RESEARCH ARTICLE

Genetic variant in TNF-α gene and its plasma level in relation to obstructive sleep apnoea in the Pakistani population

Ambreen Qamar[1], Muhammad Hanif[2], Zeba Haque[3], Sitwat Zehra[4], Saifullah Baig[5], Abdul Samad[6]

Abstract

Objective: To assess the link between tumour necrosis factor-alpha -308 guanine/adenine polymorphism and tumour necrosis factor-alpha plasma levels in relation to obstructive sleep apnoea.

Method: The cross-sectional study was conducted from December 2018 to March 2021 at the sleep clinic of Dow University Hospital, Karachi, on obstructive sleep apnoea patients and healthy controls. Epworth Sleep Scale score was used to determine daytime sleepiness, while full-night polysomnography was carried out for obstructive sleep apnoea confirmation and categorisation according to severity. Blood sample collection was followed by deoxyribonucleic acid extraction and plasma tumour necrosis factor-alpha measurement using enzyme-linked immunosorbent assay. Genotype distribution and allelic frequency were assessed. Data was analysed using SPSS 20.

Results: Out of the 225 subjects, with a mean age of 47.68±9.88 years, 132 (58.7%) were males, and 93 (41.3%) were females. Among them, 150 (66.7%) were patients, and 75 (33.3%) were controls. Heterozygous tumour necrosis factor-alpha -308 guanine/adenine genotypes were significantly higher among the patients ($p<0.05$). Minor allele -308 adenine showed an association with obstructive sleep apnoea, its severity, higher tumour necrosis factor-alpha levels, neck circumference, excessive daytime sleepiness and the presence of hypertension ($p<0.05$).

Conclusion: Tumour necrosis factor-alpha -308 adenine allele and higher tumour necrosis factor-alpha levels were found to be linked with obstructive sleep apnoea. The polymorphism also showed an association with hypertension in obstructive sleep apnoea patients.

Key Words: Tumour necrosis, polysomnography, alleles, sleep apnea, obstructive, hypertension, genotype, somnolence, enzyme, immunosorbent assay, adenine, guanine

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Introduction

Sleep is a normal biological activity, with a recurrent, reversible state of decreased responsiveness to external stimuli[1]. It has a huge impact on our mental and physical health, performance of tasks, and on overall quality of life (QOL).

Inadequate or disrupted sleep is responsible for increased risk to mental and physical health and general safety[2]. Sleep-related breathing disorders (SRBDs) are a collection of disorders characterised by breathing issues during sleep. Among the SRBDs, obstructive sleep apnoea (OSA) is a condition with serious cardiovascular and other complications, and consequently necessitates early diagnosis and treatment[3].

[1]Department of Physiology, Dow University of Health Sciences. [2]Karachi Institute of Radiotherapy and Nuclear Medicine, [3]Department of Biochemistry, Dow International Medical College, Dow University of Health Sciences, [4]Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering, [5]Department of Pulmonology, Dow University of Health Sciences, [6]Department of Physiology, Dow University of Health Sciences, Karachi, Pakistan.

Correspondence: Ambreen Qamar. Email: ambreen.qamar@duhs.edu.pk

ORCID ID: 0009-0001-1818-9521

OSA is a disorder in which partial (hypopnoea) or complete pharyngeal obstruction (apnoea) occurs during sleep, with each episode lasting at least 10 seconds involving reduced respiratory flow or complete interruption of airflow, followed by arousal from sleep[4]. Persons with OSA are barely aware of their respiratory problems during sleep even upon awakening. The severity of OSA can be defined by the number of hypopnea/apnoea per hour of sleep, which is called the Apnoea/Hypopnea Index (AHI), recorded through polysomnography (PSG), which is the gold standard diagnostic test for OSA[4]. OSA has drawn more attention in the last few years due to a substantial increase in prevalence rate; 23.4% and 49.7% in females and males, respectively[5].

OSA is increasingly recognised as a major contributor/cause of metabolic dysregulation, hypertension, diabetes, stroke and other cardiovascular diseases (CVDs) as well as mortality It is also the cause of serious neurocognitive problems, such as impaired snoring, excessive daytime sleepiness (EDS), diminished mental alertness, compromised QOL, and road accidents[6,7]. Findings are not conclusive for the aetiology

and pathophysiology of OSA and its related comorbidities. Previously, the picture of the association between OSA and CVDs was not adequately clear due to the presence of confounding factors, like obesity, but recent data supports this link independent of obesity[8]. Certain studies described that OSA triggers the similar pathways as does obesity[9].

Multiple intermediary mechanisms have been suggested for OSA and its associated comorbidities, like persistent sympathetic stimulation, endothelial damage, oxidative stress, elevated inflammatory mediators, etc.[10]. Recent data reinforced that inflammation is a significant correlate of OSA, and several OSA comorbidities can be causally described by underlying inflammatory processes[7]. The concept of inflammation as an intermediate mechanism is strengthened by the fact that OSA subject show changes in the mucosal and muscular layers of the upper airway that can affect the airways contractility, stiffness and collapsibility[11].

OSA patients represent a wide range of clinical variability in their phenotype because some patients with severe OSA do not display severe symptoms, while some patients with mild OSA have different cardio-metabolic or neurocognitive symptoms[7]. This considerably different clinical presentation of OSA and end-organ morbidities describe multifactorial pathophysiology which could involve the presence of genetic predisposition and environmental factors. Grandner MA et al.[2] defined inflammation as a correlate of OSA, and a connecting mechanism for apnoea and CVDs. Some inflammatory markers, like tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and IL-6, have shown a relationship with sleep disruption in animal models.[2]

TNF-α is an important cytokine in inflammatory responses. It acts as an immunostimulant and immunosuppressant. Irregularity in its production and distribution has been associated with various diseases[7]. Researches have shown that both increase and decrease in TNF-α levels are associated with sleep disturbance[7].

The TNF-α gene, with four exons and three introns, is positioned on chromosome 6p21.31. Studies show that TNF-α 308 guanine/adenine (G/A) has a significant impact on the TNF- α transcription levels. This polymorphic site could have two alleles; the G allele is considered the major allele, and the A allele is considered the minor allele[12]. The minor allele at this location has been suggested to stimulate transcription action and is associated with numerous immune-related diseases.

Studies have described the association of TNF-α with higher incidence and severity of OSA in some populations[13]. Kheyrandish at el. showed that TNF-α induced encouragement of the nuclear factor kappa B (NF-kB) pathway, followed by the stimulation of nitric oxide (NO) synthase, cyclooxygenase 2 (COX-2), and adenosine A1 receptors (A1ARs), all of which are related to the regulation of sleep[7]. A study on American children with OSA discovered the significance of the TNF-α -308G/A polymorphism in increasing serum TNF-α levels and linking this to the presence of EDS symptoms[14]. Another study investigated the Indian population and reported that the frequency of the minor allele (-308A) was twice as high in OSA subjects as in controls[15]. Studies described the activation of NF-κB in OSA, which is associated with TNF-α and might be responsible for the aggravation of CVD symptoms in these subjects [16].

This is the gene that has previously shown a relationship with CVDs[9] so there might be a connection between OSA and associated comorbidities. However, there are many differences in the existing data for the role of this gene and cytokine levels, which demonstrate a significant role of genetics as well as environmental factors and lifestyle influences in different populations, and which, to our knowledge, has not been evaluated in Pakistani population yet.

Although the exact mechanisms responsible for the initiation and progression of OSA and the intermediate mechanisms by which OSA causes comorbidities are not fully understood, one of the proposed mechanisms that speak of TNF-α gene involvement[15] needs to be evaluated in the local population. The current study was planned to assess the link between TNF-α -308 G/A polymorphism and TNF-α plasma levels in relation to OSA.

Patients and Methods

The cross-sectional study was conducted from December 2018 to March 2021 at the sleep clinic of Dow University Hospital, Karachi. After approval from the institutional ethics review board, the sample size was calculated using OpenEpi online calculator[17]. The sample was raised using sequential sampling technique. Those included were patients with snoring, symptoms of apnoea with an indication of PSG, or daytime sleepiness with ESS score >9. Epworth Sleep Scale (ESS) scores tell the excessive daytime sleepiness and therefore the probability of apnoea[18]. Patients were selected after verification of apnoea by PSG (Alice 6 LDX, Philips Respironics, United States). Controls with matching age and gender were selected from among Dow University of Health Sciences (DUHS) and Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN) faculty and employees as well as from

the general population. Those who were pregnant, had recent surgery, or with a serious illness were excluded. PSG tests reveal the characteristics of sleep stages and AHI. OSA cases were classified as mild (AHI: 5-15), moderate (AHI: 15-30) and severe (AHI > 30) apnoea[9].

Medical and demographic information was compiled using a pre-designed form.

Written informed consent was obtained from all the participants. After PSG recording, 5mL of venous blood was collected at 6-8am in the morning using vacuum tubes containing ethylenediaminetetraacetic (EDTA) acid. The sample was processed in a centrifuge machine at 2000rpm for 20 minutes for plasma separation, followed by storage at -80°C until the next run. Commercial enzyme-linked immunosorbent assay (ELISA) kits (DIA site, S.A. Belgium) were used, according to the manufacturer`s protocol for temperature control and procedure steps. Dichromatic readings were obtained, followed by the average of repeated detections. Deoxyribonucleic acid (DNA) extraction was performed using a kit (Thermo-Scientific Gene JET Genomic DNA Purification Kit, USA; Pub. No: MAN0012663). The purity and yield of the DNA were tested by spectrophotometry at 260-280nm. Absorbance ratio A260/280 ranged 1.8 to 2.0. Allele-specific polymerase chain reaction (PCR) was performed on an analyser (Bio-Rad CFX96 Real-Time System Analyser, USA) using the relevant kit (SYBR Green quantitative PCR kit, Thermo-Scientific, USA). The primers used (Table 1) included: Common forward primer: CTGCATCCCCGTCTTTCTCC; Wild reverse primer: ATAGGTTTTGAGGGGCATCG; Mutant reverse primer: ATAGGTTTTGAGGGGCATCA.

The extracted DNA samples were run separately on PCR using wild-type (WT) reverse and mutant reverse primers. The PCR products were then analysed for the presence of the selected single nucleotide polymorphisms (SNPs). Gel electrophoresis was performed using agarose gel prepared with Tris/Borate/EDTA (TBE) buffer. The resolved product was analysed based on its size with 850bp and the ladder size was 1kb.

Data was analysed using SPSS 20. Shapiro-Wilk test was used to check data normality. Parametric tests, such as independent sample t-test, analysis of variance (ANOVA) and chi-square test, were used as appropriate. The confidence interval (CI) was 95%, and p≤0.05 was considered significant.

Results

Out of the 225 subjects, with a mean age of 47.68±9.88 years, 132 (58.7%) were males, and 93 (41.3%) were females. Among them, 150 (66.7%) were patients, and 75 (33.3%) were controls. Detailed characteristics of both the groups were noted (Table 2). Hypertension was the most common comorbidity in OSA patients 83(55.3%) compared to controls 16(21.3%). TNF-α -308 G/G was the

Table-1: PCR reaction conditions.

PCR Reaction Condition for Tumour necrosis factor-α gene −308G/A (rs1800629)

Product Size:	**850 base pair**	
Step	Temperature and Time	No. of cycles
Initial denaturation	95 o C for 3 minutes	1
Denaturation	95 o C for 45 second	(30 cycles)
Annealing	55 o C for 80 seconds	(30 cycles)
Extension	72 o C 120 seconds	(30 cycles)
Final Extension	72 o C 7 minutes	1

PCR: Polymerase chain reaction.

Table-2: Anthropometric and clinical characteristics of the participants (n=225).

Characteristics	**Total Sample Mean ± SD**	**Controls Mean ± SD**	**Cases Mean ± SD**	**p-value***
Age (years)	47.68 ± 9.88	46.35 ± 11.18	48.35 ± 9.13	0.152
Weight (kg)	88.60 ± 20.25	71.43 ± 14.75	97.18 ± 16.92	<0.001
Height (cm)	165.83 ± 9.28	166.42 ± 10.46	164.91 ± 8.63	0.251
BMI (kg/m2)	32.13 ± 7.33	25.65 ± 5.15	35.37 ± 5.99	<0.001
Neck Circumference (cm)	40.94 ± 4.62	37.21 ± 3.45	42.77 ± 3.96	<0.001
Waist Circumference (cm)	106.57 ± 19.91	94.33 ± 13.58	112.62 ± 19.81	<0.001
Hip Circumference (cm)	112.62 ± 19.48	102.53 ± 11.91	117.60 ± 20.57	<0.001
Systolic BP (mmHg)	132.58 ± 11.47	125.20 ± 10.60	136.37 ± 10.05	<0.001
Diastolic BP (mmHg)	80.56 ± 8.62	80.33 ± 6.84	80.67 ± 9.40	0.785
Epworth sleepiness scale score	11.54 ± 6.64	4.07 ± 2.63	15.27 ± 4.55	<0.001
Apnoea Hypopnoea Index (n=150)	36.76 ± 19.89	36.76 ± 19.89	-	-
Tumour necrosis factor-α (pg/ml)	9.28 ± 5.27	3.74 ± 1.62	12.06 ± 4.15	<0.001

*p≤0.05 was considered significant.

SD: Standard deviation, BMI: Body mass index, BP: Blood pressure.

Table-3: Genotype and allelic frequencies for tumour necrosis factor alpha (-308)) between the groups.

Gene TNF-α -308 rs1800629	**SNP Genotype**	**Controls n (%)**	**Cases n (%)**	**p-value***
	GG	73 (97.3)	129 (86.0)	0.008
	GA	2 (2.7)	21 (14.0)	
	AA	-	-	
	Alleles			
	G	148 (98.6)	279 (93.0)	0.009
	A	2 (1.4)	21 (7.0)	

*p≤0.05 was considered significant.

SNP: Single nucleotide polymorphism, TNF-α: Tumour necrosis factor-alpha.

Table-4: Comparison of tumour necrosis factor-alpha (TNF-α) levels with genotype variations between the groups.

TNF-α Genotype	TNF-α Plasma levels			
	Controls		Cases	
	Mean ± SD	P-value	Mean ± SD	P-value*
GG	3.65 ± 1.54		11.50 ± 3.99	
GA	7.01 ± 0.74	0.003	15.50 ± 3.45	<0.001

*p≤0.05 was considered significant. SD: Standard deviation.

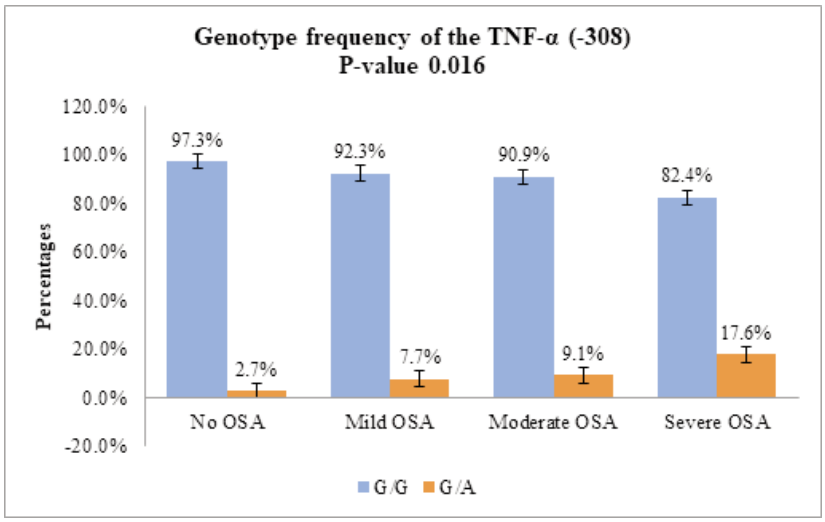

Figure: Genotype distribution for tumour necrosis factor-alpha (TNF-α(-308 gene according to obstructive sleep apnoea (OSA) severity (p= 0.016).

Table-5: Association of tumour necrosis factor-alpha (TNF-α) gene polymorphism with anthropometric and clinical characteristics (n=150).

Characteristics		G/G n (%)	G/A n (%)	p-value*
Hypertension	Absent	67 (51.9)	0 (0.0)	<0.001
	Present	62 (48.1)	21 (100.0)	
Diabetes	Absent	106 (82.2)	18 (85.7)	0.691
	Present	23 (17.8)	3 (14.3)	
Asthma	Absent	96 (74.4)	15 (71.4)	0.772
	Present	33 (25.6)	6 (28.6)	

Characteristics	G/G Mean ± SD	G/A Mean ± SD	p-value
Weight (kg)	96.06 ± 17.17	104.05 ± 13.74	0.045
Height (cm)	164.95 ± 8.62	164.60 ± 8.91	0.863
BMI (kg/m2)	35.01 ± 6.09	37.55 ± 4.94	0.072
Neck Circumference (cm)	42.41 ± 4.01	44.93 ± 2.99	0.007
Waist Circumference (cm)	44.12 ± 7.84	45.71 ± 7.59	0.388
Hip Circumference (cm)	46.14 ± 8.15	47.28 ± 7.89	0.552
Systolic BP (mmHg)	135.39 ± 9.59	141.67 ± 11.32	0.008
Diastolic BP (mmHg)	79.77 ± 9.37	86.19 ± 7.73	0.003
Epworth sleepiness scale score	14.89 ± 4.59	17.62 ± 3.58	0.010
Apnoea Hypopnea Index	35.53 ± 19.59	44.29 ± 20.57	0.061
Tumour necrosis factor-α (pg/ml)	11.50 ± 3.99	15.50 ± 3.45	<0.001

*p≤0.05 was considered significant. SD: Standard deviation, BMI: Body mass index.

most common genotype, but was more common in controls compared to OSA (p=0.008) (Table 3). The incidence of heterozygous genotype G/A was 5 times higher in the OSA group compared to the controls. There was no homozygous A/A genotype in the subjects. The frequency of minor allele A was 5 times higher in the OSA group. The association of heterozygous G/A genotype with OSA severity was significant (p=0.016) (Figure). The presence of minor alleles was associated with higher levels of its related cytokine (Table 4). Minor allele A was positively associated with hypertension, daytime sleepiness, and serum TNF-α levels (Table5).

Discussion

The participants of the current study were from the cosmopolitan, multi-ethnic city of Karachi. Both groups were matched for age and gender. Hypertension was the most common comorbidity in the OSA group, with more than half of them having high blood pressure (p<0.001). The current findings described a considerably higher prevalence of heterozygous G/A variant in the OSA group with a 5 times higher frequency of a minor allele (Table 2). The heterozygous G/A variant was also found to be associated with OSA severity (Figure).

Wilson et al. found that substitution of G to A at position -308 was more prominent in OSA[19]. According to Riha et al., the 308A allele was more common in British OSA subjects compared to controls (28% vs. 18%)[20]. Almpanidou et al. analysed population samples from Greece and reported similar findings[21]. The present findings were in line with these studies.

Khalyfa et al. in Chicago[15] examined 4 TNF-α SNPs, rs1800629 (-308), rs361525 (-238), rs2228088 (-256), and rs30993665 (-948), and discovered the significance of the TNF-α -308G/A substitution to affect TNF-α levels and its link with EDS. No association was reported for the other SNPs tested. Similarly, the current study explained the link of elevated TNF-α levels with heterozygous TNF-α -308G/A genotype and with daytime sleepiness.

Bhushan et al. scrutinised this link in 207 Indian subjects and found minor allele to be twice as high in OSA patients as in those without OSA (28% vs 13%). TNF-α -308G/A OSA carriers had higher serum TNF-α levels compared to WT carriers of OSA[15]. SNP -308G/A was described to have involvement in the alteration and regulation of the cell and the specific regulation of TNF-α transcription[14].

There are studies showing that the TNF-α gene was not associated with OSA. The frequency of the -308 A allele in the Polish group (n=179) was 14% and 12% while in the Turkish group, it was 13% and 11% (n=111) in OSA and controls, respectively[22]. None of these studies believed that this SNP played any significant role in OSA in their respective populations.

Behboudi et al. presented contrary results, with higher frequency of TNF-α -308 A allele (27% in controls compared to 23% in cases) in the Swedish subjects, and suggested that these results were due to the presence of confounding diseases, such as diabetes, hypertension and heart disease, in the controls, but they did not report positive conclusion for this association[13]. However, the current findings explained the link between the minor allele and OSA, and evaluated that OSA subjects with G/A genotype had higher ESS scores compared to OSA subjects with the G/G variant (Table 4).

A 2016 study with a large sample of the Chinese population, including 750 OSA cases and 800 controls, identified the association of minor allele A in TNF rs1800629 -308 with OSA susceptibility, but not with OSA severity[23]. Though, the present findings also explained the relationship between minor alleles with severity (Figure 1), the study did not find A/A homozygosity at position -308 in the subjects. Similar findings were reported by Popko et al. for the Polish population, but the frequency of G/A genotype was not higher in OSA[22]. Saba N. et al. also described the rare occurrence of the homozygous minor allele A/A in a Pakistani study with asthmatic patients[24] while there was no association of the minor allele with asthma in the current population.

It is noteworthy that 91.3% G/A carriers had OSA and all (100%) had hypertension, reflecting the strong link of minor alleles with the presence of hypertension in OSA.

It can be said that genetic factors predispose people to OSA, followed by negative conditions, such as apnoea, hypopnoea, sleep fragmentation and sympathovagal imbalance, as well as genetic predisposition, exacerbated hypertension, and other comorbidities.

OSA is a common cause of many CVDs and a well-known risk for EDS-related driving and occupational accidents. The prevalence of this disease is steadily increasing, and research is needed now more than ever to prevent OSA or decrease its severity. Synergistic approaches that combine different types of genetic analysis, such as genome scans, genome-wide association studies, microarrays, etc., are needed[25].

PSG is an expensive, time-consuming method and cannot be tolerated by many patients. Measurement of plasma TNF-α can be considered before PSG to assess high-risk individuals. Larger samples are recommended for longitudinal studies. Based on the available findings, it seems necessary to investigate the role of other inflammatory genes.

Conclusion

The was a strong association of OSA with the presence of minor alleles TNF-α -308A (rs1800629). Also, the degree of expression of the SNP significantly correlated with OSA and its severity in the Pakistani population. OSA patients with G/A genotype had higher TNF-α levels compared to OSA subjects with G/G genotype. The SNP was also linked to OSA-related co-morbidity, like hypertension. Plasma TNF-α may be helpful in the identification of OSA patients requiring immediate intervention.

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