Relationship between IL-1β polymorphisms and obstructive sleep apnea syndrome

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Abstract. – **OBJECTIVE:** To explore the relationship between obstructive sleep apnea syndrome (OSAS) and polymorphism in the IL-1β gene.

PATIENTS AND METHODS: We recruited 164 OSAS patients to the observation group and 146 healthy people to the control group during the same period. Using RFLP (Restriction Fragment Length Polymorphism), we detected higher G/G and C/C rates on locus 154 of the IL-1β gene in the OSAS patients compared with the control group.

RESULTS: Quantitative fluorescence PCR results showed no significant differences in the mRNA expression between the OSAS patients and the healthy people. ELISA results showed that levels of IL-1β in people with the G/G and C/C genotypes were lower than those with the G/C genotype. The frequencies of T/A and T/T on locus 468 of IL-1β was increased in the OSAS patients compared with the control group. ELISA and Western blot results indicated that individuals with the T/T and T/T genotypes at locus 468 had lower expression of IL-1β than those with A/A.

CONCLUSIONS: This observation suggests that IL-1β polymorphisms at 154 and 468 contribute to the incidence of OSAS, possibly, by altering the protein expression of IL-1β.

Key Words:
Obstructive sleep apnea syndrome (OSAS), IL-1β, Gene polymorphism, Relationship, Locus-specific.

Introduction

Obstructive sleep apnea syndrome (OSAS) is a disease characterized by pausing of breathing during sleep[1]. Clinical observations found that the causes of OSAS were complicated and are associated with many factors, such as oral diseases, ENT (ear, nose, throat) diseases, and respiratory diseases[2]. Follow-up surveys indicated that OSAS patients often developed heart and cerebrovas-cular diseases[3], endocrine system diseases, and other respiratory conditions[4]. In another word, OSAS has a significant impact on health. And it has become the focus of research on human sleep quality and the respiratory system. Epidemiological statistics showed that as of 2015, about 13 million people in China suffered from OSAS[5]. Also, surveys of people over the age of 60 showed that up to 46.27% had OSAS[6], indicating that OSAS has become an important disease in the elderly. In recent years, the research found that OSAS was mainly caused by oxygen deficiency resulting from respiratory upper airway collapse. Meanwhile, the main reasons for upper airway collapse were lung diseases and inflammations in the body[7]. Interleukin-1β (IL-1β) played an important role in pulmonary fibrosis[8]; for example, IL-1β had a significantly lower expression in patients with pneumoconiosis than in healthy people[9]. Moreover, IL-1β could activate the immune systems as well as a variety of immune cells to initiate the response to inflammations[10]. Polymorphisms of the *IL-1β* gene have been shown to be related to the pathogenesis of many diseases. For example, the *IL-1β* locus 192 was mainly C/C in healthy people, whereas it was mainly G/G and C/G in patients with colon cancer, suggesting a relationship between *IL-1β* polymorphism and disease risk[11]. Here, we investigated the relationship between OSAS and *IL-1β* polymorphisms to understand the contribution of *IL-1β* to OSAS risk and provide an experimental basis for the treatment of OSAS.

Patients and Methods

Patients

We recruited 164 OSAS patients treated in our hospital from January 2013 to January 2015 as research subjects (observation group). This group had 79 males and 85 females with a mean

age of 57.2 ± 8.3 years old. We also recruited 146 healthy individuals as the control group with 66 males, 80 females and a mean age of 58.2 ± 4.2 years old. Inclusion criteria: (1) Patients were diagnosed with OSAS according to the "Clinical Diagnosis and Treatment of Respiratory Diseases"; (2) Patients had no other diseases; (3) Patients were older than 50 years of age. Exclusion criteria: (1) Patients had other respiratory diseases; (2) Patients suffered from other inflammatory diseases; (3) Patients were younger than 50 years of age. The study was approved by the Ethics Committee of Zhejiang Hospital. Signed written informed consents were obtained from all participants before the study.

Genomic DNA Extraction

The animal genome extraction kit was purchased from AXYGEN (Tewksbury, MA, USA). Cell lysis buffer A was added to 0.2 g of tissue followed by vigorous vortex for 2 min. Lysis buffer B was added and the mixture was slightly mixed for 30 sec. Lysis buffer C was added and, after vigorous vortex, the samples were centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a collection tube and centrifuged at 12,000 rpm for 5 min. The columns were washed with 75% ethanol twice and centrifuged each time for 1 min at 12000 rpm. The genomic DNA was eluted with 50 μl elution buffer and stored at 4°C[12].

PCR-restriction Fragment Length Polymorphism (RFLP) of IL-1β

The ExTaq enzyme, dNTPs, and $6 \times$ buffer were purchased from AXYGEN (Tewksbury, MA, USA). The primers used in this study (Table I) were synthesized by Shanghai Sango (Shanghai, China). The agarose and GODVIEW were purchased from Soledad Biological Technology. The *Eco*R72 and *Not*l restriction enzymes were purchased from Thermo (Waltham, MA, USA). The remaining chemical reagents were purchased from Shanghai Sango (Shanghai, China). The *IL-1β* gene was amplified by PCR. The restriction enzymes *Eco*R72 and *Not*I were used to digest the PCR products on the predicted cleavage sites in the primers[13]. The PCR products were separated by agarose gel electrophoresis and extracted from the gel. Then, the PCR products were introduced into the T-vector. The recombinant plasmids were transformed into DH5α cells and verified by colony PCR. The positive colonies were submitted to Shanghai Sango for sequencing.

Table I. IL-1β primer sequences.

Primer name	Sequences
IL-1β-F	GTCGATGCGCTAGCTAGCATC
IL-1β-R	CGTAGGGATGCTTAGCTAGCTGC

Quantitative Fluorescence PCR

RNA extraction: Total RNAs was extracted from the blood of the observation and control groups. The quantity and quality of the RNA were measured in a spectrophotometer (ABI, USA). Quantitative fluorescence PCR: The mRNA expression of *IL-1β* was detected by quantitative fluorescence PCR. cDNAs were obtained by reverse transcription of RNAs and used as templates. Primers for the quantitative fluorescence PCR are shown in Table II. Quantitative fluorescence PCR machine (ABI, Vernon, CA, USA).

Enzyme-linked Immunosorbent Assay (ELISA)

Protein homogenates from both groups were quantified by the Coomassie blue staining. 2.5 μg of protein from each sample was used for ELISA experiments. 100 μl samples were added to a 96-well plate, and then 35 μl test solution were added. The operations followed the manufacturer's instructions, and the absorbance values were measured at 455 nm in a Multifunctional microplate reader (Bio-Rad, Hercules, CA, USA)[14].

Western Blotting

Samples were stored at -80°C. 150 mg tissues were homogenized in liquid nitrogen. The homogenates were transferred to 1.5 ml tubes. 300 μl protein extraction buffer and 10 μL protease inhibitors were added and the mixture was incubated for 30 min on ice, followed by 12,000 rpm centrifugation for 15 min. The supernatant was collected for the study. Western blotting was used to detect the protein expression. 10 μl supernatant was mixed with loading buffer and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electro-

Table II. Primer sequences for quantitative fluorescence PCR.

Primer name	Sequences
q IL-1β-F	TGCTAGCTGATCGATCGATCGTCG
q IL-1β-R	CGTAGCTGATCGATGCTAGCTAGC
GAPDH-F	TGCTAGGCTAGGACGCTAGCTAC
GAPDH-R	CTGGGCTAGATCGACGAGAGCTC

phoresis (Liuyi, Beijing, China). Then, proteins were transferred to a nylon membrane, and the membrane was blocked for 1 h at room temperature. The incubation with the anti-IL-1β antibody (1: 250) was done at 4°C overnight. After that, the horseradish peroxidase-conjugated secondary antibody (1: 1250) was added and the incubation was done at room temperature for 1 h with shaking. The membrane was washed 3 times before it was developed with diaminobenzidine. The image was taken with the Fluorchem 9900 image system. The relative amount of IL-1β protein was calculated based on the integrated optic density (IOD) values of the protein bands[15].
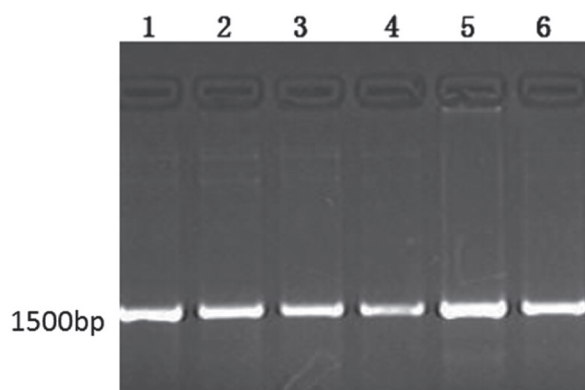
Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post-hoc test (Least Significant Difference). *p*-values < 0.05 were considered statistically significant.
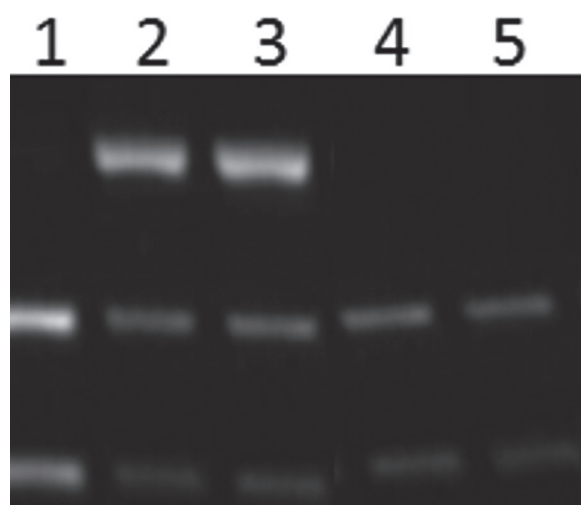
Results

PCR Amplifications and RFLP of Different Genotypes of the IL-1β Gene

The complete sequence of the *IL-1β* gene was amplified by PCR from the genomic DNA extracted from individuals in the observation and control groups. The PCR products were separated by agarose gel electrophoresis, which showed that the size of *IL-1β* was about 1,500 bp (Figure 1). Then, we digested the PCR products with

Figure 2. *Eco*R72 digestion of the IL-1β gene PCR products. Lanes 2, 3: Digestion of the PCR products from healthy people by *Eco*R72. Lanes 1, 4, 5: Digestion of the PCR products from OSAS patients by *Eco*R72.

*Eco*R72 and detected three bands in the healthy population: 750, 300, and 450 bp (Figure 2). In contrast, *Eco*R72 digestion of *IL-1β* from the OSAS patients produced a single band (750 bp) or two bands (300 and 450 bp). Digestion of *IL-1β* from the healthy people with *Not*1 displayed only two bands (500 bp, 400 bp) (Figure 3). The PCR products from the OSAS patients generated three bands (600 bp, 500 bp, 400 bp) or two bands (500 bp, 400 bp).

Sequencing of IL-1β

IL-1β was amplified by PCR from the genomic DNA of healthy and OSAS groups. The PCR products were introduced into the T-19

Figure 1. Gel electrophoresis of the IL-1β gene PCR products. Lane1-3: PCR products from healthy people; 4-6: PCR products from OSAS patients.
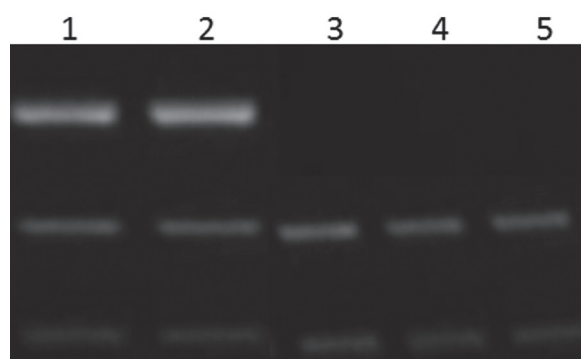

Figure 3. *Not*I digestion of the IL-1β gene PCR products. Lanse1, 2, 3: Digestion of the PCR products from OSAS patients by *Not*I. Lanes 4, 5: Digestion of the PCR products from healthy people.

vector for sequencing. The healthy group had G/C on locus 154, whereas the OSAS group had G/G or C/C genotype on locus 154 (Figure 4). At locus 468, the healthy population had A/A, but the OSAS patients had T/A or A/A (Figure 5). These results suggested the existence of different polymorphisms at loci 154 and 468 of *IL-1β* among the healthy and the OSAS groups.

Statistics of the Allele Distribution in the Two Groups

In the healthy group, the frequency of G/C at locus 154 was 75.4% (Table III). In the OSAS group, the frequency of G/C was only 17.7%, whereas the frequencies of G/G and C/C 40.05 and 42.2%, respectively (Table III). Polymorphism analysis at the locus 468 showed that in the control group dominated A/A genotype with 80.3%, whereas only 12.1% in the OSAS group were A/A (Table IV). Compared with the healthy population, OSAS patients had significantly higher frequencies of the T/A and T/T genotypes (Table IV).

IL-1β mRNA Expression

We used quantitative fluorescence PCR to detect the mRNA expression levels of *IL-1β* from total RNAs extracted from both groups. We detected no significant differences in *IL-1β* mRNA expression between the healthy population and the OSAS patients (Figure 6).

Protein Expression in the Different IL-1β Genotypes

Next, we analyzed IL-1β expression by the sensitive and quantitative ELISA. Then, we scored the values according to the genotype at loci 154 and 468 (Figure 7A). Expression of IL-1β in the patients carrying G/C at 154 was 12.3 µg/l, which was significantly higher than in C/C and G/G (1.52 µg/l both) (Figure 7B). Similarly, expression of IL-1β in the patients carrying A/A at 468 was significantly higher than in A/T and T/T (Figure 7). These results indicated a relationship between the protein expression of IL-1β and genotypes at loci 154 and 468, with the typical genotypes in OSAS patients showing significant decrease in IL-1β expression.

IL-1β Expression by Western Blot

Finally, we extracted total protein from both groups and analyzed the expression of IL-1β by Western blot. Surprisingly, the OSAS group had significantly lower level of IL-1β compared to the healthy group (Figure 8). This result was different from the mRNA expression analysis, suggesting that the differences between the groups are due to the posttranslational regulation of IL-1β translation or stability.

Discussion

OSAS is a multifactorial disease with broad effect on the human body. For example, follow-up

Table III. Statistics of the genotypes on locus 154.

| Group | Number (N) | Genotype frequency (%) | | | χ^2 | *p* |
		CC	CG	GG		
Control	146	19.5	75.4	5.1	16.054	0.000
Observation	164	42.2	17.7	40.05		

Table IV. Statistics of the genotypes on locus 468

| Group | Number (N) | Genotype frequency (%) | | | χ^2 | *p* |
		TT	TA	AA		
Control	146	12.8	6.9	80.3	16.054	0.000
Observation	164	39.6	48.3	12.1		
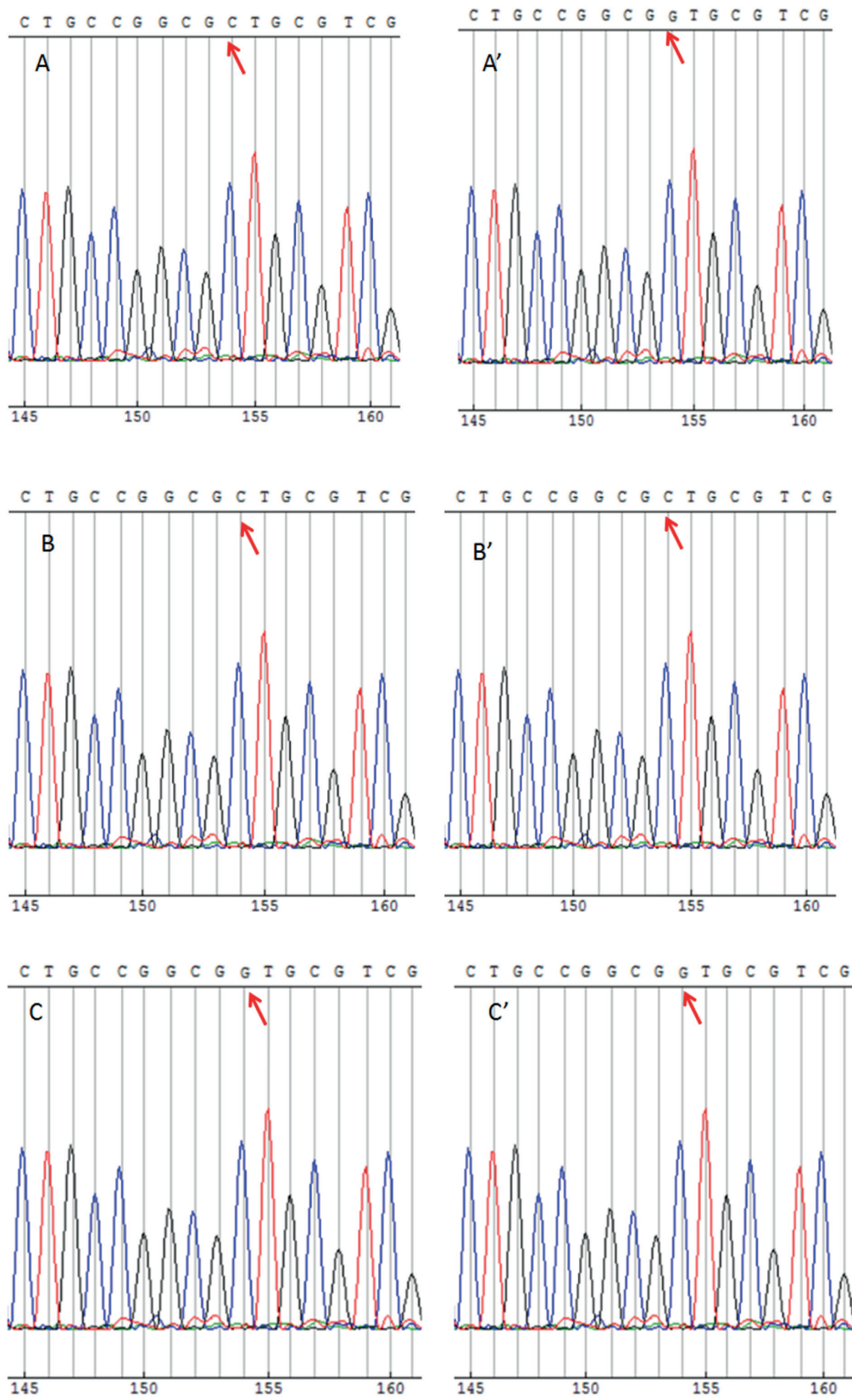
Figure 4. Sequencing results showing polymorphism of locus 154 of IL-1β. **A-A′,** Sequencing results of the IL-1β PCR products from healthy people. **C-D′**, Sequencing results of the IL-1β PCR products from OSAS patients.
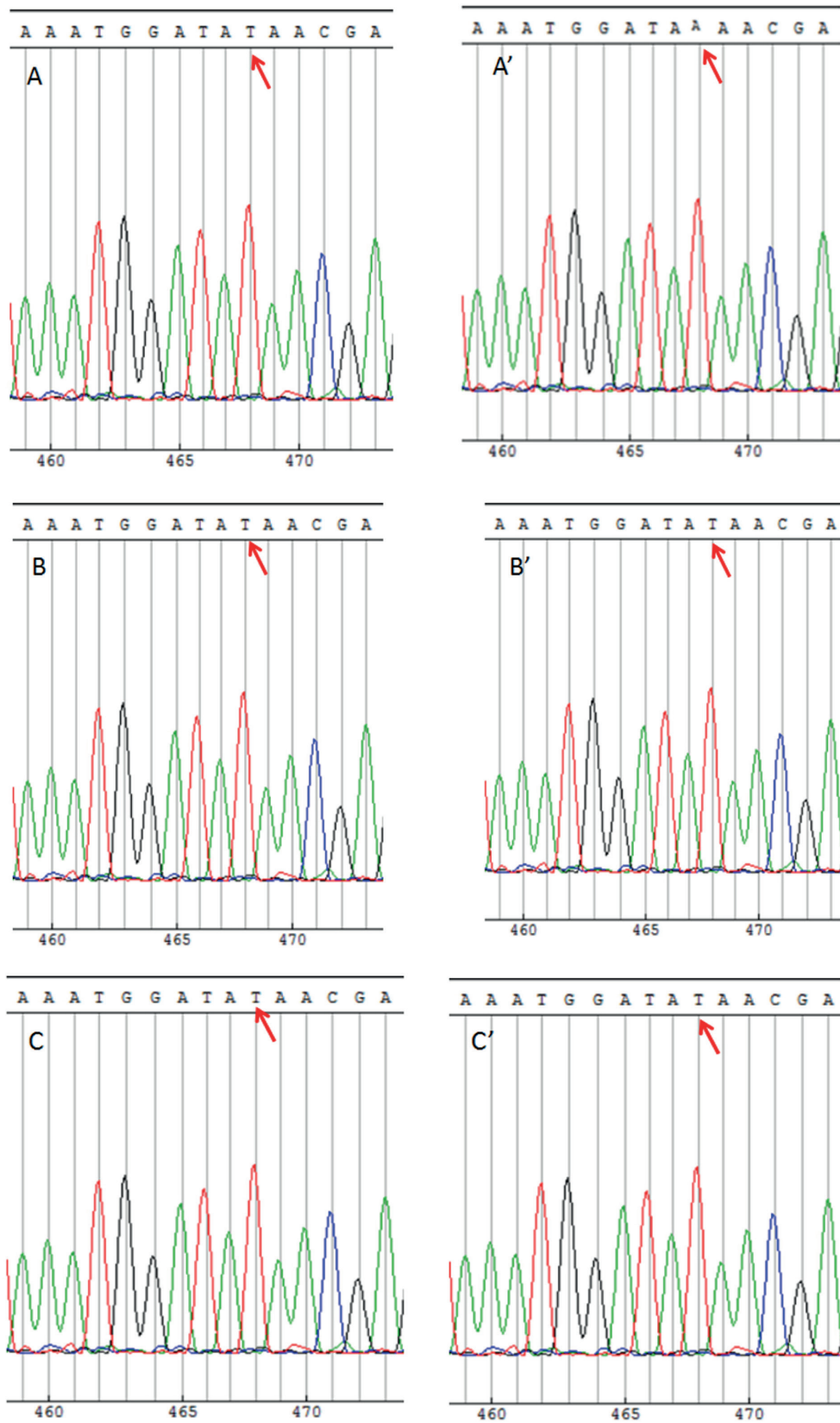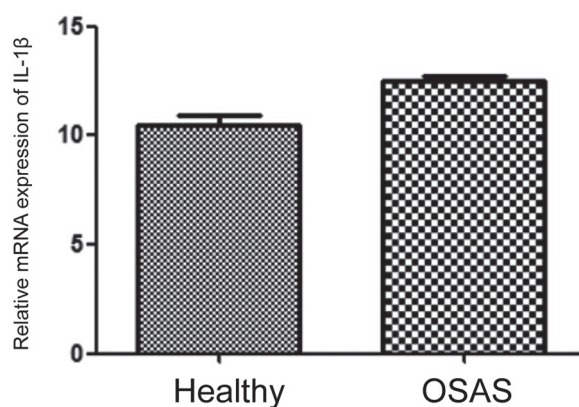
Figure 5. Sequencing results showing polymorphism of the locus 468 of IL-1β. ***A-A′.*** Sequencing results of the IL-1β PCR products from healthy people. ***C-C′***, ***D-D′*** : Sequencing results of the IL-1β PCR products from OSAS patients.
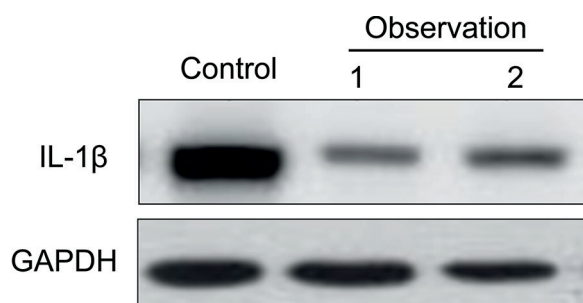
Figure 6. Quantitative fluorescence PCR results of the different genotypes of IL-1β.

A

B
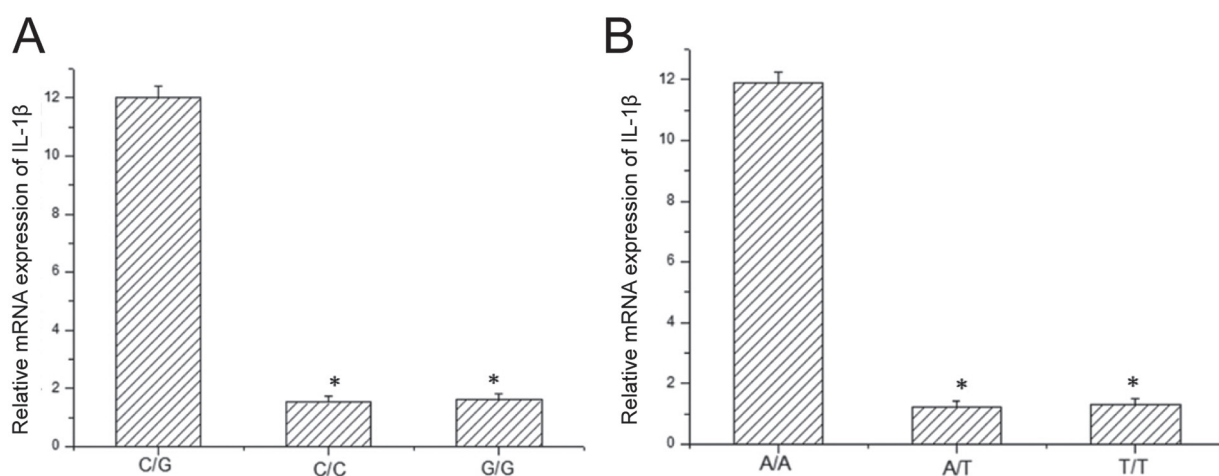
Figure 7. Protein expression of the different genotypes of IL-1β. **A,** Protein expression of IL-1β genes with polymorphism on the locus 154. **B,** Protein expression of IL-1β genes with polymorphism on the locus 468. *Indicated significant difference.

surveys found that patients with OSAS had higher risks of respiratory diseases and cardiovascular diseases[16,17]. Further, research of cardiovascular, cerebrovascular, and other diseases found that they could also lead to OSAS. Major clinical symptoms of OSAS were nocturnal snoring, awakenings and daytime sleepiness[18]. Also, varying degrees of respiratory obstruction occurred at night, often accompanied by poor nighttime respiration or even death. Recent studies have found[19] that expression of tumor necrosis factor-α (TNF-α) was significantly higher in OSAS patients than in healthy people. TNF-α prevents tumor cell proliferation by inhibiting cell metabolism[20] and chromosome replication, and limiting energy supply. Thus, it was suspected that the higher TNF-α expression in OSAS patients also inhibited cellular energy metabolism, that is, inhibiting cell oxygen intake. Clinical findings indicated that OSAS patients usually also had

varying degrees of inflammation[21]. For example, the occurrence of upper respiratory diseases and nasal inflammations in patients suffering from OSAS was significantly higher than that

Figure 8. Western blotting results of the different genotypes of IL-1β gene. Observation 1: Protein expression of IL-1β genes with polymorphism on the locus 154. Observation 2: Protein expression of IL-1β genes with polymorphism on the locus 468.

in healthy people. Studies in recent years have shown that IL-1β was critical in the development of inflammation. IL-1β could prevent the worsening of inflammations by activating the immune system and activating macrophages to engulf inflammatory cells. IL-1β contributed to the elimination of inflammatory responses and the maintenance of homeostasis. Researchers also found that hypoxia would lead to different levels of oxidative stress, while excessive reactive oxygen would induce inflammation by damaging cell membranes and organelle membranes. Currently, it was proposed that inflammatory responses and OSAS were related. In this study, we investigated the relationship between OSAS and polymorphisms in *IL-1β*.

Conclusions

Our results indicated that the protein expression level of IL-1β was significantly lower in the OSAS patients than in the healthy group. Sequencing results of samples from different individuals discovered that polymorphisms on the loci 154 and 486 significantly increased the risks of OSAS and affected IL-1β protein expression. However, we did not conduct in-depth research to understand the underlying mechanisms of the interactions between OSAS and IL-1β, which will be the focus of future studies.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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