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Cyclic intermittent hypoxia induces apoptosis through upregulation of LncRNA GAS5 expression in cardiomyocyte

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ABSTRACT: Obstructive Sleep Apnea-Hypopnea Syndrome (OSAHS) is a common disorder that disrupts breathing during sleep, with known links to several heart-related conditions. Investigating the underlying mechanisms can guide the creation of new treatments. We focused on the role of specific genetic regulators, known as long non-coding RNAs (lncRNAs), which recent evidence suggests may play a part in heart disease resulting from OSAHS. Our study explored how a simulated OSAHS condition, Cyclic Intermittent Hypoxia (CIH), affects heart cells. We found that CIH increases levels of the lncRNA GAS5 and an enzyme called TRIM11, while reducing the levels of Bcl-2, a molecule that prevents cell death, thus leading to increased heart cell apoptosis. We also demonstrated that a microRNA, miR-205-5p, is a key player in this process, as it can modify the effect of GAS5 on TRIM11. Introducing miR-205-5p mimics lessened the impact of GAS5 on TRIM11. Our findings reveal a novel lncRNA GAS5/miR-205-5p/TRIM11 pathway by which OSAHS may cause heart damage. This research offers fresh insights into the molecular dynamics of heart injury induced by OSAHS and may inform future therapeutic strategies.

KEYWORDS: sleep disorder syndrome, cyclic intermittent hypoxia, LncRNA GAS5, TRIM family protein, micro RNA

INTRODUCTION

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is a prevalent sleep disorder characterized by repetitive episodes of complete or partial obstruction of the upper airway during sleep, leading to chronic intermittent hypoxia and sleep fragmentation. This condition has been associated with an increased risk of cardiovascular diseases, including myocardial injury, hypertension, and heart failure [1]. It has been reported that the prevalence of OSAHS has increased from 6% in men and 4% in women in 1993 to 83.8% in men and 60.8% in women in 2013 [2]. A better understanding of the molecular mechanisms underlying OSAHS-induced myocardial damage is critical for the development of targeted therapeutic strategies.

Long non-coding RNAs (lncRNAs) are crucial regulators of gene expression, influencing genetic pathways through mechanisms such as epigenetic modification and interaction with transcriptional machinery. In the context of OSAHS-related myocardial injury, lncRNAs can modulate cardiomyocyte function and survival, directly impacting disease pathogenesis. Specifically, the GAS5/miR-205-5p/TRIM11 axis may serve as a therapeutic target, altering the cellular response to hypoxic stress and offering potential for novel treatment strategies. Recent studies have highlighted the role of lncRNAs in the regulation of various cellular processes, including those involved in the pathogenesis of cardiovascular diseases [3]. Growth arrest-specific transcript 5 (GAS5) is a lncRNA that has been implicated in the regulation of cell growth, apoptosis, and inflammation [3, 4]. The mechanisms through which GAS5 regulates cellular processes are various. One of them is that GAS5 interacts with microRNAs to regulate various cellular functions [5, 6]. MicroRNAs (miRNAs) are small non-coding RNAs that play crucial roles in the post-transcriptional regulation of gene expression. miR-205-5p is a miRNA that has been reported to be involved in various biological processes such as cell proliferation, migration, and apoptosis [7]. Many of the studies are done in cancer.

Tripartite motif-containing protein 11 (TRIM11) is a member of the TRIM protein family. It has a RING finger domain, two B-box domains, and a coiled-coil domain. Trim11 has been found to be expressed in multiple tissues or organs, including brain, heart, liver and skeletal muscles [8]. Like other TRIM family proteins, TRIM11 functions as an E3 ligase, mediating degradation of its substrates, thereby regulating various cellular functions. Deregulation of TRIM11 has been found under different cellular conditions, such as hypoxia [9], and in different diseases, such as cancer [10]. There have been reports claiming that Trim11 regulates the level of anti-apoptotic protein Bcl-2 in cells, thereby regulating cell apoptosis in cardiomyocytes [11].

In this study, we aim to explore the molecular mechanisms underlying myocardial injury induced by OSAHS. We hypothesize that the long non-coding RNA (lncRNA) GAS5 is upregulated in cardiomyocytes under cyclic intermittent hypoxia (CIH), which mimics

the hypoxic conditions of OSAHS. We will investigate how GAS5 interacts with miR-205-5p and affects the expression of TRIM11, a protein linked to cellular apoptosis. Through gene expression microarray screening, we will assess the changes in lncRNA GAS5 expression in AC16 cardiomyocyte cells under CIH conditions. We anticipate that the results will help us understand the potential regulatory mechanisms of GAS5 in the context of OSAHS and guide the development of targeted therapeutic strategies.

MATERIALS AND METHODS

CIH treatment

AC16 cells were purchased from Suzhou Beina Chuanglian Biotechnology Co., Ltd., China (BNCC337712) and used in the study to establish a cyclic intermittent hypoxia (CIH) cell model. Cells were cultured in DMEM/F12 (Sigma Cat. No. D6434, USA) containing 2 mm L-Glutamine (EMD Millipore Cat. No. TMS-002-C, USA), 12.5% FBS (EMD Millipore Cat. No. ES-009-B) and 1X Penicillin-Streptomycin Solution (EMD Millipore Cat. No. TMS-AB2-C). The cells were cultured in an incubator containing 95% air, 5% CO_2. Cells were grown to 60% confluency and were put into hypoxic incubator for CIH treatment. Cells grown in standard culture condition was used as control. All samples are triplicated.

For CIH treatment establishment, set up the hypoxic incubator as the following: put a 6-well plate containing 3 ml medium/well into the hypoxic incubator at 37 °C and containing 95% air, 5% CO_2. The plate was covered by an air permeable film (1150Q05, Thermo Fisher Scientific, USA). The oxygen concentration in the incubator was rapidly reduced from 20% to 5%. The dissolved oxygen partial pressure in the culture medium was monitored, and an oxygen partial pressure-time curve was plotted. Determination of hypoxia-reoxygenation time: Based on the oxygen partial pressure-time curve, when the oxygen partial pressure in the culture medium reached 56 ± 4 mmHg, maintain at this level for 10–40 seconds. Subsequently, the oxygen concentration in the incubator was increased from 5% to 20%, and the time required to reach normal oxygen partial pressure was recorded. The intermittent hypoxia-reoxygenation time was then finally determined. The parameters for the finally established CIH model in this study were: 20% O_2/5% CO_2, decrease the O_2 concentration to 5%, maintain it for 90 s, then increase the O_2 concentration back to 20%, maintain for 60 s. This is one cycle. 30 cycles were used to treat cells. After CIH treatment method establishment, cells were treated by CIH. Basically, cells were cultured in a 6-well plate, covered with an air permeable filter, and was put into the CIH treatment hypoxic incubator to receive CIH treatment. After treatment, cells were harvested appropriately for subsequent analysis. Cells cultured in the incubator with regular culture conditions were used as control.

RNA extraction

Total cellular RNA was extracted by using TRIzol® reagent (Thermo Fisher Scientific) following manufactures' instruction, and a reverse transcription kit (superscript III, Invitrogen, Thermo Fisher Scientific) was used to reverse transcribe the total RNA into cDNA. The cDNA was used for real time PCR.

Western blot

Total proteins were extracted by using RIPA buffer (containing protease and phosphatase inhibitor, protease cocktail, Roche), and quantified using a BCA method (Thermo Fisher Scientific). Then the equal amount of proteins were resolved by 4–20% SDS-PAGE electrophoresis, transferred to polyvinylidene fluoride membranes (HATF00010, Millipore), blocked in non-fat milk for 1 h at room temperature, and then incubated with primary and corresponding secondary antibodies. The protein was visualized by ECL method (Thermo Fisher Scientific). GAPDH was used as internal control (Cell Signaling Technology, USA). Antibodies used in the study were: Anti-TRIM11 (Ab111694) from Abcam, USA (1:500), Bcl-2 antibody (SC7382) from Santa Cruz Biotechnology, USA (1:500), and anti-GAPDH (#5174, CST) antibodies from Cell Signaling Technology (1:500).

LDH assay

LDH levels were measured by using an LDH detection kit (A020-2) (Nanjing, Jiancheng Biotechnology Research Institute, China) following manufacturer's instruction. Each sample was triplicated.

Apoptosis assay

TUNEL staining was used to detect apoptosis following manufacturer's instruction (Ab66108, Abcam). Flow cytometry was used to detect apoptotic cells, and the percentage of apoptotic cells was calculated by BD Accuri C6 software (V. 1.0.264.21, BD Biosciences, USA).

siRNA, miRNA mimics transfection, qRT-PCR

GAS5 siRNA was purchased from Invitrogen (#1299001), a universal control siRNA was used. miR-205-5p mimic was purchased from GenePharma (Shanghai, China). Lipofectamine (Invitrogen, Thermo Fisher Scientific) was used to do transfections. 48 h after transfection, cells were either harvest appropriately depending on the analysis or treated with CIH or control condition. After treatment, cells were then harvested for analysis. Primers used for qRT-PCR are listed in the Supplementary Table S1.

Statistical analysis

Each result was evaluated from 3 independent experiments, all data in this study were expressed as mean ± standard deviation (SD) and were analyzed using GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed using an unpaired Student's t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Expression of lncRNA GAS5 increased in CIH-treated cells

First, AC16 cells were treated with CIH conditions. Cells cultured under routine conditions were used as control. After treatment, control cells and CIH-treated cells were harvested to exact total RNAs. Based on the GSE103731 database, an expression microarray was performed, and the results showed that lncRNA GAS5 was upregulated in CIH-treated cells compared to the control cells ($p < 0.001$, log FC = 3.35). To confirm the increased level of GAS5 in CIH-treated cells, a qRT-PCR was used to check GAS5 expression level. As shown in Fig. 1a, GAS5 expression increased in CIH-treated cells to 4 folds as the one in the control cells. Meanwhile, we also found an E3 ligase, TRIM11, was upregulated by CIH treatment (Fig. 1b). Next, we tested if CIH treatment caused cell damage. Apoptosis assay was performed in both control cells and the CIH-treated cells and the results showed that CIH treatment decreased cell viability (Fig. 1c). LDH level was upregulated by CIH treatment as well (Fig. 1d), suggesting cell damage induced by CIH treatment. We also explored the expression of TRIM11 and a well-characterized anti-apoptotic protein, Bcl-2 at protein level. The result showed that TRIM11 was upregulated, while Bcl-2 level was decreased in CIH-treated cells (Fig. 1e).

Knockdown of GAS5 attenuated CIH induced cell apoptosis

Next, we investigated whether manipulating GAS5 expression will affect the expression of TRIM11 and Bcl-2. GAS5 siRNA was used to knockdown the expression of GAS5. As shown in Fig. 2a, GAS5 expression was downregulated by GAS5 siRNA while control siRNA did not. Knockdown of GAS5 attenuated CIH treatment-induced TRIM11 upregulation (Fig. 2b), resulting in the partial rescue of cell viability (Fig. 2c).

Then, we tried to explore the mechanisms underlying the regulation of TRIM11 by GAS5. It has been reported that GAS5 can act as competitive endogenous RNA (ceRNA) or a sponge of miRNA-205-5p [12, 13], to regulate its target gene expression. We checked the TRIM11 3UTR region and found that there is a potential binding site for miR-205-5p (Fig. 3).

Therefore, we tested whether miR-205-5p mediated regulation of TRIM11 expression by GAS5. First, in CIH-treated cells, miR-205-5p was downregulated compared to control cells (Fig. 4a). This is also true in miR-205-5p mimic transfected cells (Fig. 4b). Transfection of miR-205-5p attenuated CIH induced TRIM11 upregulation (Fig. 4c), and cell viability (Fig. 4d).

Our findings revealed that under intermittent hypoxic conditions (CIH), the expression of TRIM11 was significantly increased at both the mRNA and protein levels. In contrast, Bcl-2 expression was reduced, and the level of lactate dehydrogenase (LDH) was elevated, suggesting cell damage and decreased cell viability. These results suggested a GAS5/miR-205-5p/TRIM11/Bcl-2 signaling pathway mediating cell apoptosis in the context of hypoxia, which may contribute to the underlying mechanisms of myocardial injury in OSAHS.

DISCUSSION

Our study aimed to elucidate the molecular mechanism of myocardial damage caused by OSAHS and found that the GAS5/miR-205-5p/TRIM11 axis was involved in the regulation of cell apoptosis in cardiomyocytes under CIH condition. The results of this study provides novel insights into the potential regulatory mechanisms underlying OSAHS-induced myocardial damage, which may contribute to the development of targeted therapeutic approaches for patients with OSAHS and associated cardiovascular complications.

Under intermittent hypoxic conditions, we observed an upregulation of TRIM11 and lncRNA GAS5 expression. These findings indicate a potential relationship between GAS5 and TRIM11 in the context of hypoxia-induced cellular changes. Previous studies have reported that lncRNA GAS5 acts as a competing endogenous RNA (ceRNA) or a sponge of miRNAs, such as miR-205-5p [4, 9, 12]. This interaction may play a crucial role in various cellular processes, including apoptosis, inflammation, and cell growth. In our study, we found that miR-205-5p could be able to bind to both molecules, the lncRNA GAS5 and TRIM11, suggesting its role in the regulation of TRIM11 by lncRNA GAS5.

Following the knockdown of GAS5, we observed a decrease in TRIM11 expression, an increase in Bcl-2 expression, improved cell viability, and reduced LDH content. These results suggest that the downregulation of GAS5 has a significant impact on TRIM11 and Bcl-2 expression. The observed increase in cell viability upon GAS5 knockdown could be attributed to the upregulation of Bcl-2, an anti-apoptotic protein [13]. This finding supports the notion that GAS5 may function as a crucial regulator of myocardial injury in OSAHS by modulating the expression of TRIM11 and Bcl-2. In fact, it has been reported that in ischemia/reperfusion treated cardiomyocytes, overexpression of TRIM11 at-

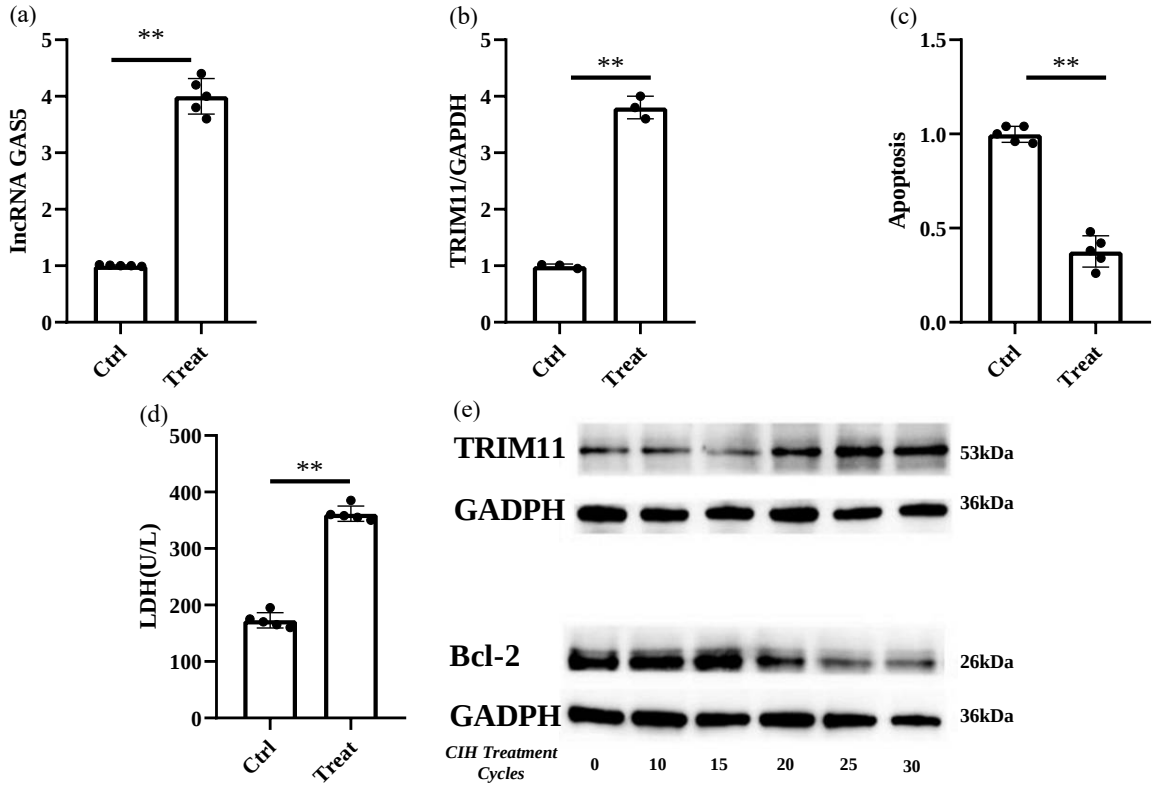

Fig. 1 The effect of CIH treatment on certain gene expression, protein level, and cell viability. The effect on (a) GAS5 expression, (b) TRIM11 expression, (c) cell viability using an apoptosis assay, (d) LDH level, and (e) TRIM11 and Bcl-2 protein levels. Ctrl = Control, Treat = Treatment. **, $p < 0.001$.

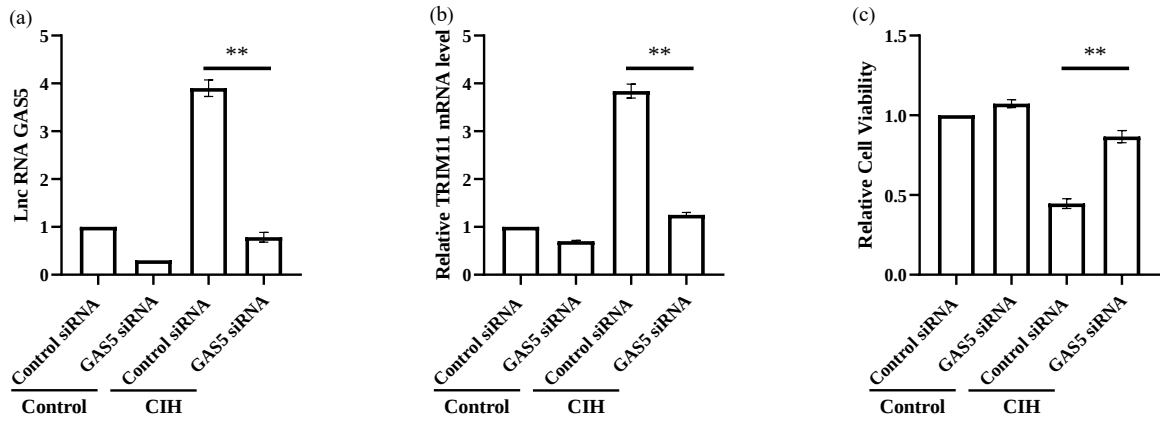

Fig. 2 Knockdown of GAS5 attenuated CIH-induced TRIM11 upregulation, Bcl-2 downregulation, and apoptosis upregulation. The expression level of indicated molecules were examined by qRT-PCR or western blot. Relative viability was calculated by subtracting the percentage of apoptotic cells from 1. (a) Expression levels of GAS5 after GAS5 siRNA transfection; (b) expression levels of TRIM11; (c) viability in untreated or CIH-treated cells.

54 ENSG00000154370 (TRIM11)		Has-mIR-205-5p	0.725648590794194	
Region	Binding type	Transcript position	Score	Conservation
UTR3	7mer	947-971	0.0266028195096098	2

Fig. 3 Predication of interaction between TRIM11 3UTR and miR-205-5p.

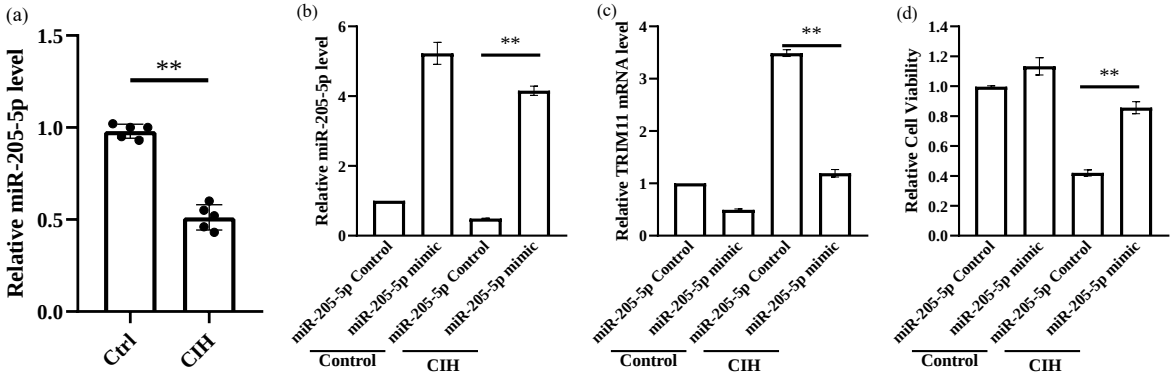

Fig. 4 Overexpression of miR-205-5p in CIH-treated and untreated cells. Expression of indicated molecules were detected by qRT-PCR or western blot. Relative viability was calculated by subtracting the percentage of apoptotic cells from 1. (a) Expression of miR-205-5p in CIH-treated and untreated cells without miR-205-5p overexpression; (b) expression level of miR-205-5p in treated or untreated cells with miR-205-5p transfection; (c) TRIM11 expression; (d) relative viability in CIH-treated and untreated with miR-205-5p overexpression.

tenuated the expression of Bcl-2 [9, 13]. By regulating TRIM11 level in cardiomyocytes, OSAHS causes myocardial injury.

Moreover, our study revealed that under CIH conditions, miR-205-5p levels were reduced while GAS5 expression was increased. This inverse relationship between miR-205-5p and GAS5 suggests that GAS5 may serve as a sponge for miR-205-5p, consequently affecting the expression of TRIM11. The overexpression of miR-205-5p mimics resulted in a decrease in TRIM11 expression, further supporting the hypothesis that GAS5 and miR-205-5p are involved in the regulation of TRIM11 expression.

Our study demonstrated a positive relationship between myocardial damage caused by OSAHS and the GAS5/miR-205-5p/TRIM11 signaling pathway. The interaction between GAS5, miR-205-5p, and TRIM11 may play an important role in the regulation of apoptosis, inflammation, and cell growth in the context of OSAHS-induced myocardial injury. Our findings contribute to the understanding of the molecular mechanisms underlying OSAHS-induced myocardial injury and provide a basis for the development of novel therapeutic strategies targeting the GAS5/miR-205-5p/TRIM11 axis.

In comparing our findings [8, 10, 14], we note that while the role of lncRNAs in OSAHS-associated cardiomyopathy is emerging, our study is the first to elucidate the CIH-induced upregulation of GAS5 and its downstream effects. Unlike the broader lncRNA profiles discussed [15, 16], our research pinpoints a specific regulatory axis involving miR-205-5p and TRIM11. This novel insight has significant implications for targeted therapeutic strategies. Furthermore, the use of CIH in our cardiomyocyte model advances upon traditional hypoxic studies by more accurately simulating the intermittent hypoxia characteristic of OSAHS [5, 6]. This methodological refinement may explain some of the variance between our results and those of prior studies which did not replicate the intermittent nature of hypoxia. While these unique contributions mark a step forward in understanding OSAHS-related heart damage, they also raise new questions about the cell-type specificity of the GAS5/miR-205-5p/TRIM11 axis, which warrants further investigation.

Future studies should focus on further validating the functional role of the GAS5/miR-205-5p/TRIM11 axis in myocardial injury associated with OSAHS using *in vivo* models, as well as exploring the therapeutic potential of targeting this axis for the treatment of OSAHS-related cardiovascular complications.

Another area of interest is the examination of potential crosstalk between the GAS5/miR-205-5p/TRIM11 axis and other known signaling pathways involved in myocardial damage, such as the AMPK/mTOR, NF-κB, and MAPK pathways. This could offer a deeper understanding of the molecular landscape and reveal additional targets for therapeutic intervention

of OSAHS induced myocardial injury.

Our research delineates the GAS5/miR-205-5p/TRIM11 axis as a pivotal element in the development of myocardial injury related to OSAHS. Future studies should substantiate this axis's *in vivo* functionality, scrutinize its therapeutic promise, and map its interplay with various molecular constituents and signaling cascades. Advancements in this direction could forge new targeted treatments for those suffering from OSAHS with concurrent cardiovascular issues, enhancing patient prognosis and diminishing the health burden imposed by this widespread sleep disorder.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.061.

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Appendix A. Supplementary data

Table S1 Primers used for qRT-PCR.

Gene	Primer
GAS5	F: 5′-ATTGGGTTTTGGTCTGGACA-3′ R: 5′-GCTCTGCCATCAGAATCGTT-3′
miR-205-5P	F: GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACGAACAGA R: TGCGGCCCAGTGTTTAGACTATC
TRIM11	F: 5′-GCCTTCTGTGGCGACGAG-3′ R: 5′-GCATCCTGCATCTGCTTCC-3′
GAPDH	F: 5′-AATCCCATCACCATCTTC-3′ R: 5′-AGGCTGTTGTCATACTTC-3′

qRT-PCR conditions were set as the following: 95 °C for 4 min; 40 cycles of 95 °C for 25 s, 60 °C for 25 s, and 72 °C for 25 s. The result was analyzed using CFX Maestro qPCR Analysis Software (Bio-Rad, USA).