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THE Trp64Arg POLYMORPHISM OF THE BETA (3) ADRENERGIC RECEPTOR IS ASSOCIATED WITH INCREASED BODY MASS INDEX IN PATIENTS WITH SLEEP APNEA[1,2,3,4]

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[4] Running title: ADRB3 polymorphisms in OSAS

ABSTRACT

Background: Obesity is an important risk factor for obstructive sleep apnea syndrome (OSAS), insulin resistance and cardiovascular disease. The Trp64Arg polymorphism (Arg variant) of the β_3-adrenergic receptor (ADRB3) has been associated with obesity. In this study we evaluated the prevalence of the Trp64Arg ADRB3 polymorphism in a large group of patients with OSAS and its association with body mass index (BMI), insulin resistance and hypertension.

Methods: We determined the ADBR3 genotypes in 387 patients with OSAS and 137 healthy subjects recruited from three tertiary Spanish hospitals.

Results: The distribution of the ADRB3 genotypes was similar in OSAS and controls and, in a multivariate model, the risk of OSAS was not associated with the presence of the Arg variant in the ADRB3 gene. However, in those patients with OSAS who carried this genetic variant BMI was higher than in those with the Trp64Trp one ($p<0.01$). Further, a linear trend for higher BMI was found in those with the Arg variant (56%, 75% and 100% in Trp/Trp, Trp/Arg and Arg/Arg, respectively, p for trend= 0.001). Insulin resistance, blood pressure values and serum levels of lipids and glucose were not associated with the presence of the Arg variant in the ADRB3 gene.

Conclusion: The presence of the Trp64Arg allele in the ADBR3 gene does not increase the risk for OSAS but it is associated with the development of obesity in those patients who suffer OSAS.

INTRODUCTION

The obstructive sleep apnea syndrome (OSAS) is a common disorder characterized by excessive daytime sleepiness, repeated episodes of upper airway obstruction during sleep and nocturnal hypoxemia [1]. Obesity is a risk factor for OSAS [2,3] and it has been hypothesized that genetic variants that predispose to obesity might facilitate the development of OSAS [4,5].

The β_3-adrenergic receptor (ADRB3) is mainly expressed in adipose tissue and contributes to variations in energy expenditure and body fat distribution [6-8]. Polymorphisms of the ADRB3 have been suggested to participate in the pathogenesis of obesity [7,9]. In particular, a single nucleotide polymorphism in the ADRB3 gene (Trp64Arg) has been associated with obesity, insulin resistance, abnormal lipid profile and arterial hypertension [10-15]. Yet because the prevalence of Trp64Arg mutation differs among ethnic groups [7] other studies have failed to show any relation between this polymorphism and obesity [16,17]. Further, it has been suggested that the association of this ADRB3 polymorphism with body weight and obesity-related phenotypes may be dependent on presence of other susceptibility genes and/or exposure to other environmental factors [7].

The potential role of this polymorphism of the ADRB3 gene in OSAS had not been explored until recently, when it has been shown to be associated with obesity in male Chinese patients [18]. However, because the prevalence of Trp64Arg mutation differs among ethnic groups [7], we designed a study that sought: *(1)* to estimate the prevalence of the Trp64Arg ADRB3 polymorphism in Spanish patients with OSAS; and, *(2)* to examine whether this gene variant was associated with other clinical phenotypes frequently present in patients with OSAS such as obesity, insulin resistance and arterial hypertension.

METHODS

Subjects and ethics

We designed a case-control study in which we determined the presence of the Trp64Arg mutation in the ADRB3 gene and their association with body mass index (BMI), lipid profile, glucose metabolism and the presence of arterial hypertension in a group of Spanish individuals with (n=387) and without OSAS (n=137) Participants were recruited and studied at sleep units of three university tertiary hospitals in Spain (Palma de Mallorca, Sevilla and Vitoria). The diagnosis of OSAS (n=387) was established by full polysomnography (E-Series Compumedics, Abbotsford, Australia) and included recording of oronasal flow, thoracoabdominal movements, electrocardiography, submental and pretibial electromyography, electrooculography, electroencefalography and trancutaneous measurement of arterial oxygen saturation. The apnea-hypopnea index (AHI) was defined as the sum of the number of apneas plus hypopneas per hour of sleep. OSAS was diagnosed when the AHI was 10
hour $^{-1}$ or greater. In controls (n=137), the diagnosis of OSAS was excluded by conventional polysomnography (in subjects whose BMI was >30 Kg/m^2) or by a cardiorespiratory sleep study that recorded nasal flow, thoracic movements, heart rate, snoring, body position and transcutaneus oxyhemoglobin saturation (Edentec, MN, USA). Exclusion criteria (both in cases and controls) were presence of chronic obstructive pulmonary disease (COPD), liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders.

Arterial hypertension was diagnosed if systolic blood pressure (SBP) was > 140 mm Hg or diastolic blood pressure (DBP) was > 90 mm Hg. Similarly, the threshold for diabetes was a glucose level >126 mg/dL, for insulin resistance a Homa index >4, for hyperlipidemia a total cholesterol level > 200 mg/dl and for obesity a BMI > 30kg/m^2.

The study was approved by the Ethics Committee of the participating institutions, and all participants signed their consent after being fully informed of its goal and characteristics.

Measurements

Blood samples (10 ml) were obtained between 8 and 10 am after an overnight fast into tubes containing EDTA and without anticoagulant for biochemical determinations. After centrifugation serum and plasma were immediately separated in aliquots and stored at –80ºC. Blood in EDTA tubes and aliquots of serum and plasma were transported in dry ice from Txagorritxu Hospital (Vitoria) and Virgen del Rocio Hospital (Sevilla) to Son Dureta Hospital (Palma de Mallorca) and stored at -80ºC until centralized analysis at the latter.

Biochemical analysis

Glucose, triglycerides, cholesterol and HDL concentrations were determined by standard enzymatic methods on a Hitachi 917 biochemical analyzer (Roche Diagnostics, Indianapolis, USA). The plasma concentration of insulin was measured by a commercial chemiluminiscent assay on an Immulite 2000 analyser (DPC, Los Angeles, USA). Insulin resistance was calculated using the Homeostasis Model Assessment (HOMA) [19].

DNA extraction and genotyping

DNA extraction of each blood sample was made using a DNA extraction Kit (Wizard Genomic, UK). The ADRB3 W64R polymorphism was genotyped using a polymerase chain reaction (PCR) The PCR primers were 5'-CAATACCGCCAACACCAGTGGG-3' and 5'-GGTCATGGTCTGGAGTCTCG-3'. PCR was carried out in a volume of 30 µL containing 30 ng of genomic DNA, 2.0 mM $MgCl_2$, 200 µM dNTP´s, 300 nM of each primer, 0.025 U EuroTaq DNA polymerase (Euroclone Pero, Italy), and 1x reaction buffer. Amplified products were digested with 3 U of BstNI (New England Biolabs, Beverly, MA) and the resulting fragments were separated on 3% agarose gels and visualized with ethidium bromide staining under ultraviolet illumination (Syngene Gen Genius, Synotopics Group, USA)

Statistical Analysis

Results are presented as mean ± SD. Comparisons between groups were performed with independent ANOVA tests for quantitative variables and Fisher's exact Chi-squared test for proportions. Correlations between variables were explored using the Spearman test. The Hardy-Weinberg equilibrium for allelic distribution was tested using the formula: $1= p^2 + 2pq + q^2$ where p and q are the allele frequencies for the Trp and Arg variants respectively [20]. The risk of being a carrier of the Arg variant in the ADRB3 polymorphism was quantified with an odds ratio in patients with OSAS versus controls and its 95% confidence interval was calculated. Finally, a multivariate logistic regression analysis was conducted including carrier status and a number of co morbidities. A p-value <0.05 was considered statistically significant.

RESULTS

We studied 387 patients with OSAS and 137 healthy controls. Table 1 shows the main demographic and clinical characteristics and the biochemical profile of all subjects studied. Patients with OSAS were older and had significantly higher BMI and systolic and diastolic blood pressure, glucose, triglycerides and the HOMA index values than controls (all $p< 0.05$). Differences in insulin levels were of borderline significance ($p=0.056$) and there were no differences in cholesterol levels.

Table 2 presents the distribution of genotypes and allele frequencies of the observed ADBR3 variants in all participants. The genotype frequency distributions were in Hardy-Weinberg equilibrium both in patients with OSAS cases and in control subjects, and there were no differences between them ($\chi^2 = 0.414$). Further, being a carrier of the Arg variant in the ADRB3 polymorphism did not increased significantly the risk of suffering OSAS (OR 1.24; 95% C.I. 0.73-2.11, $p=0.514$), (Table 2). However, we found that patients with OSAS carrying the Arg variant in the ADRB3 gene had higher BMI than those with the Trp variant only ($p<0.01$) (Table

4). By contrast, in control subjects BMI was not different according to the presence or absence of the Arg variant (28.5±4.6 vs 28.6±5.1 Kg/m^2, respectively, p=0.731).

In patients with OSAS the prevalence of obesity (BMI>30kg/m^2) increased linearly in carriers of the Arg variant in the ADRB3 gene (56%, 75% and 100% in Trp/Trp, Trp/Arg and Arg/Arg, respectively, p for trend = 0.001, p χ^2 = 0.005. The corresponding BMI values were 31.5 ± 5.7, 33.5 ± 6.9 and 33.9 ± 3.1 in Trp/Trp, Trp/Arg and Arg/Arg, respectively, p for trend = 0.038. Finally, we used a multivariate model to investigate if carrier status influenced the clinical phenotype of OSAS, but we could not demonstrate an independent effect of the Arg variant of the ADRB3 gene upon the prevalence of obesity, insulin resistance or hypertension

DISCUSSION

This study provides two findings of interest. First, the genotypes and allele frequencies for the observed ADBR3 gene variants observed in Spanish patients with OSAS were not different from those observed in healthy controls of the same ethnic origin. Second, BMI was significantly greater in those patients with OSAS who carried the Arg variant in the ADRB3 gene (Trp64Arg and Arg64Arg genotypes) than in those with theTrp64Trp genotype. These two observations suggest that the Trp64Arg polymorphism is not associated with the development of OSAS in this Spanish population but it may favour the development of obesity in patients who already suffer OSAS. Consistent with this hypothesis, a previous study has recently shown an independent effect this polymorphism on BMI in Chinese patients with OSAS [18].

The ADRB3 is mainly expressed in adipose tissue and contributes to population variations in energy expenditure and body fat distribution [6]. A missense mutation of the gene (Trp64Arg) has been associated with increased BMI and an enhanced capacity to gain weight [8]. However, studies carried out in different ethnic groups have reported conflicting results about the effect of the Trp64Arg ADRB3 variant on body weight [14,21-23]. In general, meta- analysis are consistent with a small effect of the Arg64 mutation on BMI [17,24]. Although the relevance of

this polymorphism to human obesity is still under debate an important finding is that Trp64Arg polymorphism is common in diverse populations. Thus, even if the biological effect is small in absolute terms it may still account for significant population-based attributable risk of obesity [7].

There are several ways to explore the influence of a genetic background on the phenotypic expression of a given trait. The study of the allelic frequency and the prevalence of a given single nucleotide polymorphisms (SNP) are among the most commonly used ones. In our study the allelic frequency of the Arg64 allele was similar between patients with OSAS and controls (Table 2). The fact that it was similar in obese and non obese patients (vs obese and non obese controls, respectively; Table 3), suggests that this polymorphism is not a major determinant of OSAS. However, the observation that patients with OSAS carrying the Trp64Arg variant had higher BMI than patients with the Trp64Trp variant is consistent with this polymorphism facilitating the development of the obesity in patients who, for other reasons, have developed OSAS.

The molecular mechanisms underlying the association of the Trp64Arg genotype and various physiological phenotypes are still unclear [7]. Recent evidence suggests that TR64Arg ADRB3 variant has additive and interactive effects with a number of other candidate gene variants such as uncoupling protein-1 (UCP1) and UCP2 genes or lipoprotein lipase gene [25-27]. Further, it has been suggested that the sleep disruption that characterizes OSAS may influence the expression of these genes and thus the relative importance of a variant at these loci in determining the obesity risk [5].

Studies that have examined the association between the Trp64Arg polymorphism and different comorbidities associated to obesity such as insulin resistance and hypertension have produced inconsistent results [13-15]. We found that the Trp64Arg polymorphism was not associated with glucose levels, lipid concentrations and insulin resistance in patients with OSAS. Furthermore,

the AHI and blood pressure values were not different between genotypes. These results are in keeping with previous studies that reported that the Arg64 mutation can be associated with obesity independent of blood lipids or glucose levels [12].

Potential limitations

Studies carried out in different ethnic groups have reported conflicting results about the effect of the TR64Arg ADRB3 variant on body weight [14,21-23]. Although our results are in concordance with other studies in Mediterranean Spanish population [27], our findings may be confounded by inadequately controlled risk factors such as nutritional status, physical activity or the interaction between the ADRB3 gene with other gene variants. Thus, further studies in different sample populations are needed to determine the independent or possible synergistic effect of these polymorphisms on obesity in OSAS.

In conclusion, our results do not support a role of the Arg64 allele of ADRB3 gene in the pathogenesis of OSAS buy they show that its presence in those patients who have already developed OSAS is associated with a greater tendency to develop obesity

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Table 1: Characteristics of patients with OSAS and controls (values are mean±SD unless otherwise stated).

	OSAS (n=387)	**Controls** (n=137)
Age (years)	50±11**	47±11
Male, %	87%**	76%
AHI (events/h)	51±24***	2±2
BMI (Kg/m^2)	31.9±5.9***	28.6±4.7
SBP (mmHg)	133±17***	125±14
DBP (mmHg)	83±13 ***	78±10
Glucose (mg/dL)	108±23 ***	97±16
Triglycerides (mg/dL)	163±118**	133±67
Cholesterol (mg/dL)	212±41	216±38
HDL (mg/dL)	51±13	53±12
Insulin (μU/mL)	17±13	14±14
HOMA index	4.6±3.9*	3.5±4.2
Obesity %	60%***	36%
Hypercholesterolemia %	62%	66%
Diabetes %	13%**	4%
Insulin resistance %	46%***	20%
Hipertension %	40%***	9%

*p<0.05, **p<0.005, ***p<0.001 versus healthy controls

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, AHI: apnea-hypopnea index, HDL: HDL cholesterol

Table 2: Genotypic and Allelic frequencies of the ADBR3 gene in patients with OSAS and controls

	OSAS (n=387)	**Controls** (n=137)
Genotype Trp/Trp	316 (81.7)	116 (84.7%)
Genotype Trp/Arg	67 (17.3%)	21(15.3%)
Genotype Arg/Arg	4 (1%)	0 (0%)
Allele Trp	699 (90%)	253 (92%)
Allele Arg	75 (10%)	21(8%)

Table 3: Gene-by-obesity interaction of the Arg variant of the ADBR3 gene in patients with OSAS and controls

	Non-obese			**Obese**	
	OSAS	**Controls**		**OSAS**	**Controls**
	(n=155)	(n=88)		(n=231)	(n=49)
Arg variant carrier	17	14	Arg variant carrier	54	7
Non-carrier	138	74	Non-carrier	177	42
	RR=0.65 (0.30-1.39)			RR=1.83 (0.78-4.30)	
	P=0.318			P=0.186	

Table 4: Clinical and biochemical characteristics of OSAS patients according the presence of the Arg variant of the ADRB3 gene (values are mean ± SD).

	No Mutation (n=316) (Genotype Trp/Trp)	**Mutation** (n=71) (Genotype Trp/Arg and Arg/Arg)
AHI	51 ± 23	50 ± 27
BMI	31.5 ± 5.6	33.5 ± 5.7 *
SBP (mmHg)	133 ± 17	133 ± 15
DBP (mmHg)	83 ± 12	83 ± 13
Glucose (mg/dL)	107 ± 23	108 ± 23
Triglycerides (mg/dL)	162 ± 120	167 ± 109
Cholesterol (mg/dL)	212 ± 42	210 ± 38
HDLc (mg/dL)	51 ± 13	49 ± 10
Insulin (μU/mL)	16 ± 14	18 ± 10
HOMA index	4.5 ± 4	4.8 ± 2.9

* $p<0.01$.

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, AHI: apnea-hypopnea index. LDLc: LDL cholesterol, HDLc: HDL cholesterol