Serotonin-2A and 2C Receptor Gene Polymorphisms in Japanese Patients with Obstructive Sleep Apnea

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Abstract

Objective The serotonin (5-HT) 2A and 2C receptor subtype plays an important role in the maintenance of upper airway stability and normal breathing in obesity. Polymorphisms in the 5-HT 2A receptor gene (HTR2A) and 5-HT 2C receptor gene (HTR2C) are associated with various diseases. The aim of this study was to investigate whether or not the HTR2A/C genotypes are associated with obstructive sleep apnea (OSA).

Methods The PCR-restriction fragment length polymorphism method was used to determine genotypes of the HTR2A/C gene. The genotype distributions and allele frequencies were statistically analyzed.

Subjects We studied 177 consecutive male patients with excessive daytime somnolence and an apnea plus hypopnea number [apnea-hypopnea index (AHI)] of greater than five per hour of sleep established by full polysomnography. One hundred Japanese men in whom OSA was clinically excluded were randomly selected as a control group.

Results Genotypes and allele frequencies of 102T/C polymorphism of the HTR2A and 796G/C polymorphism of the HTR2C did not differ between controls and patients with OSA. HTR2C polymorphism was considered inappropriate for association studies because of low frequency of the mutant allele. Multiple regression analysis showed that age and body mass index (BMI) were significantly associated with OSA, but HTR2A polymorphisms were not. HTR2A polymorphisms had no significant relationship with AHI or BMI, although further study with more samples will be needed for powerful statistical analyses.

Conclusions These results indicate that age and BMI,

not these polymorphisms, are associated with OSA in this population.

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Key words: genetic susceptibility, obstructive sleep apnea, body mass index

Introduction

Obstructive sleep apnea (OSA) is a common form of sleep disordered breathing (SDB) and has been identified as an independent risk factor for several cardiovascular disease processes (1–4). The pathogenesis of OSA involves a collapsible upper airway dependent upon the upper airway dilator muscles for patency and normal respiration (5). While ventilation is normal in waking in patients with OSA, sleep-related reductions in motoneuronal activity of the upper-airway dilator result in collapse of the upper airway and apneas (6). Serotonin [5-hydroxytryptamine (5-HT)] is an important neurotransmitter that has a key role in controlling a large variety of sensory and motor functions. Genetic association has been reported between a synonymous 102T/C polymorphism in the gene encoding 5-HT 2A receptor (HTR2A) and a number of clinical phenotypes including schizophrenia (7, 8), clozapine response (9), psychotic symptoms in Alzheimer's disease (10), and certain features of depression (11). A human 5-HT 2C receptor gene (HTR2C) 796G/C polymorphism is related to CSF monoamine metabolite concentrations and DSM-III-R psychiatric diagnosis (12), clozapine response in schizophrenia (13), bipolar disorder (14), tardive dyskinesia in chronic schizophrenia (15), and the personality trait of reward dependence (16). However, the relationships

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between 796G/C polymorphism and behavior and drug response are still controversial.

Most functional studies show that 5-HT and serotonergic neurons have a significant excitatory effect on respiratory motoneurons, including upper airway motoneurons, both *in vivo* and *in vitro*, and that serotonergic neurons show maximal activity during wakefulness and minimal activity during REM sleep (17–19). Sleep-related withdrawal of 5-HT at motoneurons contributes to the collapse of the upper airway and obstructive breathing (20, 21). We therefore hypothesized that these polymorphisms may be associated with OSA. Accordingly, we studied the polymorphisms of HTR2A and HTR2C in Japanese subjects with OSA.

Methods

Subjects

We studied 177 consecutive male patients with excessive daytime somnolence and with an apnea plus hypopnea number [apnea-hypopnea index (AHI)] of greater than five per hour of sleep. AHI score was established by full polysomnography (PSG). We excluded patients with renal failure, thyroid dysfunction, and psychiatric disorders. We also excluded patients 65 and older because the severity of SDB defined by obstructive events decreased and the prevalence of central sleep apnea increased past age 65 years (22).

One hundred Japanese men with a mean age of 38.7 years, who had requested annual physical examinations, were randomly selected as the control group. They did not have any abnormalities based on physical examination, chest radiography, electrocardiogram, urinalysis, and routine laboratory blood testing. In control subjects, OSA was excluded clinically by the criteria of Kapuniai et al (23). The control population consisted of unrelated ethnically matched subjects from the same area of Japan. The mean age and body mass index (BMI) of control subjects were lower than those of patients with OSA (Table 1).

All the subjects gave their written informed consent to participate in this study. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and the study was approved by the Committee of Ethics of Niigata University.

Polysomnography

Polysomnography [PSG (Somnostar, SensorMedics, Yorba Linda, CA)] was performed including measurements of electroencephalogram, electromyogram, electrooculogram, electrocardiogram, airflow by oronasal thermistor, chest and abdominal wall movements, oxygen saturation by pulse oxymeter, snoring sounds by tracheal microphone, and body position. PSG data were scored according to the standard criteria. The severity of sleep apnea was assessed by AHI, lowest oxygen saturation, cumulative percentage of sleep time with oxygen saturation below 90%, and the average number of arousals per hour of sleep. AHI was used for association studies as a representative of severity of OSA.

Determination of HTR2A and HTR2C genotypes

DNA was extracted from whole peripheral blood by the standard method. For the HTR2A and HTR2C receptor genotyping, the polymerase chain reaction (PCR)-restriction fragment length polymorphism method was performed (24, 25). The 102T/C polymorphism in the HTR2A was detected using the oligonucleotide primers 5'-TCTGCTACAAGTTC TGGCTT-3' and 5'-CTGCAGCTTTTCTCTAGGG-3' to amplify a 342-bp segment covering the polymorphic site. PCR was performed using denaturation at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 3 minutes (DNA Thermal Cycler 9600; Perkin Elmer-Cetus, Norwalk, CT). The PCR products were digested with 2.0 U of *Msp I* (New England Biolabs, Beverly, MA) at 37°C for 2 hours, and run on a 2% ethidium bromide-agarose gel. *Msp I* digests the 342 bp DNA segment from CC homozygous individuals into 126 and 216 bp fragments. DNA from TT homozygous individuals was not cut and DNA from CT heterozygous individuals shows the expected fragments at 342, 216, and 126 bp. For the polymorphism in the HTR2C, PCR primers: 5'-CTGCCATGATCA CAA-3' and 5'-GGCCTATTGTTTGGCCAT-3' were employed. PCR was performed using denaturation at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 3 minutes. The PCR products were digested with 2.0 U of *Nla III* (New England Biolabs) at 37°C for 2 hours, and run on a 4% ethidium bromide-NuSieve GTG (BioWhittaker Molecular Applications, East Rutherford, NJ) agarose gel. The restriction pattern was as follows: wild-type 130, 30, 20, and 4 bp; mutant 150, 30, and 4 bp.

Statistical analysis

The genotype distributions and allele frequencies in two groups were analyzed with the chi-square test. The Mann-Whitney U test or Kruskal-Wallis test was used for unadjusted comparisons of age, BMI, and AHI between two or among three groups. The BMDP Statistical Package program (BMDP; Los Angeles, CA) was used for multivariate adjustments for all covariates, simultaneously, using the Cox regression analysis. The nQuery Advisor software (Statistical Solutions, Saugus, MA) was used to calculate power for the chi-square analyses. P values less than 0.05 were considered significant.

Results

Characteristics of study subjects are presented in Table 1. Age and BMI were significantly different between controls and patients with OSA. Genotype and allele frequencies of two polymorphisms in controls and OSA are shown in Table 2. As for HTR2A genotype of the 100 control subjects, 29 had the CC type, 46 the CT type, and 25 the TT genotype. Of the 177 OSA subjects, 40 had the CC type, 90 the CT type, and 47 the TT genotype. The frequencies of C allele in con-

Table 1. Characteristics of Study Subjects

	Controls (n=100)	Patients (n=177)	<i>p</i> value*
Age, yr			
Mean±SD	38.7±11.1	48.3±10.1	<0.001
Range	21–63	23–64	
AHI, events/h	NA	46.1±25.8	
BMI, kg/m ²			
Mean±SD	21.7±2.3	28.3±5.8	<0.001
Range	17.5–27.3	19.4–77.0	

*Mann-Whitney U test. AHI: apnea hypopnea index, BMI: body mass index.

trol and OSA groups were 52.0% and 48.0%, respectively. The genotype distribution and allele frequencies were not different between the groups. As for HTR2C genotype, of the 100 controls, 5 had the CC type, none the CG type, and 95 the GG genotype. Of the 160 OSA subjects, 6 had the CC type, none the CG type, and 154 the GG genotype. The frequencies of C allele in those groups were 5.0% and 3.8%, respectively. The genotype distribution and allele frequencies were not different between the groups. The 796G/C polymorphism of the HTR2C gene was considered to be inappropriate for association studies because of the low frequency of the mutant allele in this population.

Multivariate analysis using Cox regression analysis on AHI was made with regard to the factors of age, BMI, and HTR2A genotype. Independent factors for AHI were age ($p<0.001$) and BMI ($p<0.001$). The HTR2A polymorphism was not included as a predictive variable (Table 3).

We then studied the relationships between HTR2A polymorphism and the severity of OSA, and between the polymorphism and BMI. HTR2A genotypes and alleles in mild and moderate OSA and severe OSA subjects according to the severity criteria are shown in Table 4 (26). Genotype and allele frequencies for HTR2A polymorphism in nonobese (BMI<25) and obese ($25\leq$ BMI) subjects are summarized in Table 5 (27). The genotype distribution and allele frequencies were not different between the subjects grouped by severity of OSA or BMI. We next studied AHI and BMI in each genotype of the HTR2A (Table 6). Age, AHI, and BMI were not different among 40 CC type, 90 CT type, and 47 TT type of the HTR2A.

We finally calculated the statistical power of chi-square analyses in this study. The power of each analysis was less than 50% indicating that sample numbers were not sufficient to confirm a negative association of HTR2A polymorphism with OSA.

Discussion

The present study showed a 102T/C polymorphism of the HTR2A and a 796G/C polymorphism of the HTR2C in Japanese subjects with OSA. Genotypes and allele frequen-

Table 2. Genotype and Allele Frequencies of Two Polymorphisms in Controls and OSA*

Polymorphism	Control (n=100)	OSA (n=177)	<i>p</i> Value [†]
5HT2A 102T/C			
Genotypes			
CC	29 (29.0)	40 (22.6)	0.493
CT	46 (46.0)	90 (50.8)	
TT	25 (25.0)	47 (26.6)	
Alleles			
C	104 (52.0)	170 (48.0)	0.369
T	96 (48.0)	184 (52.0)	
5HT2C 796G/C [‡]			
Genotypes			
CC	5 (5.0)	6 (3.8)	0.626
GG	95 (95.0)	154 (96.2)	
Alleles			
C	10 (5.0)	12 (3.8)	0.491
G	190 (95.0)	308 (96.2)	

*Values in parentheses are percentages. [†]Chi-square analysis.

[‡]Genotypes of HTR2C were not determined in 17 OSA subjects for technical reasons. OSA: obstructive sleep apnea, HTR2A: serotonin-2A receptor gene, HTR2C: serotonin-2C receptor gene.

Table 3. Multiple Regression Model on AHI for All Subjects

Factors	Coefficient	SE of regression	<i>p</i> value
Age, year	0.096	0.020	<0.001
BMI, kg/m ²	0.663	0.091	<0.001
Constant	-19.963	2.519	<0.001

OSA: obstructive sleep apnea, SE: standard error, BMI: body mass index.

Table 4. Severity of AHI and HTR2A Genotypes and Alleles*

	5≤AHI<30 (n=59)	30≤AHI (n=118)	<i>p</i> Value
AHI (Mean±SD)	18.2±7.6	46.1±25.8	
BMI (Mean±SD)	27.6±7.9	28.3±5.8	0.002 [†]
Genotypes			
CC	14 (23.7)	26 (22.0)	0.946 [‡]
CT	29 (49.2)	61 (51.7)	
TT	16 (27.1)	31 (26.3)	
Alleles			
C	57 (48.3)	113 (47.9)	0.940 [‡]
T	61 (51.7)	123 (52.1)	

*Values in parentheses are percentages. [†]Mann-Whitney U test.

[‡]Chi-square test for difference in genotypic or allelic proportions between mild and moderate OSA and severe OSA subjects. AHI: apnea hypopnea index, HTR2A: serotonin-2A receptor gene, BMI: body mass index.

Table 5. HTR2A and Genotypes and Alleles in Nonobese and Obese Subjects*

	BMI<25 (n=48)	25≤BMI (n=129)	p Value
BMI (Mean±SD)	23.2±1.2	30.2±5.7	
AHI (Mean±SD)	34.8±20.3	50.3±26.5	0.007 [†]
Genotypes			
	CC 11 (22.9)	29 (22.5)	0.990 [†]
	CT 24 (50.0)	66 (51.2)	
	TT 13 (27.1)	34 (26.3)	
Alleles			
	C 46 (44.3)	124 (48.9)	0.981 [†]
	T 50 (55.7)	134 (51.1)	

*Values in parentheses are percentages. [†]Mann-Whitney U test.

[†]Chi-square test for difference in genotypic or allelic proportions between nonobese and obese subjects. HTR2A: serotonin-2A receptor gene, BMI: body mass index, AHI: apnea hypopnea index.

Table 6. AHI and BMI in Each Genotype of the HTR2A Gene

	HTR2A genotype			p Value*
	CC (n=40)	CT (n=90)	TT (n=47)	
Age	48.5±9.7	48.6±10.5	47.7±9.9	0.8606
AHI	51.7±30.6	43.9±23.6	45.6±25.4	0.5082
BMI	28.4±4.6	28.6±6.8	27.7±4.3	0.8340

Means and standard deviations are indicated. *Kruskal-Wallis test. AHI: apnea hypopnea index, BMI: body mass index, HTR2A: serotonin-2A receptor gene.

cies of the SNPs were not different between controls and OSA patients. Multivariate analysis on AHI revealed that independent factors for AHI were age and BMI and the HTR2A polymorphism was not a predictive variable.

OSA is characterized by recurrent sleep-induced collapse of the pharyngeal airway leading to hypoxemia and hypercapnia. Variables tending to promote pharyngeal collapse include negative pressure within the airway (eg, during inspiration) and positive pressure outside the airway (eg, fat deposition, small mandible) (6). Patency of the human upper airway is maintained mostly by muscle activation and soft tissue structures. The activity of the muscles responsible for maintaining patency of the upper airway is increased during inspiration, thus stiffening and dilating the upper airway and acting to counteract the collapsing influence of negative airway pressure (28). During sleep there is loss of both tonic premotor input (and neuromuscular compensation) and reflex driven muscle activation leading to a large decrement in electromyogram and ultimately airway collapse. 5-HT plays an important role in the patency of the upper airway. 5-HT excites adult upper airway dilator motor neurons (17, 18) and provides intrinsic excitation at brainstem motor neurons in

unanesthetized animals (19). The activity of neurons supplying 5-HT to motor neurons declines in sleep (20, 21). Furthermore, pretreatment of upper airway dilator motor neurons with 5-HT reduces sleep state-dependent suppression in upper airway dilator muscle activity (29).

5-HT acts through a large family of receptors (30). The 5-HT 2A/2C receptor subtype plays an important role in the maintenance of upper airway stability and normal breathing in obesity. 5-HT 2A is the predominant excitatory 5-HT receptor subtype at the hypoglossal motor neurons (31). Polymorphisms in the HTR2A and HTR2C are associated with various diseases. There was, however, no association of HTR2A 102T/C and HTR2C 796G/C polymorphisms and the development of OSA in the present study. A recent study employing a highly accurate quantitative allele-specific primer extension assay suggests that the HTR2A locus is unlikely to contain common polymorphisms or epigenetic modification that alters HTR2A mRNA levels in adult brain (32). Although 5-HT 2A receptor predominantly contributes to 5-HT excitation within a representative upper airway motor nucleus, lack of common polymorphisms or epigenetic modification that alters HTR2A mRNA levels might be the reason for the absence of an association of the 102T/C polymorphism with AHI in this population. The serotonin 2C excitatory effects are of lower magnitude (31). Although 796G/C polymorphism of the HTR2C was functional (33), the effect of an active allele of the polymorphism might not be sufficient for the SNP to be associated with AHI because of less dominance of the subtype in the hypoglossal nucleus.

The variability in airway size is probably determined by genetic influences on bony structure, tongue size and tonsillar tissue as well as acquired factors such as obesity. Obesity may affect pharyngeal size by fat deposition around the upper airway or by altering muscle orientation and function. Narrowing of the pharyngeal airway as a result of alterations in craniofacial morphology has also been suggested in the etiology of OSA (34). A number of factors combine to determine airway size which has an impact on the propensity for pharyngeal collapse and apnea during sleep. Although genetic polymorphisms of the HTR2A and HTR2C genes may play an important role in the activity of the pharyngeal dilator muscles, other variables such as cephalometric features and soft tissue structures might be determinants for patency of the human upper airway during sleep.

Kadotani and colleagues reported an association between the ApoEε4 allele and SDB in a study of 791 Caucasian adults in Wisconsin (35). However, others could not replicate their results in different genetic populations (36, 37). Recently, a whole genome scan for OSA and obesity in African-American families identified a candidate region on chromosome 8q for linkage to the AHI (38). HTR2A and HTR2C are on chromosome 13q and Xq, respectively, and not on chromosome 8q. Comparison of Far-East Asian men with Caucasian men demonstrated that Asian men had greater severity of OSA and less obesity suggesting the etiology of sleep disordered breathing in Asians may differ

from that of Caucasians (39). Therefore, the results of the whole genome scan should be further reconfirmed in patients with SDB of different races.

This is the first report to study genetic association of HTR2A and HTR2C with SDB. There are some limitations to our study. The present study was not powerful enough to confirm a negative association between the HTR2A polymorphism and OSA. Our controls have not undergone PSG and OSA was clinically excluded by the criteria of self report scores by Kapuniai et al (23), which can correctly identify 70–76% of sleep apnea cases with AHI >5 with predictive accuracy of 88% for AHI >10. Our control group might represent Japanese male subjects without OSA, but a further study with a larger number of patients with OSA and control subjects whose AHI was proved to be less than five by PSG should be performed.

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