A Functional Variation in the Hypocretin Neuropeptide Precursor Gene May Be Associated With Obstructive Sleep Apnea Syndrome in Japan

Wael A. Ahmed, MD, PhD; Makiko Tsutsumi, PhD; Seiichi Nakata, MD, PhD; Terumi Mori, MSc; Yoichi Nishimura, MD; Toshiyuki Fujisawa, MD; Ichiro Kato, MD; Mayuki Nakashima, MD, PhD; Hiroki Kurahashi, MD, PhD; Kenji Suzuki, MD, PhD

OBJECTIVES/HYPOTHESIS: To evaluate the association of hypocretin neuropeptide precursor gene (HCRT) variations with obstructive sleep apnea syndrome (OSAS) in a cohort of Japanese patients and to further evaluate whether the significant HCRT variations have potential functional consequences on HCRT expression.

STUDY DESIGN: Case-control genetic association study.

METHODS: We studied the genetic variations within the HCRT gene. The study population consisted of 100 OSAS patients and 100 control subjects. The HCRT gene was amplified by polymerase chain reaction in all study subjects followed by direct sequencing and analysis of sequencing data.

RESULTS: Two genetic variations within the HCRT intron, IVS1+16T>C (rs9902709) and IVS1–69G>C, were identified with significant differences between patients and controls (P < .05). A reporter gene assay using HeLa cells showed that the construct containing the C allele of the rs9902709 variation had significantly higher luciferase activity compared with the construct containing the T allele (P = .002). Furthermore, enzyme immunoassay revealed that subjects with T/C and C/C genotypes for rs9902709 had 1.4-fold and 1.5-fold increases in sera levels of orexin-A, respectively.

CONCLUSIONS: Our genetic association study, followed by functional and quantitative phenotyping assays, demonstrated a functional locus within the HCRT gene, which may act to increase HCRT expression and lead to a protective effect against the development of OSAS.

KEY WORDS: Obstructive sleep apnea syndrome, genes, hypocretin neuropeptide precursor gene, reporter gene assay, functional variation.

LEVEL OF EVIDENCE: 2b


INTRODUCTION
Obstructive sleep apnea syndrome (OSAS) is a relatively common sleep disorder that is characterized by recurrent episodes of partial or complete collapse of the upper airway during sleep, usually in association with loud snoring and daytime sleepiness.1 Physiologically, such episodes are often associated with arousals, sleep fragmentation, intermittent hypoxia and hypercapnia, and nocturnal hypertension. Diagnosis is based on standard clinical criteria and is generally validated by an overnight sleep study with measurement of the apnea-hypopnea index (AHI), which is the number of apneas and hypopneas per hour of sleep.2 Estimates of OSAS prevalence are approximately 3% to 7% for adult men and 2% to 5% for adult women in the general population.3 OSAS is associated with substantial comorbidity, including obesity, hypertension, diabetes, and cardiovascular diseases.4,5 Thus, accurate diagnosis and proper management are crucial.

The pathogenesis of OSAS is not fully understood at the molecular level. Several genetic and epidemiologic studies have indicated that the risk of OSAS is related to multiple genetic, environmental, and developmental factors.6–8 Identification of susceptible genes for OSAS is expected to elucidate new pathophysiologic mechanisms of the disease and to lead to the development of novel preventive and curative measures. There are four primary (intermediate) pathogenic pathways through which genetic factors might act to increase the
susceptibility to OSAS.9 These pathways include upper airway anatomy and craniofacial form, ventilatory control and upper airway collapsibility, body fat distribution, and sleep-wake control.

Hypocretin-1 and -2 (also known as orexin-A and -B) are newly discovered neuropeptides processed from a common precursor, preprohypocretin (also known as prepro-orexin),10,11 which is encoded by the hypocretin neuropeptide precursor gene (HCRT). Hypocretin-containing cells are located exclusively in the posterolateral hypothalamus, with widespread projections to the entire neuroaxis. The linkage of OSAS to the hypocretin/orexin system is complex and based on different pathophysiological mechanisms. The peptides of that system have pleiotropic effects on a wide range of physiologic and behavioral processes in mammals.12,13 Some of these processes, such as feeding and sleep regulation, muscle tone, and autonomic and neuroendocrine functions, may be of relevance for the pathogenesis of OSAS. Furthermore, close proximity of hypocretin/orexin system neurons to central respiratory control centers facilitates potential interactions between arousal and respiratory systems.

Several clinical studies have shown abnormally low levels of circulating hypocretin-1 (orexin-A) in patients with OSAS, independent of the level of somnolence and/or presence of obesity.14–16 and it was concluded by those studies that the low hypocretin-1 levels might be related to the pathogenesis of OSAS. However, to our knowledge, there has been no relevant study concerning genetic variations in HCRT in OSAS.

In this study, we investigated the association of HCRT variations with OSAS in a cohort of Japanese patients, and we further evaluated whether the significant HCRT variations have potential functional consequences on HCRT expression.

MATERIALS AND METHODS

Subjects
A total of 100 consecutive unrelated Japanese patients with OSAS (94 men, 6 women; mean age ± standard deviation [SD] = 47.89 ± 10.82 years) were involved in the study. Mean body mass index (BMI) was 27.44 kg/m². The diagnosis of OSAS was based on history including the Berlin questionnaire adopted by Netzer et al.17 All of them were shown to have a high risk for OSAS, otorhinolaryngologic examination including fiberoptic endoscopy while patient was awake and during drug-induced sedation, and overnight polysomnography. Patients who had AHI > 15 were selected. The mean ± SD of AHI was 41.68 ± 18.11 events per hour.

Another group was comprised of 100 healthy Japanese subjects (90 men and 10 women; mean age ± SD = 45.21 ± 9.61 years) who were recruited as a control group. Mean BMI was 26.3 kg/m². They were chosen from among university staff and volunteers. The control subjects were chosen on the basis of the following criteria: no symptoms related to disordered breathing during sleep; matching sex, age, and BMI to the patient group; no abnormal findings during routine otorhinolaryngologic examination; and according to the Berlin questionnaire, they were shown to have a low risk for OSAS.

All patients and control subjects were from central Japan. Written informed consent was obtained from all participants after they had received full written and verbal information, and the patients’ anonymity was preserved. The study was approved by the Ethics Review Board for Human Genome Studies at Fujita Health University (approval number 49).

DNA Analysis
Peripheral blood leucocytes or exfoliated buccal cells were obtained as a source of genomic DNA from all study subjects. Either Puregene DNA Purification Kit (Gentra, Minneapolis, MN) or QuickGene DNA Whole Blood Kit (Fuji Photo Film Co. Ltd., Tokyo, Japan) was used for DNA extraction from blood leucocytes. QIAamp DNA Mini Kit (Qiagen K.K., Tokyo, Japan) was used for DNA extraction from buccal cells.

Both sense (5'-CGATTTCAACCCGAAAAGCTAAG-3') and antisense (5'-CAGACAAACGCGAAGTCTAAGTACG-3') oligonucleotide primers were designed to amplify a DNA fragment having a length of 1,628 base pairs (bp) covering the genomic region between 64 bp upstream of exon 1 and 232 bp downstream of exon 2 of the human HCRT (GenBank accession no.AF118885) (Fig. 1). Polymerase chain reaction (PCR) was performed under standard conditions. Unincorporated primers and excess deoxynucleotides were removed by incubation of PCR products with exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT; USB Corp., Cleveland, OH). The genotypes were determined by sequencing the amplified products using a commercial kit and analyzer (Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit; ABI PRISM 3100 Genetic Analyzer; PE Applied Biosystems, Foster City, CA) with sequencing primers (5'-GCTGTCTCTCCGCAAGACG-3', 5'-GCAATTCTGGAGTCTTGCG-3', and 5'-CTCAAGCGCTGAGCTCCTC-3').

RNA Analysis
A genomic region including the exon 1, intron 1, and exon 2 of the HCRT gene was amplified using appropriate primers. The PCR templates were obtained from the genomic DNA of a subject known to be heterozygous for the IVS1+16T>C variation as well as from the genomic DNA of a subject known to be heterozygous for the IVS1-69G>C variation. The resulting PCR products were cloned into the pFLAG-CMV-2 expression vector (Sigma, St. Louis, MO) in the same orientation as that of the original genes.

Three different constructs were selected for subsequent transfection. These constructs include a wild construct (TG construct), which had the major alleles of both variations (IVS1+16T>C and IVS1-69G>C) and two variant constructs (CG and TC constructs).
Each of the variant constructs had the minor allele of either IVS1-16T>C or IVS1-69G>C variations, respectively. The selected constructs were transiently transfected into SH-SY5Y or COS-7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer instructions. After 48 hours, the cells were harvested, and total RNA extracts were isolated with RNeasy Kit (Qiagen). After treatment with DNase I, the first strand cDNAs were synthesized with the use of an oligo (dT) primer, and Superscript III (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using primers designed with vector sequences. The PCR products were then resolved in 2% agarose gels and evaluated with Image J software.

Gene Reporter Assay

The luciferase assay was used as a reporter system to assess functional consequences of the identified variations to the transcriptional activity. The HCRTR1 intronic region (from +22 to +837) was amplified from the genomic DNA of a subject known to be heterozygous for the IVS1-16T>C variation as well as from genomic DNA of a subject known to be heterozygous for the IVS1-69G>C variation. The resulting PCR products were subcloned into the firefly luciferase expression vector, the pGL3-Promotor Vector containing an SV40 promoter (Promega, Madison, WI), in the same orientation as that of the original gene. Three different constructs were selected for subsequent transfection. These constructs include a wild construct (TG construct), which had the major alleles of both variations (IVS1-16T>C and IVS1-69G>C), and two variant constructs (CG and TC constructs). Each of the variant constructs had the minor allele of either IVS1-16T>C or IVS1-69G>C variations, respectively. The selected constructs were transiently transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer instructions. The Renilla luciferase expression vector pRL-TK (Promega) was co-transfected as an internal standard. After 48 hours, the cells were harvested and the luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Each experiment was independently repeated three times, and each sample was studied in triplicates.

Measurement of Serum Hypocretin-1 (Orexin-A) Concentration

Serum concentrations of hypocretin-1 (orexin-a) were measured in nine subjects. They were classified according to the transcriptional activity.

RESULTS

TABLE I. Genotype Distributions, Allele Frequencies, and Dominant or Recessive Model for the Identified HCRT Variations in Control Subjects and Patients With Obstructive Sleep Apnea Syndrome.

<table>
<thead>
<tr>
<th>Variations</th>
<th>Control, No. (%)</th>
<th>Patients, No. (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS1+16 T&gt;C (rs 9902709)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>89 (89)</td>
<td>89 (89)</td>
<td>.08</td>
</tr>
<tr>
<td>T/C</td>
<td>7 (7)</td>
<td>11 (11)</td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>4 (4)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td>.32</td>
</tr>
<tr>
<td>T</td>
<td>185 (92.5)</td>
<td>189 (94.5)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15 (7.5)</td>
<td>11 (5.5)</td>
<td></td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td>.04</td>
</tr>
<tr>
<td>T/T + T/C</td>
<td>96 (96)</td>
<td>100 (100)</td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>4 (4)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>IVS1-69G&gt;C</td>
<td></td>
<td></td>
<td>.02</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>95 (95)</td>
<td>100 (100)</td>
<td></td>
</tr>
<tr>
<td>G/C</td>
<td>5 (5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td>.02</td>
</tr>
<tr>
<td>G</td>
<td>195 (97.5)</td>
<td>200 (100)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5 (2.5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td>.02</td>
</tr>
<tr>
<td>G/G</td>
<td>95 (95)</td>
<td>100 (100)</td>
<td></td>
</tr>
<tr>
<td>C/C + G/C</td>
<td>5 (5)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

All statistical comparisons were done with the \( \chi^2 \) test.

Statistical Analysis

Comparisons of genotype distributions and allele frequencies between the OSAS patients and control subjects were done with a \( \chi^2 \) test by using SNPAlyze software version 6.0 (Dynamome, Yokohama, Japan).

For luciferase reporter gene assay, the unpaired Student \( t \) test was applied to compare relative luciferase activity between each type of variant constructs and the wild construct. For comparison of sera concentrations of hypocretin-1 (orexin-A) between studied subjects, the Mann-Whitney \( U \) test was used. Tests were done using the Statistical Package for Social Sciences (SPSS) version 16 (SPSS Inc., Chicago, IL) for Windows system. All statistically significant \( P \) values were set at <.05.
To investigate the effects of these variants on the function of the HCRT gene, we first examined how the rs9902709 and IVS1–69G>C variations affect splicing events during HCRT transcription. We constructed expression vectors encoding HCRT gene with and without these variations. Then these vectors were introduced into two different types of cultured cell lines. RT-PCR analysis revealed no aberrant HCRT-PCR products due to abnormally spliced transcripts (data not shown).

Next, to determine whether these two identified variations in HCRT would affect its expression level, we constructed three plasmids with a genomic fragment (HCRT intron) containing both variations upstream of a luciferase gene transcriptional unit. The construct containing the minor allele of the rs9902709 variation (CG construct) showed a significant increase in the luciferase activity (P = .002) compared to the wild construct (TG construct). On the other hand, the construct containing the minor allele of the IVS1–69G>C variation (TC construct) did not show any significant difference (P = .2) in luciferase activity when compared with the wild construct (Fig. 2). These results suggest that the C allele of the rs9902709 variation may be associated with increased transcriptional activity of the HCRT gene.

Finally, we performed quantitative phenotypic assessment to evaluate whether the different genotypes of the rs9902709 variation would affect the serum concentration of hypocretin-1 (orexin-A). We collected sera samples from nine control subjects who were classified into three groups according to the rs9902709 genotype and compared the sera concentrations of hypocretin-1 between different groups. Although we did not notice a significant difference among the three groups, we found that subjects with C/C (minor allele homozygous) and T/C (heterozygous) genotypes had higher sera concentrations of hypocretin-1 by 1.3-fold and 1.4-fold, respectively, than the subjects with T/T (major allele homozygous) genotype (Fig. 3).

**DISCUSSION**

Hypocretin/orexin is a neurotransmitter known to have pleiotropic effects on several aspects of the OSAS phenotype. It has important effects on regulation of food intake and sleep/wake regulation, and recently it has been implicated in influencing upper airway neuromuscular activity. Circulating hypocretin levels in OSAS patients have been reported to be significantly lower than in normal subjects and to be inversely correlated with OSAS severity.

The present study systematically investigated the relationship between HCRT variations and OSAS through direct gene sequencing, and demonstrated two significant variations (rs9902709 and IVS1–69G>C). The C/C genotype of rs9902709 as well as the G/C
genotype IVS1–69G>C were associated with a decreased risk for OSAS (i.e., identified only in control subjects). Neither of the variations showed effects upon normal splicing in our assay system using two different types of cell lines. However, the construct containing the C allele of rs9902709 showed significant increase in gene expression compared with other constructs in the luciferase assay in vitro. Furthermore, subjects with T/C and C/C genotypes (i.e., C allele carriers) had 1.4-fold and 1.5-fold increases in sera levels of orexin-A, respectively. These evidences from both genotypic and phenotypic analyses in our study may suggest a role for hypocretin/orexin agonists as a novel therapy for management of OSAS.

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**BIBLIOGRAPHY**