ABSTRACT

Aim To investigate the relationship of IL-1β and IL-6 cytokine gene polymorphisms with obstructive sleep apnea syndrome (OSAS) in 61 patients admitted to the neurology clinic in Kafkas University Hospital with insomnia problem who were diagnosed with OSAS in sleeping labs, and 80 healthy subjects not associated with the syndrome.

Methods Blood samples were taken to isolate DNA from patients diagnosed with OSAS based on polysomnography results and healthy controls. DNA amplification of the genes was performed with PCR. Amplification products were cut with the restriction enzymes in order to determine IL-1 gene (TaqI) and IL-6 gene (Lwel) polymorphisms. The cut DNA fragments were carried out in agarose gel electrophoresis, and RFLP analysis was performed by utilizing the images with gel imaging system. PCR products were sequenced with an Applied Biosystems Automated Sequencer.

Results Polymorphic changes were observed for IL-1β gene in 26 of 62 patients (41.9%), and 16 of the 80 (25.8%) in the control group. The incidence of polymorphic changes in IL-6 gene was seen in seven (of the 62 patients) (11.3%), and in the 16 (20%) controls.

Conclusion The findings on the genomic level in OSAS may provide an important contribution to diagnosis of obstructive sleep apnea syndrome in clinical practice, as well as it helps to obtain the results easily about environmental and genetic interaction of OSAS patients.

Key words: OSAS, cytokine genes, RFLP, Turkey
INTRODUCTION

Sleep is an essential part of health and sleep-breathing disorders can cause serious health problems, economic losses both social and personal. The most common type of sleep apnea is defined as obstructive disorders (1). Obstructive sleep apnea syndrome (OSAS) disease constitutes a risk in people with certain anatomical features such as obesity, advanced age, gender and short neck (2). Daytime sleepiness, snoring, airway obstruction are the main findings of apnea (3). Sleep apnea is a risk for the development of many systemic diseases and is involved in the etiopathogenesis of various cardiovascular and disease progression (3). Obstructive sleep apnea syndrome and obesity are disorders common in individuals (2-3). Disorders associated with obesity have become some of the most serious social problems that triggered the formation of OSAS in recent years. Obstructive sleep apnea syndrome is a disease characterized in the upper respiratory tract in adults and in middle-aged individuals; it often leads to hypoxia and affects the sympathetic nervous system, metabolic reactions may also lead to an increase in blood pressure (3-4). As the obesity is a multifactorial disease, a significant relationship between genetic factors, obesity and OSAS has been defined (5). In OSAS, the reason of the airway obstruction is that the soft tissue slumps down the trachea during sleep. In each apnea event, enough oxygen does not get to the brain and sleep of persons with syndrome is broken down due to suffocation, and sleep is of poor quality and fragmented (6). Obstructive sleep apnea syndrome, in general, is common in overweight adults, in patients with diabetes. Risk factors include male gender, obesity, and age. Sleep apnea may be seen at any age, or even in children (6-7).

Cytokine genes of interleukin-1β (IL-1β) are shown physiological insomnia roles (6). IL-1 concentration in the cells plasma of healthy human was assumed to be the highest during awakenings and infusion and at the beginning of sleep (7). The most stable IL-1β levels in plasma with cytokine cycle variation can affect patients with narcolepsy, and interleukin-6 (IL-6) and hypersomnia (3-4). The significant increases in cytokine concentrations can be measured by OSAS and narcolepsy, and average sleep having correlation with the intensity of sleep, sleep can be measured as latency. It is associated with IL-6 levels and body mass index (BMI) (8-10). Obesity is one of the serious consequences leading to uneven course of sleep. This interaction can lead to increases in inflammatory cytokines (9). IL-1β and IL-6 genes can alter the levels of protein synthesis. IL-6 functions are known as lipid metabolism and energy expenditure (11). Polymorphism in the promoter region of the IL-6 gene influences the level of 174 (G174C) interleukin 6. Also in the arrangement of body mass interleukin -1β is known to be effective (12). The gold standard method in the diagnosis of sleep disordered breathing is the polysomnography method (10). Currently, the connection of OSAS syndromes with genetics is investigated (12). In our study, the genomic aspects of IL-1β and IL-6 gene polymorphisms from cytokine genes in patients with obstructive sleep syndrome were examined. The most common form of polymorphism is the single nucleotide polymorphism consisting of a single base pair change in genomic DNA. Allelic and genotypic distribution analysis of cytokine genes were evaluated in individuals with obesity and individuals with sleep apnea syndromes visiting outpatient clinics. The aim of the study was to evaluate the clinical importance of cytokine genes which have been found to be linked with OSAS. In this sense, genotypic studies polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) method was used. PCR products were sequenced with an Applied Biosystems Automated Sequencer.

PATIENTS AND METHODS

Patients and controls

During three years (2011-2014) 1000 people having problems of sleepiness disease reported to the sleep laboratory in Kafkas University, Turkey. Patients diagnosed with OSAS were selected for the research by the results of polysomnography (PSG), and were studied at the sleep laboratory at Kafkas University Medicine Faculty Research and Application Hospital (apnoea hypopnoea index, AHI of < 5-30). A control group of 80 voluntarily subjects with an
apnoea hypopnoea index (AHI) <5 were selected randomly (general population without any classification). The population in this region shows trends toward obesity resulting from poor nutrition habits and the long winter season. In addition, sleep disease diagnoses in the region exceed the country’s average (p<0.05). For the patients and controls an ethical approval was received from the Medical Ethics Committee of Ataturk University (Erzurum).

**Polysomnography**

Full-night polysomnographic recording was performed using an Embla N7000 system (Medcare; Reykjavik, Iceland). The following parameters were recorded: electroencephalography, electrocardiography, electrooculography, submental and anterior tibialis muscle electromyography, nasal pressure, oronasal airflow by thermal sensor, snoring, oxygen saturation by finger oxymeter, and respiratory effort by thoracic and abdominal inductance plethysmography (13). Sleep disordered breathing events were scored manually by the same investigator, according to the American Academy of Sleep Medicine criteria (14). Obstructive apnea was defined as a drop in the peak oronasal thermal sensor excursion by ≥90% from baseline for at least 10 s. Hypopnea was defined as at least a 50% drop in airflow for at least 10 s despite respiratory efforts and at least a 3% decrease in oxyhemoglobin saturation. Patients were diagnosed with OSAS if the AHI was ≥5. Grading was conducted according to mild OSAS with an AHI of 5-14, moderate OSAS with an AHI of 15-29, and severe OSAS with an AHI ≤30 (13-14). The lowest O2 saturation value was measured throughout the night for each patient. Polysomnographic results of the patients and controls were recorded in the hospital sleep laboratory using the 24-hour duration as a baseline with the laboratory PSG system. The heights of the patients and controls were measured in cm, while weights and body mass index (BMI) values were calculated in kg/m².

**Determination of genotypes the Interleukin-6 and Interleukin-1**

For genotyping 10 mL blood samples were taken from each patient and the control group into tubes with EDTA. DNA was extracted using commercially available Invitrogen genomic DNA extraction mini kits (CS11010, London, UK) DNA purification kit according to the manufacturer’s instructions. The isolated DNA samples were measured at the Nanodrop Spectrophotometer (Thermo ND1000 Wilmington, USA) and kept at -20 °C.

The primers used to identify Interleukin-6 (IL-6) were F: 5’-TGA CTT CAG CTT TAC TCT TGT T-3 and R: 5’-CTC AGG TGT CCT CGAAGAAAT CAAA-3’ the Interleukin-1β genes (IL-1β) specific primers were: F 5’-GCT TTT TTG CTG TGA GTC CCG-3’ and R 5’-CTC AGG TGT CCT CGAAGAAAT CAAA-3’ (15). A final volume 25 μL PCR protocol that included 2.5 μL Taq polymerase buffer solution, 2.5 μL magnesium chloride (2 mM), 2 μL dNTP mix (0.2 mM), 1 μL forward primer (10 pmol), 1 μL reverse primer (10 pmol), 2 μL genomic DNA (100 ng/μL), 1 μL DNA taq polymerase enzyme (5 u/μL), and 13 μL distilled water; a total volume of 25 μL was used, PCR conditions were as follows: an initial denaturation for 5 min at 94 °C, then 35 cycles at 94°C for 45 s, at 63°C for 45 s, at 72°C for 55 s, and a final extension at 1 cycle 72°C for 7 min. The PCR products were detected by agarose gel electrophoresis (at 90V, 300 A for 1.5 h) on 2% agarose gel containing ethidium bromide, and the fluorescent intensity of each band was evaluated with a UV transilluminator (Gel Logic Pro 2200, Montreal, Canada) (16). For the Interleukin-1β polymorphism, the PCR amplification bands were observed as 195 bp, and the Interleukin-6 amplification bands as 198 bp. Amplified products were digested: Interleukin-1β with 5U Thermus aquaticus (TaqI), and Interleukin-6 was digested with 5U Listeria welshimeri (LwelI) (New England Biolabs, INC UK) (17). Digestion products were visualized, and resulting fragments were separated on 2.5% agarose gel and with ethidium bromide staining under ultraviolet illumination (Gel Logic Pro 2200, Canada). The single amplicon of 195 bp as a result of the section of the Interleukin-1β polymorphism with the restriction enzyme (TaqI) was separated into two DNA fragments as 110 bp and 85 bp. As a result of the section of Interleukin-6 with enzyme (LwelI), the 198 bp
bands were observed as having 110 bp + 88bp. The PCR products were then isolated using agarose gel electrophoresis (15-18).

All of the genomic analyses were conducted in the laboratory of molecular genetics in Department of Bioengineering, Kafkas University. In addition, Bigdye Cycle Sequencing kit v.3.1, Applied Biosystems and approximately 5 μL true of PCR products were sequenced with an Applied Biosystems Automated Sequencer (ABI 3130 XL Genetic Analyzer, Foster City, CA 94404 USA). Restrictions mapping and SNP bioinformatic analysis was done as Vector NTI Software (Life Technologies).

Statistical analysis

For each polymorphism, deviation of the genotype frequencies in the controls from those expected under Hardy-Weinberg equilibrium was assessed using the standard χ2 test. Genotype frequencies in cases and controls were compared by χ2 tests. The genotypic-specific risks were estimated as odds ratios (ORs) with associated 95% intervals (CIs) by unconditional logistic regression. p<0.05 was considered to be significant.

RESULTS

The patients diagnosed with the OSAS disease from Kafkas University, School of Medicine Neurology Clinic, between August 2011- July 2014, were examined according to polysomnography data (Table 1).

Table 1. Demographic characteristic of obstructive sleep apnea syndrome (OSAS) patients and control group

<table>
<thead>
<tr>
<th>Age</th>
<th>OSAS patients (n=62)</th>
<th>Control group (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>25-40</td>
<td>3 (9.6)</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>41-60</td>
<td>10 (32)</td>
<td>10 (32.6)</td>
</tr>
<tr>
<td>&gt;61</td>
<td>16 (59)</td>
<td>16 (58.1)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (50)</td>
<td>31 (50)</td>
</tr>
</tbody>
</table>

The percentage of OSAS patients having BMI more than 30 kg/m² was 66% (41 out of 62), 23 (74.1%) females and 18 (58%) males. In the control group, 42.5% (34 out of 80) of examinees had BMI less than 25 kg/m². Patients with OSAS mostly had AHI >30, 32 (51.6%), of which 14 (45.16%) were females and 18 (58.07%) males (Table 2).

Table 2. Distribution of patients with obstructive sleep apnea syndrome (OSAS) and control group according to body mass index (BMI) and apnoea hypopnoea index (AHI)

<table>
<thead>
<tr>
<th>BMI</th>
<th>OSAS patients (n=62)</th>
<th>Control group (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>28-30</td>
<td>8 (25.8)</td>
<td>13 (41.9)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>23 (74.1)</td>
<td>18 (58.0)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (50)</td>
<td>31 (50)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AHI</th>
<th>OSAS patients (n=62)</th>
<th>Control group (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>5-14</td>
<td>7 (22.58)</td>
<td>0</td>
</tr>
<tr>
<td>(medium)</td>
<td>10 (32.25)</td>
<td>13 (41.93)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>14 (45.16)</td>
<td>18 (58.07)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (50)</td>
<td>31 (50)</td>
</tr>
</tbody>
</table>

There were some similarities between males and females in terms of frequency of gene polymorphism. An increase of BMI resulted in more IL-1β gene polymorphism change. Genotype of IL-1β gene polymorphism showed CC genotype in nine (14.5%), CT in 26 (41.9%), TT in 27 (43.5%) in OSAS patients, and CC in eight (10%), CT in 16 (20%) and TT genotype in 56 (70%) controls. In patients IL-1β polymorphic change rate was observed in 26 of 62 patients (41.9%), and the same polymorphic change rate was observed in 16 of 80 (25.8%) controls. The prevalence of polymorphic changes in IL-6 gene was 11.3% (seven of the 62) patients, and 20% (16 of 80) controls (p<0.05 for IL-1B C / T and IL-6 G174C polymorphic regions of cytokines) (Table 3).

Table 3. Genotypic and allelic results of IL-1β and IL-6 gene polymorphisms in the obstructive sleep apnea syndrome (OSAS) patients and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Interleukin-1β</th>
<th>OSAS (n=62)</th>
<th>Control Group (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequencies of genotypes</td>
<td>9</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Alleles</td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Frequencies of alleles</td>
<td>22</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>Genotypes</td>
<td>GG</td>
<td>CC</td>
</tr>
<tr>
<td>Frequencies of genotypes</td>
<td>48</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Alleles</td>
<td>G</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>Frequencies of alleles</td>
<td>51</td>
<td>11</td>
<td>64</td>
</tr>
<tr>
<td>Alleles</td>
<td>82.3 (17.7)</td>
<td>80 (20)</td>
<td></td>
</tr>
</tbody>
</table>
The most important result of DNA sequence of IL-1β gene proved restriction enzymes (TaqI) cleavage sites for both patients and control group (Figure 1).

The most important result of DNA sequence of IL-6 gene was shown as base pair’s changes from GG in the control group to GC and CC in the OSAS patients, in other words SNP. Furthermore, IL-6 gene restriction enzymes (Lwe I) cleavage sites were proven for both patients and control group after sequence resulting. In the SNP regions and restriction enzyme (Lwe I) cleavage site were detected and are given in Figure 2.

Figure 1. Sequencing IL-1β gene; A) the restriction enzyme cleavage sites are provided in control individuals (TaqI); B restriction enzyme cleavage site is provided in obstructive sleep apnea syndrome (OSAS) patients as sequencing results (MvaI) (Gok I, 2014)

Figure 2. In the IL-6 gene sequencing results: A) SNP region is shown normal (T/T) in control individuals; B) and C) sequencing result of obstructive sleep apnea syndrome (OSAS) patients (SNP) region the change (G/T) is provided (Gok I, 2014)
DISCUSSION

In various countries around the world, OSAS is different in terms of clinical signs and the prevalence of incidence is increasing between 40-65 years. Generally, the disease in populations was found to be 4% in males and 2% in females (5,19). There is sleep apnea syndrome in approximately 0.9-1.9% of Turkey’s population (20-21). Yang et al. in 2013 in China gave researches importance of its genetic aspects; until now many genes and polymorphism region related to the disease have been identified (22). The diversity of genes which are responsible for the formation of respiratory-related diseases and of polymorphic region located in this gene was expected to be made a criterion for the diagnosis of OSAS and genomic studies were carried out. In addition, an increase of BMI results in a change in cytokine gene polymorphisms (17,23). In this study, according to polysomnography results in the population of Northern Anatolia, IL-1β, IL-6 polymorphism genotypes distribution was investigated in individuals diagnosed with OSAS: 87% of patients with OSAS included in the study were over 40 years of age, and 66% of the cases with BMI >30 were aged above 30 years, and our results support this relationship.

In the study by Popko et al. in 2008 in Poland it was found that IL-1β polymorphism was a risk factor for obesity, IL-6 polymorphism was associated with interleukin receptor function in carrying the C allele, but there was no significant relationship with obesity in a group of 140 women, 50% of which had BMI <30 kg/m² (others had MBI >25 kg/m²(15)). Our research is not in compliance with the research of Popko et al. regarding IL-6 polymorphism, however it correlates with IL-1β polymorphism.

Riha et al. in 2009 in Germany and the UK, investigated IL-1 polymorphism in normal, overweight and obese man and women, and they found that polymorphisms of IL-1β can be effective on the development of obesity in European society (8). In other study, Arnardottir et al. in 2012 in Iceland, confirmed that the BMI increase resulted in IL-6 gene polymorphism change, and obesity and other respiratory disease triggers of the OSAS (23).

In this study we observed an increase in body mass index of those bearing the genotypes of IL-6 and alleles of IL-1β; BMI values of 41 of 62 individuals (66%) with OSAS whose two polymorphisms in the IL-6 and IL-1β gene were examined in our study were observed of greater than 30. Our findings are similar to the results of the Mannarino et al. (14). In a study conducted by Buck and colleagues in 2010 for the IL-6 gene in Northern Europe, authors have found no relationship between IL-6 genotype and obesity (12). In this research that was carried out for North East Anatolia region, the IL-6 gene was not associated with obesity, which is in line with the findings of Buck et al. In research conducted in various European countries in a similar manner, the effect of IL-6 gene was found to be associated with metabolism and energy consumption (24-25).

In our study, we suggested that IL-6 gene polymorphism had no direct relationship with OSAS, but if the BMI is greater than 30 in patients with OSAS and controls, it may increase the risk of obesity. A changing of the balance of IL-1β gene polymorphism could induce metabolism, and environmental interactions of genes may trigger the formation of OSAS (15). There is a number of genetic variants of this gene polymorphism affecting the OSAS progress of obesity or vice versa, as a cause there are or there will be probably susceptibility variants affecting the development of obesity of OSAS but also affecting pleiotropism. Identification of such genes and understanding their function will contribute to new perspectives in the partners pathogenesis of these diseases. Finally, saying that genetic polymorphisms may influence susceptibility to each disease may not be very accurate; the possibility should not be ignored. Similarly, the use of such a model with information about the genetic aspects of obesity will be able to allow for a more comprehensive way to understand how OSAS can affect the obesity.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.
REFERENCES


