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FREQUENCY OF DISTRIBUTION OF INFLAMMATORY CYTOKINES IL-1, IL-6 AND TNF-α GENE POLYMORPHISM IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA

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Obesity is one of the most commonly identified factors for the obstructive sleep apnea syndrome (OSAS). Adipose tissue is the source of many cytokines, among them there are IL-6, IL-1, and TNF-α. The level of inflammatory cytokines increases in people with OSAS and obesity. The aim of this study was to evaluate the distribution of genotypes in inflammatory cytokine genes in people with obesity-related OSAS. The examined group consisted of 102 person with obesity related-OSAS and 77 normal weight person without OSAS. Genotyping of DNA sequence variation was carried out by restriction enzyme (IL-1: Taq I, IL-6: Lwe I, TNF-α: Nco I) analysis of PCR amplified DNA. The study revealed a significant correlation between polymorphism located in the promoter region of inflammatory cytokine genes and obesity-related OSAS.

Keywords: *inflammatory cytokines, IL-6, IL-1, TNF-α, gene polymorphism, obesity, OSAS*

INTRODUCTION

Obesity-related disorders became one of the most serious social problems, reaching a form of civilization-related disease. Obesity is a multifactor disease in which genetic factors play an important role. These factors account for up to 40% of causes leading to obesity. There are a great number of genes affecting food intake and energy expenditure (1, 2).

One of the consequences of obesity is the obstructive sleep apnea syndrome (OSAS). OSAS is caused by blockage of the airway, usually when the soft tissue in the rear of the throat collapses during sleep. With each apnea event, the brain briefly arouses people with OSAS in order to resume breathing, but, consequently, sleep is fragmented and of poor quality. Sleep apnea is as common as is adult diabetes. Risk factors include male gender, overweight (3), and the age over forty, but sleep apnea can strike anyone at any age, even children (4). Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) have been shown to modulate physiological sleep. Plasma IL-1 concentration is highest at the onset of sleep in humans suggesting a sleep wake cycle variation of this cytokine (5). Vgontzas *et al* (6) determined the plasma levels of IL-1, TNF-α, and IL-6 in patients with OSAS, narcolepsy, and hypersomnia. The concentration of TNF- was significantly elevated in patients with OSAS and narcolepsy, and it correlated with the intensity of sleepiness measured as the mean nap sleep latency. IL-6 levels were elevated only in OSAS and correlated with body mass index (6, 7).

Serious consequences accompanying obesity and leading to the development of OSAS may be caused by increased level of inflammatory cytokines, such as IL-1, IL-6, and TNF-α. It is possible that polymorphisms located in cytokines' genes affect the level of protein expression. It is known that IL-6 plays a role in lipid metabolism and energy expenditure (8, 9). The polymorphism found in point 174 (G174C) of a promoter region of IL-6 gene affects the level of interleukin 6 expression (10). A similar role in adipose tissue metabolism plays TNF-α. The level of it depends on the presence of polymorphism G308A in the promoter region of TNF-α gene. The presence of the A allele provokes a double increase of TNF-α gene expression and leads to higher TNF-α production (11, 12). Another inflammatory cytokine with a great impact on body mass regulation is interleukin 1 (IL-1). Similarly, to IL-6 and TNF- α, the influence of IL-1 on lipid metabolism and energy expenditure seems connected with the concentration of this cytokine. The presence of T allele in a homozygotic set causes four-fold increase of IL-1β production (13, 14).

The purpose of the present study was to evaluate the frequency of IL-1, IL-6, TNF-α gene polymorphism in OSAS adults with overweight (BMI>25) or obesity (BMI>30).

MATERIAL AND METHODS

Written informed consent was obtained from all patients and controls during the enrollment visit. The study protocol was approved by the Warsaw University Medical Ethics Committee in Warsaw, Poland.

The study group consisted of 102 patients (74 men and 28 women) (body mass index, BMI>25 kg/m^2), aged 21-77, with newly diagnosed OSAS (apnea-hypopnea index, AHI≥5) and 77 non-apneic controls (AHI <5) aged 18-65 (39 men and 38 women) and BMI-matched. To confirm the

diagnosis, all patients underwent standard polysomnography in the sleep disorder center. All patients were referred to the Lung Diseases Clinic of the Institute of Tuberculosis and Lung Diseases in Warsaw, Poland.

PCR assay

Genomic DNA was isolated using Genomic Midi AX isolation kit with ion-exchange membranes (A&A Biotechnology, Gdynia, Poland). A 1 ml blood sample was diluted with saline in v/v 1:1. After 30 min incubation at 50°C with lysing buffer and proteinase K solution, the mixture was vortexed, placed in the column, and centrifuged. The column was washed twice with buffer and transferred into a new tube. The elution buffer was added and centrifugation at 4000 rpm was applied. This step was repeated twice. The sample was centrifuged for 2 min with isopropanol. Supernatant was removed and the pellet was washed with ethanol. After centrifugation, supernatant was removed and the pellet was air-dried for 10 min. Afterward, pure DNA was dissolved in 200 µl of sterile water.

Genotyping was done using a PCR-restriction fragment length polymorphism analyses. Amplification was carried out in a 50 µl volume containing 300 ng genomic DNA, 0.1 µM of each primer (DNA-Gdansk, Gdansk, Poland):

Interleukin 1-beta (IL-1B+T3954C)

- for 5'- GCT TTT TTG CTG TGA GTC CCG-3'
  rev 5'-CTC AGG TGT CCT CGA AGA AAT CAA A-3'
- Interleukin 6 (IL-6 C174G)
  for 5'TGA CTT CAG CTT TAC TCT TTG T-3'
  rev 5'CTG ATT GGA AAC CTT ATT AAG-3'
- TNF-α (TNF-α A308T)
  for 5'- TCC TCC CTG CTC CGA TTC CG-3'
  rev 5'- AGG CAA TAG GTT TTG AGG GCC AT-3',

200 µM of each dNTP (DNA-Gdansk, Gdansk, Poland), 3 mM of magnesium chloride, and 0.2 U of Taq Gold polymerase (Applied Biosystems, Warrington, UK). Thirty five cycles were conducted in a thermocycler, Mastercycler personal (Eppendorf, Hamburg, Germany). The amplified PCR products were digested with the addition of appropriate enzymes. The digested samples were separated by electrophoresis on a 2% agarose gel.

Frequency of distribution analysis was performed with a Chi^2 square test. Statistical significance was accepted at $P<0.05$.

RESULTS

Genotype distribution and G308A allele frequency in controls and OSAS patients are shown in *Table 1*. Homozygotic A/A genotype was not found in the examined population. Statistical analysis did not show any significant difference in the frequency of distribution of G/G and G/A genotypes in the OSAS patients compared with the control group. However, 308A allele was more common in the OSAS than in control individuals; the difference reached 5-7%.

Genotype distribution and allele frequency of the G174C polymorphism in IL-6 gene differed between the OSAS patients and control subjects (*Table 2*). Genetic analyses revealed the more frequent presence of C allele (in homozygotic C/C and heterozygotic G/C sets) in the OSAS patients, analyzed as a whole

Table 1. Frequency of polymorphism G308A in TNF-α gene in OSAS patients and in the control group.

TNF-α	OSAS whole group		Control group		P
	n	%	n	%	
G/G	73	71.6	59	76.6	
G/A	29	28.4	18	23.4	0.4
TNF-α	OSAS females		Control females		P
G/G	19	67.9	28	73.7	
G/A	9	32.1	10	26,3	0.6
TNF-α	OSAS males		Control males		P
G/G	54	73	31	79.5	
G/A	20	27	8	20.5	0.44

group, compared with the controls. Significant gender differences were also observed regarding the C and G alleles of the IL-6 genotype in the group of men, but not women (*Table 2*).

No differences were observed in the frequency of IL-1 (C3954T) genotype distribution. The presence of T and C alleles was similar in the population of OSAS patients and control subjects studied (*Table 3*).

DISCUSSION

OSAS appears to result from inflammatory state with increased levels of IL-1, IL-6, and TNF-α. Cytokine level may be dependent on polymorphic changes in related genes. The results of this study support the hypothesis about the relationship between the IL-6 genotype and OSAS. The C-containing genotypes (C/C or G/C) were more frequently found in OSAS patients than in control subjects. The influence of G174C polymorphism on sleep regulation may be caused by the allele influence on the cytokine expression rate (10). The amount of IL-6 in circulation is regulated at the level of gene expression. Transcription of IL-6 DNA is tightly regulated by many factors, such as NFIL-6, NF-κB, Fos/Jun CRBP, and glucocorticoid receptors (15, 16). It is suggested that part of the promoter region 180 to 123 plays a key role in transcription induction by viruses, IL-1, TNF-α, PDGF, and EGF. Activation of the IL-6 promoter involves synergism between the transcription factors NFIL-6 (158 to 145) and NF-κB (73 to 64). Additionally, the region 225 to 164 containing the G174C polymorphism has been reported to demonstrate a negative regulatory effect on gene expression (16, 17).

Studies on the repression of the IL-6 promoter demonstrate binding of the glucocorticoid receptor to a region around -201. The G174C polymorphism seems located close enough to this side, may influence binding of the glucocorticoid receptor, and may affect its ability to repress transcriptional activation (17). It is also possible that the change of G into C creates the potential

Table 2. Frequency of polymorphism G174C in IL-6 gene in OSAS patients and in the control group.

Genotype IL-6	OSAS whole group		Control group		P
	n	%	n	%	
G/G	20	19.6	24	31,2	0.1
G/C	50	49	36	46,7	
C/C	32	31.4	17	22,1	
G/G+G/C	70	68.6	60	77,9	0.1
C/C	32	31.4	17	22,1	
C/C+G/C	82	80.4	53	68,8	0.07
G/G	20	19.6	24	31,2	
Genotype IL-6	OSAS females		Control females		P
G/G	6	21,4	9	23,7	0.4
G/C	16	57,2	16	42,1	
C/C	6	21,4	13	34.2	
G/G+G/C	22	78,6	25	65.8	0.2
C/C	6	21,4	13	34.2	
C/C+G/C	22	78.6	29	76.3	0.8
G/G	6	21.4	9	23.7	
Genotype IL-6	OSAS males		Control males		P
G/G	14	19	15	38.5	0.007
G/C	34	45.9	20	51.5	
C/C	26	35.1	4	10.2	
G/G+G/C	48	64.9	35	89.7	0.004
C/C	26	35.1	4	10.3	
C/C+G/C	60	81	24	61.5	0.02
G/G	14	19	15	38.5	

Table 3. Frequency of polymorphism C3954T in IL-1 gene in OSAS patients and in the control group.

Genotype IL-1	OSAS whole group		Control group		P
	n	%	n	%	
T/T+C/T	40	39.2	30	39	0.9
C/C	62	60.8	47	61	
Genotype IL-1	OSAS females		Control females		P
	n	%	n	%	
T/T+C/T	11	39.3	13	34.2	0.6
C/C	17	60.7	25	65.8	
Genotype IL-1	OSAS males		Control males		P
	n	%	n	%	
T/T+C/T	29	39.2	17	43.6	0.6
C/C	45	60.8	22	56.4	

binding side for the transcription factor NF-1. This factor has inhibitory effects on IL-6 gene transcription.

The interaction between steroid hormones and IL-6 expression can explain a gender-dependent influence of G174C polymorphism on IL-6 expression observed in the present study in which the C allele was often found in obese OSAS men, but not women.

The present findings also suggest the relationship between the G308A polymorphism located in the promoter region of TNF-α gene and the development of OSAS. However, we could not substantiate any statistical significance in the frequency of the A allele occurrence in the population samples studied. The function of this polymorphism remains unclear. It is located in the DNA sequence responsible for the binding of AP-2 transcription factor (18). The role of -308A allele in the positive regulation of gene expression is supported by many earlier studies (19). This polymorphic variant has been shown to affect the promoter region of the TNF-α, the gene leading to a higher rate of transcription compared with -308G allele. The presence of polymorphism in the regulatory region of a gene may lead to differences in gene expression.

We found no correlation between the C3954T polymorphism in the IL-1 gene and OSAS. Allele frequencies were very similar in the control group and in the OSAS population. It is difficult to find a direct relationship between this polymorphism and OSAS. However, the existence of such a relationship is confirmed in a study of Um *et al* (20). These authors have found that the T-allele carriers (T/T and C/T) have a lower level of total cholesterol, TG, BMI, and WHR compared with C/C homozygotes.

The mechanism by which the IL-1β gene polymorphism influences obesity-related OSAS is still unknown. Pociot *et al* (13, 14) have reported that homozygosis for the IL-1β -3954T allele is associated with a four-fold increase in the production of IL-1β compared with the cases homozygous for the C allele. It is possible that the C3954T polymorphism located in the promoter region of IL-1β has indirect influence on obesity and OSAS development by affecting adipose tissue metabolism. IL-1β is secreted by human adipose cells (21). This secretion is regulated by TNF-α production in obesity. An increase in TNF-α production may act on adipose tissue to increase the expression and release of IL-1β. The IL-1β regulates lipid metabolism and synergizes with other effects of TNF-α (22).

The present findings led us to suggest that polymorphism of inflammatory cytokine genes has an important influence on OSAS development. It is possible that this influence is exerted *via* changes in the expression of cytokines. Shamsuzzaman *et al* (23) have reported that plasma C-reactive protein is elevated in patients with OSAS. Teramoto *et al* (24) have reported that plasma levels of IL-6 and TNF-α are elevated in patients with OSAS, which is associated with the level of hsCRP. Those results may explain the relationship between polymorphism in promoter regions of inflammatory cytokine genes and the development of OSAS.

We conclude that inflammatory cytokines gene polymorphisms may contribute to the OSAS pathogenesis. OSAS may have a strong genetic basis due to the effects from a number of genes including those for inflammatory mediators.

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