward Primer, R: Reverse Primer)

Gene	Sequence
Human_Gapdh_F	TCGGAGTCAACGGATTTGGT
Human_Gapdh_R	TTCCCGTTCTCAGCCTTGAC
Human_Timeless_F	AGGAGCGGAGTGCAGATTTT
Human_Timeless_R	TCGAGAATGCCTGTTGCCTC
Human_Clock_F	AAGTTAGGGCTGAAAGACGACGA
Human_Clock_R	GAACTCCGAGAAGAGGCAGAAG
Human_Bmal1_F	AGGATGGCTGTTCCAGCACATGA
Human_Bmal1_R	CAAAAATCCATCTGCTGCCCTG
Human_Per1_F	CTGAGGAGGCCGAGAGGAAAGAA
Human_Per1_R	AGGAGGAGGAGGCACATTTAC
Human_Per2_F	AAGCAGGTGAAAGCCAATGAAGA
Human_Per2_R	CCACCGCAAACATATCGGCATT
Human_Per3_F	TGCAGGGCATCCTCCCTTTGA
Human_Per3_R	TCCGGCTCCAGGGATTCACAA
Human_Cry1_F	CTGCGTCTACATCCTGGACC
Human_Cry1_R	GAAGCAAAAATCGCCACCTGT
Human_Cry2_F	TCCCTAGCATGTCAGCCCCTT
Human_Cry2_R	AGGATTTGAGGCACTGTTCCGA
Human_Ckl $\delta$ _F	CTCACAGAATAGCATTCCTTTCCG
Human_Ckl $\delta$ _R	TGCACGACAGACTGAAGACC
Human_Ror $\alpha$ _F	TCGCAGCGATGAAAGCTCAAAT
Human_Ror $\alpha$ _R	GTGGCATTGCTTTGCTGACT
Human_NR1D1_F	ACAGCTGACACCACCCAGATC
Human_NR1D1_R	CATGGGCATAGGTGAAGATTTCT
Human_NPAS2_F	ATCCTGCAGGCCAATATCCG
Human_NPAS2_R	CTGCTGCAGGAACATCTGGA
Human_HIF1 $\alpha$ _F	GCCGCTGGAGACACAATCAT
Human_HIF1 $\alpha$ _R	GAAGTGGCTTTGGCGTTTCA
Human_HIF1 $\beta$ _F	GACTACTGCCAACCCCGAAA
Human_HIF1 $\beta$ _R	CTCATCATCCGACCTGGCAA

higher than the subjects in the no NH group after adjusting for age and BMI ( $P = 0.022$ ,  $P = 0.033$  and  $P = 0.039$ , respectively). AHI, ODI, mean SpO<sub>2</sub>, LSpO<sub>2</sub> (%), lowest pulse oxygen saturation), and T90 (%) are greatly increased both in patients with OSA or NH.

**Table 2** General Characteristics of the Study Participants

	Control (AHI<15 Events/h, n=22)	OSA (AHI≥15 Events/h, n=21)	P value	no NH (T90<0, n=22)	NH (T90>0, n=21)	P value
Age (years)	39.0±7.8	38.9±8.3	0.969	37.5±6.9	40.4±8.8	0.238
BMI (kg/m <sup>2</sup> )	25.1±3.1	28.1±2.4	0.001	25.0±3.1	28.2±2.3	<0.001
Current smokers(n)	3	5	0.457	4	4	1.000
Hypertension (n)	5	9	0.203	4	10	0.055
AHI (events/h)	1.6 (0.6, 6.9)	42.4 (21.1, 63.1)	<0.001	1.6 (0.6, 8.6)	40.4 (18.2, 63.2)	<0.001
ODI (events/h)	1.4(0.3,4.9)	38.6(19.1,60.1)	<0.001	1.4(0.3,4.9)	38.6(17.0,60.1)	<0.001
Mean SpO <sub>2</sub> (%)	97.3 (95.6, 98.1)	95.8 (94.0, 97.4)	0.029	97.7 (95.8, 98.4)	95.1 (94.0, 96.7)	<0.001
LSpO <sub>2</sub> (%)	91.6±4.0	81.7±6.7	<0.001	92.4±2.6	80.8±5.8	<0.001
T90(%)	0(0, 0)	0.6(0.2,1.6)	<0.001	0(0, 0)	0.6(0.3, 1.6)	<0.001
ALT(U/L)	25(19, 35.5)	46(27.5, 56)	0.017	25(19, 33.2)	44(27.5, 62.5)	0.011
Cr (umol/L)	80.1±8.2	75.4±11.5	0.128	80.3±7.8	75.2±11.7	0.099
Glucose (mmol/L)	4.9±0.6	5.2±0.6	0.082	4.8±0.6	5.3±0.6	0.008
HBA1C(%)	5.2(5.0, 5.5)	5.5(5.3, 5.6)	0.025	5.2(5.0, 5.5)	5.5(5.4, 5.6)	0.006
TG (mmol/L)	1.1(0.8, 1.8)	2.4(1.5, 4.2)	0.004	1.1(0.8, 1.8)	2.4(1.5, 4.2)	0.004
TC (mmol/L)	4.7±0.7	4.8±0.8	0.722	4.7±0.7	4.8±0.8	0.965
LDL (mmol/L)	3.2±0.7	2.9±0.8	0.357	3.2±0.7	2.9±0.8	0.200
HDL (mmol/L)	1.2±0.3	1.0±0.2	0.026	1.2±0.3	1.0±0.2	0.005
UA (umol/L)	391.6±74.8	425.4±93.5	0.197	410.1±95.8	406.0±74.8	0.874
hsCRP (mg/L)	0.8(0.6, 1.1)	2.0(0.8, 2.6)	0.002	0.9(0.6, 1.8)	1.2(0.8, 2.4)	0.194

**Notes:** Data are presented as mean values and SD, counts, or medians and interquartile ranges, as appropriate. Differences tested by t-test for each OSA subset against Controls.

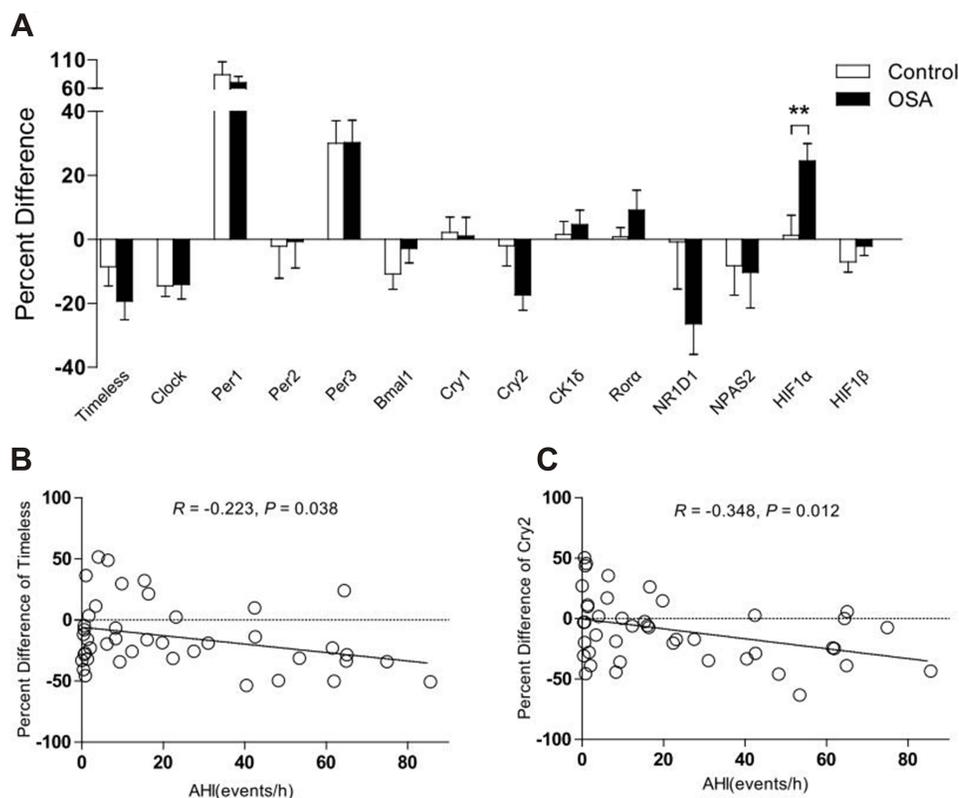
**Abbreviations:** AHI, Apnea-hypopnea index; ALT, Alanine aminotransferase; BMI, Body mass index; Cr, Creatinine; HBA1C, hemoglobin A1C; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; LSpO<sub>2</sub>, Lowest pulse arterial oxygen saturation; T90 (%), percent of time spent with SpO<sub>2</sub> below 90%; TC, Total cholesterol; TG, Triglyceride; UA, Uric acid.

## The mRNA Expression Levels of Clock Genes and HIF1 $\alpha$ According to OSA Status

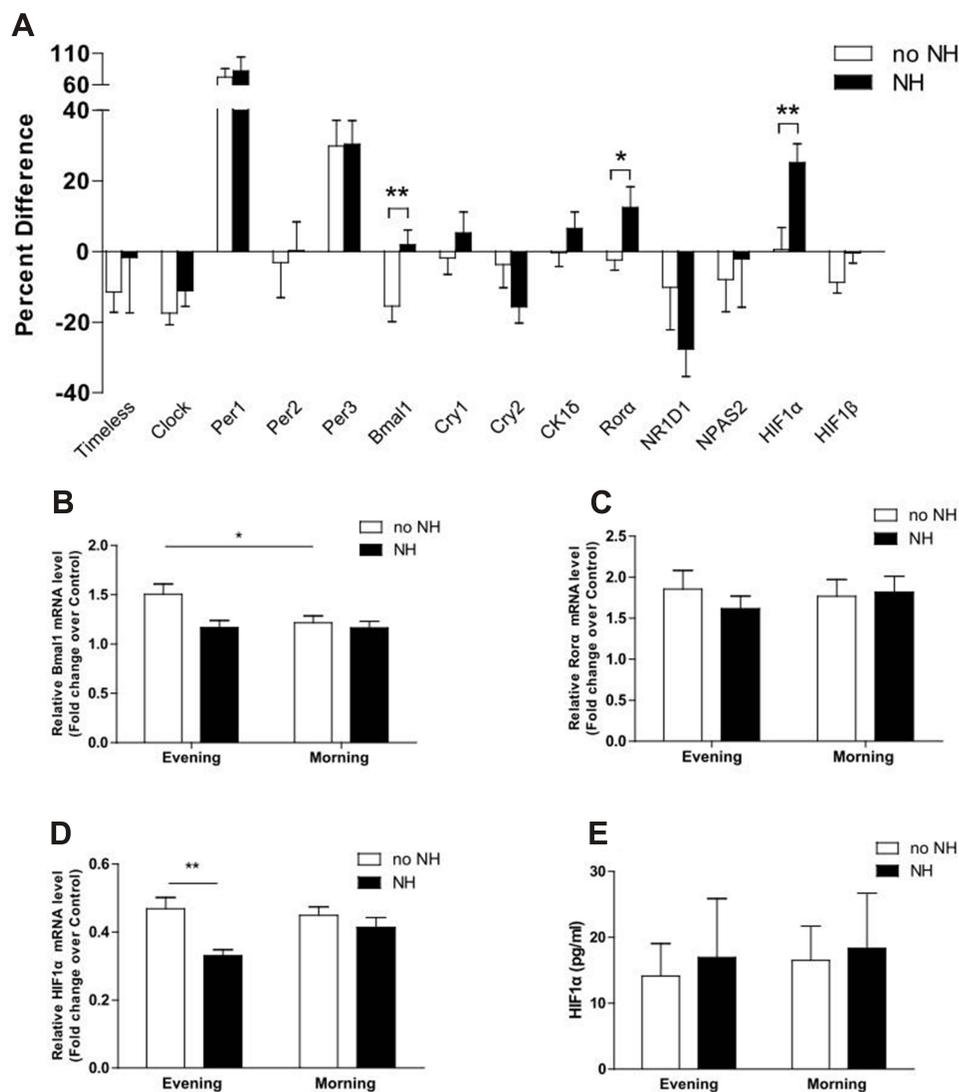
No significant differences were detected between the OSA and control group with regards to the mRNA expression levels of clock genes and HIF1 $\alpha$ ; this was the case for both the evening and morning samples ( $P > 0.05$ ). No significant differences were identified between the evening and morning samples of both groups ( $P > 0.05$ ) in terms of the mRNA levels of clock genes and *HIF1 $\alpha$*  ( $P > 0.05$ ). The MEVs of each target gene (the percentage difference for each gene) is depicted in Figure 1A. Of these, the MEV for *HIF1 $\alpha$*  mRNA expression increased by 23% in subjects with OSA when compared to those without OSA ( $P = 0.008$ ) after one night of sleep. However, no significant changes were detected in the expression levels of other clock genes when compared to control subjects. Further analysis detected a significant trend towards the downregulation of MEV for the *Timeless* gene ( $R = -0.223$ ,  $P = 0.038$ ) and *Cry2* gene ( $R = -0.348$ ,  $P = 0.012$ ) as AHI values increased (Figure 1B and C).

## The mRNA Expression Levels of Clock Genes and HIF1 $\alpha$ According to NH Status

In the NH group, the MEVs of *Bmal1*, *Rora*, and *HIF1 $\alpha$*  were upregulated by 16% ( $P = 0.006$ ), 14% ( $P = 0.027$ ), and 25% ( $P = 0.005$ ), respectively, when compared with the no NH group (Figure 2A). The mRNA expression of *Bmal1* mRNA in the morning decreased significantly after sleep in the no NH group ( $P = 0.039$ ), but not in the NH group (Figure 2B). A similar trend was also observed for the expression of *Rora*, although without statistical significance (Figure 2C). The mRNA levels of *HIF1 $\alpha$*  increased after a single night of sleep in the NH group but this change was not statistically significant. Analyses also showed that the evening levels of *HIF1 $\alpha$*  mRNA were significantly higher in the no NH group than in the NH group ( $P = 0.003$ ) (Figure 2D). Consequently, the protein levels of HIF1 $\alpha$  in both groups had increased the following morning; however, these changes were not statistically significant due to wide variation and the small sample size in the NH and no NH groups at both time points (Figure 2E). No statistical differences were found for



**Figure 1** The percentage difference of morning-evening variation in clock gene expression for subjects with and without OSA. (A) expression of *HIF1 $\alpha$*  increased by 23% in subjects with OSA when compared with those without OSA ( $P = 0.008$ ) (B and C) Relative gene expression of *Timeless* ( $R = -0.223$ ,  $P = 0.038$ ) and *Cry2* ( $R = -0.348$ ,  $P = 0.012$ ) in subjects with OSA decreased with increasing AHI. Data represent means  $\pm$  SEM. \*\* $P < 0.01$ .

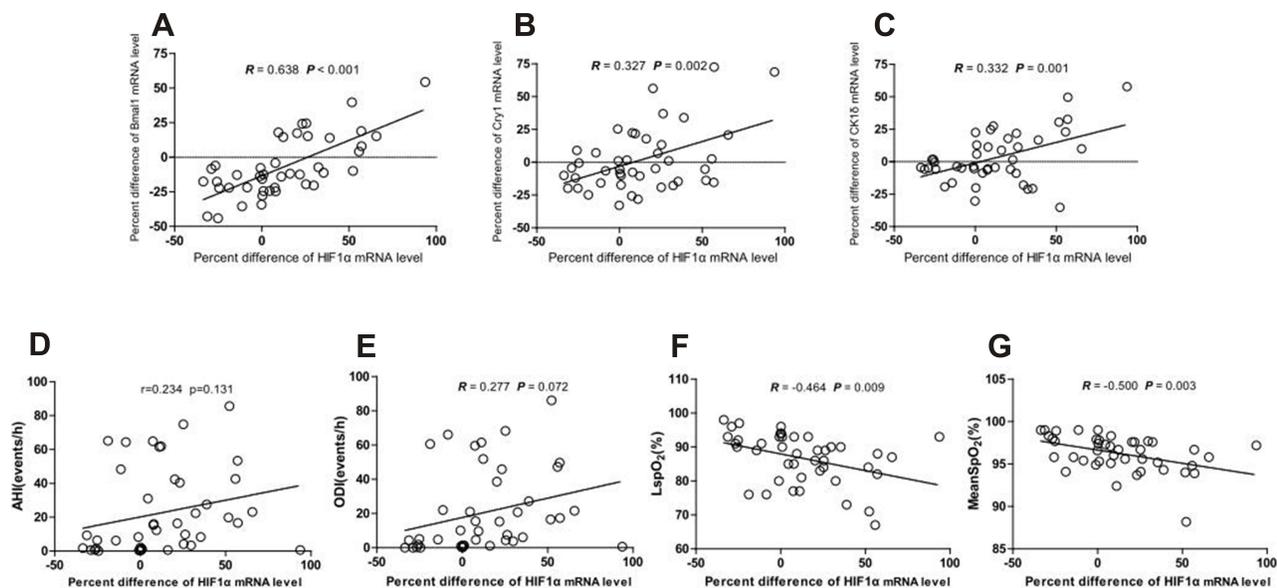


**Figure 2** The percentage difference of morning-evening variation in clock gene expression for subjects with and without NH. (A–D) The percentage difference of *Bmal1*, *Rora*, and *HIF1α* were up-regulated by 16% ( $P = 0.006$ ), 14% ( $P = 0.027$ ), and 25% ( $P = 0.005$ ), respectively, in subjects with nocturnal hypoxemia. (E) The protein concentration of *HIF1α* was increased in patients with NH after one-night of hypoxia but did not reach a significant statistical difference. Data represent means  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

the mRNA expression levels of other clock genes when comparing the NH and no NH group at both time points or when comparing evening and morning samples for both groups.

## Correlations Between the MEVs of *HIF1α* and the MEVs of Clock Gene and PSG Parameters

Next, we attempted to identify correlations between the MEVs of *HIF1α* and clock genes mRNA expression levels and found that the MEV of *HIF1α* mRNA levels was positively correlated with the MEVs of *Bmal1*, *Cry1*, and *CK1δ* mRNA levels ( $R = 0.638$ ,  $P < 0.001$ ;  $R = 0.327$ ,  $P = 0.002$ ;  $R = 0.332$ ,  $P = 0.001$ , respectively, Figure 3A–C); the MEV of *HIF1α* did not correlate with the expression of any of the other clock genes. We also investigated the MEVs of *HIF1α* mRNA levels and PSG parameters. As shown in Figure 3F and G, the MEV of *HIF1α* mRNA expression was negatively correlated with LSpO<sub>2</sub> (%) and mean SpO<sub>2</sub> (%) ( $R = -0.46$ ,  $P = 0.009$ ;  $R = -0.5$ ,  $P = 0.003$ ; respectively). There was no significant correlation between AHI, ODI, and the MEV for *HIF1α* mRNA.



**Figure 3** Correlations between morning-evening variation of *HIF-1α* and circadian genes mRNA levels (*Bmal1*, *Cry1*, and *CK1δ*), PSG parameters (AHI, ODI, LSpO<sub>2</sub>, and Mean SpO<sub>2</sub>). (A–C) Correlation between morning-evening variation for *HIF1α* and *Bmal1*, *Cry1*, and *CK1δ* mRNA level ( $R = 0.638$ ,  $P < 0.001$ ;  $R = 0.327$ ,  $P = 0.032$ ;  $R = 0.332$ ,  $P = 0.030$ , respectively). (D–G) Correlation between morning-evening variation for *HIF1α* mRNA level and PSG parameters (AHI, ODI, LSpO<sub>2</sub>, and Mean SpO<sub>2</sub>) ( $R = 0.234$ ,  $P = 0.131$ ;  $R = 0.277$ ,  $P = 0.072$ ;  $R = -0.464$ ,  $P = 0.009$ ;  $R = -0.500$ ,  $P = 0.003$ , respectively).

## Discussion

In this preliminary study, we showed that patients with OSA or NH had a significantly higher MEV for *HIF1α* mRNA expression. We also found that the MEVs for *Bmal1* and *Rora* mRNA expression were significantly increased in patients with NH. In addition, we identified positive correlations between the MEVs for *Bmal1*, *Cry1*, *CK1δ*, and *HIF1α*. Intermittent hypoxia is known to be the main contributor to OSA-related pathologies and place patients at a higher risk of developing multiple comorbidities.<sup>33</sup> Our findings suggest that an important interrelationship may exist between OSA-induced hypoxia and clock gene disturbance.

We also observed a remarkable increase in the amplitude of *HIF1α* mRNA expression levels in the mornings in patients from the OSA or NH groups after one night of sleep. A similar trend, albeit not significant, was observed for the levels of HIF1α protein in subjects from the NH group. This may be explained by changes in post-translational regulation rather than transcriptional control which leads to a mismatch between *HIF1α* expression at the mRNA and protein levels in blood serum. In addition, the MEV for *HIF1α* mRNA level was negatively correlated with LSpO<sub>2</sub> and mean SpO<sub>2</sub> but not AHI. These results further implied that more HIF1α were upregulated in response to acute hypoxia in one night than the chronic intermittent hypoxia induced by OSA. In fact, previous studies on the relationship between HIF1α and OSA are inconsistent.<sup>25,29,34</sup> Our current findings are similar to those reported by Kaczmarek et al, who showed that the mRNA expression of *HIF1α* in skin biopsies in severely hypoxemic OSA patients was significantly higher than those in control subjects or those who had mildly hypoxemic OSA.<sup>34</sup> However, other studies found that the serum levels of HIF1α protein were chronically increased in patients with OSA when compared with control patients.<sup>25,29</sup> In another study, Gabrylska et al found that the serum levels of HIF1α protein were higher in patients with OSA in both the evening and the morning; moreover, there were no significant differences between the OSA and control group with regards to *HIF1α* mRNA expression.<sup>25</sup> In comparison, we believe our findings might be due to the fact that a single night, with a reduction in the oxygen saturation of hemoglobin, may have directly affected the mRNA expression of *HIF1α* in the peripheral blood cells. However, these changes were not sufficient to cause a significant upregulation in the protein levels of HIF1α in our cohort of patients, who were relatively young OSA patients with a small BMI, and without significant complications. Another interpretation is that, during hypoxia, the overexpressed HIF1α protein translocate from the cytoplasm to the nucleus to form a transcriptionally active HIF1 complex via dimerization with HIF-1β to induce gene expression. Thus, the increase in serum HIF-1α protein was not so evident.

Our results showed no significant changes in the expression of circadian rhythm genes in OSA patients, irrespective of whether we considered evening or morning time points. These findings are somewhat different from those described in previous studies, in which the changes of circadian genes are inconsistent. A cross-sectional analysis of 49 veterans with chronic kidney disease found that the expression of *Per3* was reduced in sleep apnea, and that the expression levels of *NPAS2*, *Per1*, and *NR1D1* were reduced in subjects with NH.<sup>18</sup> Similarly, another study described the disruption of *Per1* mRNA expression levels in subjects with OSA and that CPAP treatment reversed this condition in 16 subjects.<sup>20</sup> However, Moreira et al reported statistically significant differences for the expression of clock genes in 13 patients with severe OSA when compared to 8 healthy subjects; this condition was not reversed by CPAP therapy.<sup>21</sup>

Furthermore, our results showed that the MEVs for *Cry2* and *Timeless* mRNA levels decreased with the increasing severity of sleep apnea. *Cry1* and *Cry2* are necessary for the maintenance of circadian behavioral rhythms; both circadian rhythmicity and light-induced suppression of pineal melatonin concentration were disturbed in *Cry1* and *Cry2* double-deficient mice.<sup>35,36</sup> Meanwhile, the human *Timeless* protein interacts with both *Cry2* and cell cycle checkpoint proteins and plays an important role in the DNA damage checkpoint response.<sup>37</sup> We speculated that OSA may influence the DNA repair process, or potentially apoptosis. Moreover, we identified a positive correlation between the mRNA levels of *HIF1α* and *Cry1*; this was similar to previous findings in which the evening levels of *Cry1* proteins were positively related to the levels of *HIF1α* protein.<sup>38</sup> According to our current study, the two isoforms of *Cry* protein may play opposing roles in OSA and NH.

After a single night in the presence of apnea and hypoxia, we found that the gene expression of *Bmall*, *Rora*, and *HIF1α* were enhanced in participants with NH when compared to the control group. In addition, the MEV for *Bmall* was also positively correlated with the expression of *HIF1α*. Previous reports showed that *Rora* can directly activate the transcription of *Bmall*, thereby maintaining a robust circadian rhythm in the peripheral tissues,<sup>39</sup> and the murine SCN.<sup>40</sup> Simultaneously, transcription of the *Rora* gene appeared to be upregulated by hypoxia in a panel of cell lines derived from different human tissues.<sup>41</sup> Moreover, it is known that *Bmall* and *Rora* form a regulatory loop and increase the transcriptional activity of *HIF1α* within the nucleus.<sup>42,43</sup> Consistent with this view, our data showed that the mRNA expression of *Rora* and *HIF1α* were both upregulated in the morning after one night of hypoxia. Therefore, our results provide a new insight for the function of *Rora* in the amplification of hypoxia signaling. Further research is now needed to elucidate the potential role of *Rora* in OSA. *CK1δ* is a serine/threonine-specific kinase that phosphorylates *HIF1α* at S247 to reduce its affinity for its hetero-dimerization partner *HIF-1β*.<sup>44,45</sup> Studies also showed that the silencing of *CK1δ* enhanced the activity of *HIF1* under hypoxic conditions while the overexpression of *CK1δ* inhibited *HIF1* activity under such conditions; these data suggest the importance of the *CK1δ*-dependent phosphorylation of *HIF1α*.<sup>46</sup> In the present study, the enhancement of *CK1δ* expression under hypoxic conditions may contribute to the accumulation of *HIF1α* protein in patients with OSA.

The current study had key advantages that need to be highlighted. For example, only a very limited number of human studies have been published relating to the potential association between *HIF1α* and clock gene expression in patients with OSA. Secondly, our methodology was clinically accessible and implemented efficiently. In our study, we only included males without any severe comorbidities; in this manner, we were able to reduce the potential effects of confounding factors because OSA is a heterogeneous disease with different clinical phenotypes.<sup>47</sup> However, there are some limitations that need to be considered. First, we only analyzed the effect of hypoxia on *HIF1α* and clock genes after one night; we did not consider data over longer periods of time. Further research is now needed to investigate the mRNA and protein levels of *HIF1α* at more time points during the day and determine the precise correlations between *HIF1α* expression at the mRNA and protein levels. Secondly, the relatively small sample size limits the generalizability of our conclusions; future studies should be conducted in a larger OSA cohort to investigate the expression of *HIF1α* and clock genes in a more comprehensive manner.

## Conclusions

In conclusion, the current study indicated that OSA was associated with some alterations in the expression of several clock genes, including *Timeless*, *Cry1*, *Cry2*, *Bmall*, *Rora*, and *CK1δ*. Interestingly, these disruptions in clock gene expression may be mediated by *HIF1α*; the mRNA morning–evening variation for *HIF1α* was positively correlated with

the expression of *Bmal1*, *Cry1*, and *CK1δ*. Our findings suggest that OSA and OSA-related nocturnal hypoxia may be associated with the altered expression of clock genes during the response to hypoxia. A better understanding of the direction and magnitude of the association between OSA and 24-h gene expression patterns of key clock genes may open the door for the development of novel therapeutic targets to treat OSA-related complications.

## Brief Summary

1. There is a significant lack of literature relating to circadian clock disruption in patients experiencing OSA. This study aimed to investigate the relationship between *HIF1α*, a series of circadian clock genes, and polysomnography variables.
2. Data highlight the positive association between the mRNA expression of a series of clock genes and *HIF1α*: a trend towards the downregulation of *Timeless* and *Cry2* with increasing OSA severity, and an increase in the morning-evening variation of *Bmal1* and *Rora* mRNA expression in patients with NH. Findings enhance our understanding of the complex relationship between intermittent hypoxia, circadian rhythms, and OSA.

## Abbreviations

AASM, American Academy of Sleep Medicine; AHI, apnea-hypopnea index; ALT, alanine aminotransferase; BMI, body mass index; CK1, casein kinase 1; CPAP, continuous positive airway pressure; Cr, creatinine; Cry, cryptochrome; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HbA1C, hemoglobin A1C; HDL, high-density lipoprotein; HIF1α, hypoxia-inducible factor 1α; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; LSpO<sub>2</sub>, Lowest pulse arterial oxygen saturation; MEV, morning-evening variation; NH, nocturnal hypoxemia; OSA, obstructive sleep apnea; Per, period; PSG, polysomnography; PUMCH, Peking Union Medical School of Hospital; RT-qPCR, quantitative real-time polymerase chain reaction; SCN, suprachiasmatic nucleus; SpO<sub>2</sub>, oxygen saturation; T90 (%), Sleep time spent with oxygen saturation < 90%; TBA, total bile acid; TC, total cholesterol; TG, triglyceride; UA, uric acid.

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## Disclosure

The authors declare no conflicts of interest in this work.

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