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Tumour necrosis factor-α (-308) gene polymorphism in obstructive sleep apnoea–hypopnoea syndrome

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ABSTRACT: **Patients with obstructive sleep apnoea–hypopnoea syndrome (OSAHS) have elevated circulating levels of tumour necrosis factor (TNF)-α. The hypothesis in this study was that OSAHS might be associated with the TNF-α (-308A) gene polymorphism, which results in increased TNF-α production. This hypothesis was examined in OSAHS patients, their siblings and population controls.**

A total of 206 subjects were recruited. All underwent sleep studies and clinical review, and were subsequently classified as having OSAHS or not depending on apnoea–hypopnoea frequency, sex, age and symptoms. All subjects had blood collected and genotyping was performed on DNA extracted from peripheral leukocytes. Some 192 random UK blood donors were used as population controls.

The results demonstrated a significant association for TNF-α (-308A) allele carriage with OSAHS (OR=1.8; 95% Confidence interval: 1.18–2.75) when compared with population controls. Siblings with OSAHS were significantly more likely to carry the TNF-α (-308A) allele. In addition, 21 pairs of male siblings discordant for carriage of the -308A allele showed a significant level of discordance for the OSAHS phenotype.

In conclusion, this study demonstrates an association of tumour necrosis factor-α (-308A) carriage with obstructive sleep apnoea–hypopnoea syndrome, suggesting that inflammation may be implicated in the pathogenesis of this condition.

KEYWORDS: **Genetics, obstructive sleep apnoea, tumour necrosis factor-α**

Obstructive sleep apnoea–hypopnoea syndrome (OSAHS) is the second most common respiratory condition, affecting ~0.3–4% of the middle-aged population. It is defined on the basis of symptoms of daytime sleepiness and objective measures of disordered breathing during sleep [1, 2]. OSAHS has a familial component, independent of obesity [3], and there is increasing evidence that OSAHS is associated with inflammation. Raised levels of C-reactive protein, hypercytokinaemia and tumour necrosis factor (TNF)-α have been reported in OSAHS [4–6]. The raised serum TNF-α level is independent of obesity and correlates with systemic blood pressure [5, 7, 8]. Furthermore, TNF-α also promotes sleepiness, the major feature of OSAHS [9].

Increased production of TNF-α both *in vitro* and *in vivo* has been reported to be associated with a functional TNF-α gene polymorphism, consisting of a guanine (G) to adenine (A) substitution at position -308 in the promoter region [10, 11]. It was thus hypothesised that OSAHS might be associated with the TNF-α (-308A) gene polymorphism. This was tested in OSAHS patients, their siblings and population controls.

METHODS

A case-control study, based on the recruitment of patients with OSAHS and their available siblings, was undertaken between January 1997 and December 2002 at the Scottish Sleep Centre (Edinburgh, UK). A total of 192 random anonymous UK blood donors were used as additional population controls. Approval for the study was obtained from the local research ethics committee (Edinburgh, UK).

Recruitment of subjects

A total of 557 consecutive patients attending the Scottish Sleep Centre with symptoms of OSAHS and an apnoea–hypopnoea index (AHI) ⩾15 were asked to participate in the study as index

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cases, and were requested to contact their siblings to ask them to complete a sleep questionnaire, take part in a sleep study and provide a blood sample. Out of 312 index subjects willing to participate in the study, 155 had siblings who were also available. Some 44 siblings defaulted on attendance and eight sibling pairs had technically inadequate data collected. In total, 103 sib-pairs (206 subjects) successfully completed the study. All subjects were Caucasian.

Data collection

After giving written, informed consent, all subjects had their height, weight and neck circumference and blood pressure measured, and were asked to fill out a standard sleep questionnaire (including an Epworth Sleepiness Score (ESS) [12]). Subjects had 20 mL of blood collected. All siblings had overnight polysomnography (PSG) using standard techniques [13]. Studies were performed in sound-proofed and electrically screened bedrooms at the Edinburgh Sleep Centre (UK).

Sleep was recorded using electroencephalography (EEG), electro-oculography (EOG) and electromyography (EMG), using bipolar signals from silver chloride surface electrodes. EEG was recorded from two scalp sites (Cz/Pz). Frontal EEG was also recorded using ''mixed'' channels comprising EEG/EOG signals (Cz/Fp1, Cz/Fp2). The EOG was recorded from electrodes placed at sites on the outer canthus of the eye and Fp1 and Fp2. Submental EMG was recorded using two electrodes placed on the belly of the genioglossus. A grounding electrode was placed at Fpz. Electrodes placed on the right and left anterior tibialis recorded leg EMG. All signals were recorded on the Compumedics W-series and R-series systems (Compumedics, Melbourne, Australia).

Index patients had either overnight PSG (87 out of 103) or a home (16 out of 103 cases) study using the limited sleep study system Edentrace (EdenTec Model 3711 Digital Recorder; Nellcor, Eden Prairie, Minnesota, USA), which has been extensively validated against PSG [14, 15]. This device records oronasal airflow using a thermocouple, chest wall movement by electrical impedance, heart rate using an electrocardiogram and finger pulse oximetry; no EEG is recorded.

Sleep studies were analysed by one researcher (R.L. Riha) after blinding of data using standard scoring criteria [14, 16, 17].

Phenotyping

OSAHS phenotype was defined using AHI and sleepiness, as measured by the ESS. Table 1 shows the AHI cut-off values based on normative data for Caucasian subjects [2, 18, 19]. Each subject's AHI was first scored as either definitely abnormal, indeterminate or definitely normal on the basis of sex and age. Each subject's ESS (out of a total of 24) was then scored as either sleepy (ESS $\geqslant$11 out of 24) or not abnormally sleepy (ESS $<$11 out of 24). The ESS cut-offs were based on normative data derived for Caucasian subjects [20, 21].

OSAHS was then classified as being definitely present, indeterminate or definitely absent by the algorithm represented in table 2.

Blood donors

DNA from UK Caucasian human random control DNA panels (Product No. HRC-1 96 array and No. HRC-2 96 array; European Collection of Cell Cultures, http://www.ecacc.org.uk) was used as a second set of controls. All donors gave written informed consent for their blood to be used for research purposes.

TABLE 1 Classification of apnoea–hypopnoea index (AHI) according to sex and age

	AHI·h^{-1}	
	Male	Female
Normal		
20–40 yrs	<10	<10
40–60 yrs	<15	<10
>60 yrs	<20	<15
Indeterminate		
20–40 yrs	10–15	10–15
40–60 yrs	15–20	10–15
>60 yrs	20–30	15–20
Abnormal		
20–40 yrs	>15	>15
40–60 yrs	>20	>15
>60 yrs	>30	>20

TABLE 2 Presence or absence of obstructive sleep apnoea–hypopnoea syndrome (OSAHS) phenotype using scores derived for apnoea–hypopnoea index (AHI) and sleepiness

Score for AHI	Score for sleepiness	OSAHS phenotype
Abnormal	Abnormal	OSAHS
Abnormal	Normal	Indeterminate
Indeterminate	Abnormal	OSAHS
Indeterminate	Normal	No OSAHS
Normal	Abnormal	No OSAHS
Normal	Normal	No OSAHS

Allelic discrimination analysis

DNA for recruited subjects was extracted using the Wizard® R Genomic DNA Purification Kit (TM050; Promega, Southampton, UK) or the Nucleon Extraction and Purification Protocol (for extraction of DNA from 10 mL of whole blood; Nucleon BACC3 RPN 8512; Amersham plc, Little Chalfont, UK).

The TaqMan system was used to study the -308 (A–G) single nucleotide polymorphism (SNP). Assay-by-Design® (Applied Biosystems, Foster City, CA, USA) was used to design the probe and primers.

Each 10 μL of PCR contained 10 μg of genomic DNA, 900 μM primers, 250 μM probes and 2.5 μL of TaqMan Universal PCR master mix (Applied Biosystems). The solution was pipetted into each well of a 96- or 384-well plate using an automated liquid handling robot.

Amplification was performed using the Peltier Thermal Cycler (PTC-225 DNA tetrad PCR machine; MJ Research, Waltham, MA, USA). Fluorescence in each well was measured before and after PCR using an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) and reproduced as scatter diagrams. Alleles were read and classified on the plots by two readers independently blinded to subject status.

Statistical analysis

Between-group comparisons were performed using the Chi-squared test and unpaired and paired t-tests. A logistic regression model using generalised estimating equations was used to test the association of OSAHS with TNF-α carriage on an allele-counting basis (assuming Hardy-Weinberg equilibrium and a multiplicative risk model) [22]. The significance of frequency differences of OSAHS between genotype-discordant sibling pairs was estimated using one sibling pair from each family by McNemar's test of symmetry. Association and linkage analysis of OSAHS with the TNF-α (-308) SNP was performed using formulae in the SIBASSOC programme [23]. SIBASSOC performs a Chi-squared test using the most genotypically distinct unaffected sibling as a control for each case. The FBAT [24] programme was used to examine the association of quantitative traits with genotype. Power was determined using the formulae described by MACHIN *et al.* [25] for case-controlled studies comparing two proportions and using the odds ratio (OR). The Hardy-Weinberg equilibrium was tested using the following formula:

$$1 = p^2 + 2pq + q^2 \quad (1)$$

for allelic distribution in the population, and the frequencies of minor and major alleles were compared with published frequencies.

All p-values are for two-tailed tests and considered to be significant at the $\alpha = 0.05$ level.

RESULTS

Sixty-three subjects had no OSAHS and 40 were classed as intermediate for OSAHS by criteria determined *a priori* (tables 1 and 2). Population characteristics for the study subjects with definite OSAHS and definitely no OSAHS are presented (table 3). Subjects classified as indeterminate for OSAHS (n=40) were not included in subsequent analyses.

Genotyping results for the -308 (A–G) SNP for TNF-α were available for 190 UK blood donor controls (99%) and 200 out of the 206 study subjects (97%). Of the blood donor controls, 95 (50%) were male and the mean±SD age was 38±8 yrs.

The distribution of allelic frequencies and genotypes was significantly different between subjects with a definite diagnosis of OSAHS and UK population controls (table 4). Allele distribution in the control group (n=190 blood donors) was in Hardy-Weinberg equilibrium.

Logistic regression showed a significant association for TNF-α (-308A) allele carriage with OSAHS (OR (95% confidence interval) 1.82 (1.18–2.75); p=0.006).

Analysis of quantitative traits (AHI and body mass index (BMI)) showed no association independently with a diagnosis of OSAHS or with genotype (p=0.4 and p=0.5, respectively). This was based on 35 informative sibling groups.

Forty-five pairs of siblings (males and females) were discordant for the diagnosis of OSAHS (the remainder was concordant for the diagnosis or one of the siblings had intermediate phenotype). There was a significantly higher prevalence of the -308A allele in the siblings with OSAHS (p=0.04) using SIBASSOC. Anthropometric and sleep variables in this group are shown in table 5.

Several papers have suggested that there may be sexual dimorphism in innate immune responses, including the secretion of TNF-α [26–28]. Logistic regression analysis corrected for sex still showed significant association of the -308A allele with OSAHS (1.64 (1.06–2.55); p=0.023). Further analysis was undertaken using McNemar's test in 21 pairs of male siblings

TABLE 3 Characteristics of the recruited subjects

	No OSAHS	Definite OSAHS	p-value
Subjects n	63	103	
Sex ratio M:F	33:30	83:20	<0.0001
Age yrs	51±10	52±9	0.6
BMI kg·m^{-2}	27±5	30±6	<0.0001
Neck circumference cm	37±4	41±4	<0.0001
SBP mmHg	130±17	138±18	0.007
DBP mmHg	82±12	84±12	0.6
Sleep efficiency#	76±12	73±15	0.1
REM time min	70±24	64±30	0.2
NREM time min	287±47	275±62	0.2
S_{a,O_2} % awake	97±2	96±2	0.07
Lowest S_{p,O_2} %	90±7	83±10	<0.0001

OSAHS: obstructive sleep apnoea–hypopnoea syndrome; M: male; F: female; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; REM: rapid eye movement sleep; NREM: nonrapid eye movement sleep; S_{a,O_2}: arterial oxygen saturation awake; S_{p,O_2}: arterial oxygen saturation asleep. #: as a percentage of the total time spent asleep divided by the time of the study.

TABLE 4 Allelic and genotype frequencies for tumour necrosis factor-α (-308) A–G polymorphism in subjects with definite obstructive sleep apnoea–hypopnoea syndrome (OSAHS) *versus* population controls

	Controls	Definite OSAHS	p-value
Subjects n	190	103	
G/G	130 (69)	52 (51)	0.01
A/G	52 (27)	44 (43)	
A/A	8 (4)	7 (7)	
Alleles n	380	206	
G	312 (82)	148 (72)	0.004
A	68 (18)	58 (28)	

Data are presented as n (%), unless otherwise stated.

TABLE 5 Characteristics of the 45 sibling pairs discordant for a diagnosis of obstructive sleep apnoea–hypopnoea syndrome (OSAHS)

	No OSAHS	OSAHS	p-value
Sex ratio M:F	23:22	40:5	<0.0001
Age yrs	51±10	51±9	0.6
BMI kg·m^{-2}	27±5	30±6	0.08
Neck circumference cm	37±4	42±3	<0.0001
Sleep efficiency#	77±10	72±15	0.05
REM time min	73±21	65±32	0.26
Lowest S_{p,O_2} %	91±4	82±10	<0.0001

M: male; F: female; BMI: body mass index; REM: rapid eye movement sleep; S_{p,O_2}: arterial oxygen saturation asleep. #: sleep efficiency as a percentage of the total time spent asleep divided by the time of the study.

discordant for carriage of the -308A allele. Fifteen subjects in this group who were A-allele positive had OSAHS whilst their siblings did not (p=0.001). There was no significant difference between the 21 sib-pairs with and without the -308A allele with regard to BMI (28±3 *versus* 28±5 kg·m^{-2}) or age (50±9 *versus* 51±9 yrs). Female sib-pairs in the study were either concordant for OSAHS or one of the pair had an intermediate phenotype, precluding similar analysis.

DISCUSSION

In this study, the TNF-α (-308A) allele was significantly associated with a diagnosis of OSAHS, both in cases *versus* random population controls and in siblings. The marked association between the TNF-α (-308A) allele and OSAHS in the male sib-pairs discordant for OSAHS strongly suggests that this relationship is significant. However, this allele is not required for the development of OSAHS, as its prevalence was 28% in the OSAHS group overall compared with 18% in population controls.

Inflammation in OSAHS is common and includes elevated levels of circulating pro-inflammatory cytokines. TNF-α and interleukin (IL)-6 are elevated in OSAHS, independently of obesity, and the circadian rhythm of TNF-α secretion is disrupted [29]. Additionally, other mediators of inflammation are also elevated, including intracellular adhesion molecule-1 and C-reactive protein [30]. TNF-α, C-reactive protein and IL-6 appear to produce their harmful effects by inducing endothelial dysfunction. TNF-α damages endothelial cells, causes apoptosis of these cells and triggers procoagulant activity and fibrin deposition. TNF-α also enhances the production of reactive oxygen species, including inducible nitric oxide, and decreases myocardial contractility in a dose-dependent fashion [31].

Sleep disruption in OSAHS may be one factor driving the increased susceptibility to cardiovascular diseases in this condition. A genetic propensity towards increased TNF-α production may further exacerbate these effects. The driving forces leading to elevations of pro-inflammatory cytokines in OSAHS may be related to increased activity of both branches of the autonomic nervous system, intermittent hypoxia, localised inflammation in the oropharynx leading to increased cytokine induction, and increased visceral adiposity [30].

In the present study, an independent association of the TNF-α (-308A) SNP with the diagnosis of OSAHS has been demonstrated. This suggests a disease-promoting role, manifest as increased susceptibility to symptoms (somnolence and fatigue) and exacerbation of upper airways inflammation. A further extension of this work would include measurement of serum levels of circulating TNF-α, serum-soluble TNF receptor p55 and serum-soluble receptor p75 in all subjects, which the current study design precluded.

The strengths of this study include the sibling pair design with additional population controls, the scoring of all records blind to case or allele status and the *a priori* agreed classification of case status. The study has several potential limitations. AHI was measured on a single night only, when it may vary from night to night [32]; however, a 1-night study is the standard and only realistic way of assessing sleep-disordered breathing in volunteer (or patient) studies. All study participants were investigated in the same laboratory, using the same standardised techniques for setting up, recording and scoring events. There was a high intra- and inter-rater reproducibility for analysing results, which were performed blind to subject status. The *a priori* use of an indeterminate score reduced misclassification by making a wide distinction between those with definite sleep apnoea and those definitely without it. The ESS allowed for appraisal of somnolence in daily life situations, which more objective tests may not [12]. It is the simplest, most practical and most widely validated and utilised test for general clinical and research use. Furthermore, sleepiness is the major presenting symptom of OSAHS [33].

Although population stratification is a possible explanation for the observed association between OSAHS and TNF-α, a number of factors reduce the likelihood of this. Genotype frequencies for the non-OSAHS group were in Hardy-Weinberg equilibrium, mitigating against genotyping or significant population stratification errors. Following PRITCHARD and ROSENBERG [34], who argued for the use of unlinked genetic markers to detect population stratification in association studies, two polymorphisms (serotonin receptor 2A T102C and growth hormone receptor +561G/T), not known to be in linkage disequilibrium with the TNF gene, were examined for association with OSAHS. Genotypic distributions in the entire group were in Hardy-Weinberg equilibrium and no associations with OSAHS were detected. However, the overall number of markers available was insufficient to carry out a formal test for population stratification. Numerous studies have also looked at whether stratification really is a major issue in epidemiological studies and found the error rates to be extremely small [35, 36].

TNF-α allele frequencies for the normal subjects in this study were not significantly different compared with published UK data for Caucasians, excluding bias based on ethnicity [37]. The power of the study was also adequate to determine a difference in allelic frequency (two-group continuity corrected Chi-squared test of equal proportions showed power of 99% at α=0.05 level) for the analysis between subjects with OSAHS

and blood donor controls. Although the OSAHS status of the latter group was unknown, this would tend to dilute the reported difference (by inclusion of OSAHS cases as normals) rather than strengthen it. Furthermore, blood donors in the UK are screened to be in good health and the taking of most medicines prohibits donation. Finally, lack of an independent association of genotype with quantitative traits for OSAHS using FBAT may be a reflection of low power in this population with respect to this statistical method *per se*.

TNF-α (-308A) is in linkage disequilibrium with human leukocyte antigen (HLA) class I and II alleles, the class III region which encodes several components of the complement system and the major histocompatibility class IV cluster, which includes lymphotoxin-α (one of five microsatellites within the TNF locus) and lymphotoxin-β [38]. The association of TNF-α (-308A) with OSAHS may, therefore, be due to the direct influence of the SNP in question and/or due to linkage disequilibrium with other polymorphisms within the TNF-α gene or other genes within the HLA system.

In conclusion, this is the first known study to examine the tumour necrosis factor-α -308 (A–G) single nucleotide polymorphism in subjects with obstructive sleep apnoea–hypopnoea syndrome. The increased prevalence of the -308A allele in subjects with this disease lends support to the argument that it is associated with inflammation. Further research is needed to explore the utility of pro-inflammatory cytokine gene polymorphisms in obstructive sleep apnoea–hypopnoea syndrome for risk stratification, thereby paving the way for potential therapeutic strategies, such as tumour necrosis factor-α monoclonal antibodies

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