Association Between *CLOCK* Gene Variants and Restless Legs Syndrome in Koreans

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Objective Previous studies have suggested various causes of restless legs syndrome (RLS), including iron and dopamine concentrations in the brain. Genetic influences have also been reported in many studies. There is also a possibility that circadian clock genes may be involved because symptoms of RLS worsen at night. We investigated whether *CLOCK* and *NPAS2* gene polymorphisms were associated with RLS.

Methods A total of 227 patients with RLS and 229 non-RLS matched controls were assessed according to the International Restless Legs Syndrome Study Group diagnostic criteria. Genotyping was performed using reverse transcription polymerase chain reaction and high-resolution melting curve analyses.

Results Although the genotype distributions of the *CLOCK* variants (rs1801260 and rs2412646) were not significantly different between patients with RLS and non-RLS controls, the allele frequencies of *CLOCK* rs1801260 showed marginally significant differences between the two groups (X^2=2.98, p=0.085). Furthermore, there was a significant difference in the distribution of *CLOCK* haplotypes (rs1801260-rs2412646) between patients with RLS and non-RLS controls (p=0.013). The distributions of allelic, genotypic, and haplotypic variants of *NPAS2* (rs2305160 and rs6725296) were not significantly different between the two groups.

Conclusion Our results suggest that *CLOCK* variants may be associated with decreased susceptibility to RLS.

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Keywords *CLOCK* gene; Circadian rhythm; Restless legs syndrome.

INTRODUCTION

Restless legs syndrome (RLS), also called Willis-Ekbom disease, causes unpleasant or uncomfortable sensations in the legs and an irresistible urge to move them. RLS can cause severe sleep disturbance, depression, anxiety, and poor quality of life; it can also be a risk factor for cardiovascular disease.[1-3] A recent epidemiologic study reported that the prevalence of RLS in the Korean population is 7.5%, but only 24.3% of those diagnosed with RLS are treated for their symptoms.[4,5]

Although the pathophysiological mechanism of RLS is still unknown, evidence suggests a genetic involvement in the disease. A family history of RLS has been reported in 60% of patients with idiopathic RLS,[6,7] and linkage analyses in families have identified seven genetic loci associated with RLS: *RLS1* on chromosome 12q12−q21, *RLS2* on 14q13−21, *RLS3* on 9p24−p22, *RLS4* on 2q33, *RLS5* on 20p13, *RLS6* on 19p13, and *RLS7* on 16p12.1.[8-14] Several gene variants have been found to be associated with RLS risk, most of which in genome-wide association studies (GWAS), with the strongest candidates being variants of *PTPRD*, *BTBD9*, *MEIS1*, and *MAP2K5/SKOR1* genes in Caucasian populations.[15-17]

Among these genes, *MEIS1* is the most likely cause of RLS in Caucasian populations,[18] but studies in Asia have shown a stronger link between *BTBD9* or *MAP2K5/SKOR1* genes.[19,20]

A GWAS of a Korean population reported that the *UTRN* gene showed a significant association with RLS.[20] This suggests the possibility of racial differences in the genetic influences on RLS. One essential diagnostic characteristic of RLS is the presence of circadian variation in symptoms, and the diagnostic criteria of RLS also reflect this. However, the mechanisms underlying these circadian variations remain unknown. Because dopaminergic mechanisms seem to play a central role in the pathophysiology of RLS, changes in the circadian regulation of the central dopamine system or factors affecting it are likely to cause the worsening of symptoms during the nighttime.[21] Because RLS severity varies with the circadian pattern, with the maximum number of occurrences in the late evening or at night, circadian genes can be candidate genes for RLS.

Although RLS exhibits clear circadian symptom fluctuations, circadian genes have not been reported to be associated with primary RLS in previous studies. In a study done by Kripke et al.,[22] *CLOCK*, *TEF*, and *NR1D1* were found to be associated with periodic limb movement index, number of RLS diagnostic questions answered affirmatively, and reported frequency of RLS symptoms, respectively, when the possible confounding factor of apnea was minimized by selecting participants with apnea-hypopnea index<15. Such associations were not, however, observed when answering yes to all four diagnostic criteria or not was used for group determination. There have been studies on the association between circadian genes and primary and secondary RLS in special populations: *CLOCK*, *BTBD9*, *GNB3*, and *TH* were associated with antipsychotic-induced RLS in Korean schizophrenia patients, and TEF was associated with sleep disturbances, including RLS symptoms, in Chinese Parkinson's disease patients.[23,24] Overall, an association between circadian genes and primary RLS as a distinct diagnostic syndrome in the general population has not been thoroughly examined.

CLOCK, as a component of CLOCK:BMAL1 heterodimer, plays a major role in the transcriptional feedback loop generating circadian rhythm. NPAS2 is the paralog of CLOCK and can substitute for CLOCK. *CLOCK* genes are involved in the circadian rhythmicity of melatonin synthesis and secretion, and the significant differences of *CLOCK* gene variants in Korean schizophrenia patients with RLS were postulated to be the result of melatonin's circadian modulation.[23,25] We hypothesized that the circadian rhythmicity of primary RLS is likewise under the control of circadian genes, including *CLOCK* and *NPAS2*, a functional substitute of *CLOCK* in the suprachiasmatic nucleus, via melatonin modulation.[26] Therefore, we investigated whether there was an association between circadian gene (*CLOCK* and *NPAS2*) polymorphisms and primary RLS.

METHODS

Study participants

All subjects were recruited from multiple centers, including the Korea University Hospital Sleep Clinic and two private sleep clinics in Seoul, South Korea. All subjects visited the sleep clinics because of insomnia. Sleep specialists reached a diagnosis based on the subject's signs and symptoms, medical and family histories, physical examinations, and test results. They also ruled out other conditions that may result in symptoms similar to those of RLS. Sex- and age-matched healthy controls were selected based on RLS symptom evaluation and medical and family histories. All subjects and controls were assessed using the International Restless Legs Syndrome Study Group (IRLSSG) diagnostic criteria. Finally, there were 227 patients with RLS and 229 non-RLS matched controls from the general population. Written informed consent was obtained from all study participants, including the patients with RLS and non-RLS matched controls, and approval was obtained from the Institutional Review Board (IRB) of Korea University Hospital (IRB No. IEC107014).

Symptom assessment

RLS was assessed using the IRLSSG diagnostic criteria. All subjects were asked about the following four diagnostic criteria for RLS: 1) urges to move their legs, 2) unpleasant sensations in their legs, 3) symptoms worsening during rest and relieved by movement, and 4) symptoms worsening in the evening or at night.[27] Unpleasant sensations caused by other kinds of medical, surgical, or psychiatric problems were not considered to be symptoms of RLS. All the four essential diagnostic criteria had to be met for the subjects to be diagnosed with RLS.

Non-RLS-matched controls were recruited from the general population. Controls were also assessed using the IRLSSG diagnostic criteria to exclude RLS diagnosis. RLS symptom severity was assessed using the IRLSSG rating scale.[28]

Genotyping

A total of 456 participants were genotyped for this study. Blood was collected in 5-mL EDTA vacutainers (BD, Plymouth, UK) and stored at -80°C until genotyping. Genomic DNA was extracted using a blood DNA purification kit (Cosmo Genetech Co. Ltd., Seoul, Korea). After quality assessment with a Nano-Drop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), genotyping was performed using high-resolution melting (HRM) curve analysis. PCR was performed in a volume of 20 mL per reaction with a 96-well Bio-Rad CFX96 Real Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Reaction mixtures included 1.5 mL of genomic DNA as a template, 1×Sso Fast EvaGreen SuperMix (Bio-Rad), 200 mM of each primer, and sterile H_2O. PCR primers for *CLOCK*

and *NPAS2* genes were as follows: *CLOCK* rs1801260 forward: 5'-GGGAAAGTTCCAGCAGTT-3', reverse: 5'-ATCCAGG CACCTAAAACAC-3'; rs2412646 forward: 5'-TGGAGTAA GTTGTTACACAGC-3', reverse: 5'-TGGGAAATTATAGTC AGAATTGGAA-3'; NPAS2 rs2305160 forward: 5'-GATTTG TGGGAACTTCTTGAG-3', reverse: 5'-ATGAGGACTGTT TGATGTGT-3'; and rs2305160 forward: 5'-ACTTCCAGA CCTGTGAT-3', reverse: 5'-GCTTTACATCCATTCATTCC TTC-3'. The amplification protocol was started at 98°C for 3 min, followed by 39 cycles of 98°C for 10 s and 58°C for 20 s. After an initial step of 95°C for 10 s and 65°C for 10 s, melting curves were generated from 65°C to 95°C in increments of 0.3°C/cycle. Melting profiles were analyzed using the Bio-Rad Precision Melt software. The Hardy-Weinberg equilibrium test was performed to assess the goodness of fit of the data.

Statistical analysis

The Hardy-Weinberg equilibrium was tested using the χ^2 test for goodness of fit. To compare the baseline characteristics between the patients with RLS and non-RLS controls, χ^2 and t-tests were performed for categorical variables and continuous variables, respectively. All analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA) and SNPAlyze (DYNA-COM, Chiba, Japan). Haplotype analysis was performed using the SNPAlyze software. Differences were considered statistically significant at p values less than 0.05, and those less than 0.10 were considered marginally significant. Power calculations were performed using G*Power 3.1.4 (Dusseldorf, Germany).[29]

RESULTS

Demographic and clinical features of the study participants

There were no significant differences in age or sex ratio between patients with RLS and controls (χ^2=2.52, p=0.113; t= 1.59, p=0.112). The severity of the RLS symptoms evaluated using the IRLSSG Rating Scale was on average 22.16±8.20. Among the 227 patients with RLS, 58 had a family history of RLS in the first-degree relatives (23.60%). Table 1 shows the demographic and clinical features of the study participants.

Genotype distributions and allele frequencies of the *CLOCK* and *NPAS2* genes

No deviations from Hardy-Weinberg equilibrium were observed in any of the variants of *CLOCK* and *NPAS2* genes between the patients with RLS and non-RLS controls. Table 2 shows the genotype distributions according to the general genetic model, dominant model, and recessive model, and allele frequencies of *CLOCK* and *NPAS2* variants. Although the gen-

Table 1. Demographic and clinical features of study participants: patients with RLS and non-RLS controls

	Patients with RLS (N=227)	Non-RLS controls (N=229)	p
Female/male	153/74	138/91	0.113
Age, years	49.45 (15.74)	47.21 (14.21)	0.112
Family history	58 (25.55)	-	-
RLS severity*	22.16 (8.20)	-	-

Data are presented as the N (%) or mean (SD). *assessed using the International RLS Study Group (IRLSSG) Rating Scale. RLS, restless legs syndrome

otype distributions (general genetic, dominant, and recessive models) of the two *CLOCK* variants (rs1801260 and rs2412646) were not significantly different between the patients with RLS and non-RLS controls, the allele frequencies of *CLOCK* rs1801260 were marginally significantly different between the two groups (χ^2=2.98, p=0.085). The genotype distributions (general genetic, dominant, and recessive models) and allele frequencies of the two variants of *NPAS2* (rs2305160 and rs6725296) did not show any significant differences between the two groups.

Haplotype analysis

Global haplotype analysis for the two variants of *CLOCK* (rs1801260-rs2412646) revealed a statistically significant difference in haplotype frequency between the patients with RLS and non-RLS controls (permutated p=0.013) (Table 3). We did not perform *NPAS2* gene haplotype analysis of the *NPAS2* gene because there were no significant differences in the genotype and allele distributions between the two groups.

DISCUSSION

Typically, RLS symptoms appear and worsen during the evening and at night. Due to this characteristic diurnal variation in symptom intensity, many studies have suggested that RLS symptoms may be modulated by circadian rhythmicity.[30-32] In addition, RLS has been reported to have a strong genetic component.[33,34] Specifically regarding circadian rhythm genes, a study by Jung et al.[23] found significant differences in the genotype and haplotype frequencies of *CLOCK* gene variants between patients with RLS and non-RLS controls in Korean patients with schizophrenia. In the present study, we investigated the association between RLS and *CLOCK* and *NPAS2* polymorphisms in the general Korean population. We observed genetic associations only in the *CLOCK* gene, and significant differences in allele frequency and haplotype frequency between patients with RLS and non-RLS controls. The risk for RLS tended to be lower among those with the G allele of *CLOCK* rs1801260, and especially among those with the G-T haplotype of *CLOCK*

(rs1801260-rs2412646). Therefore, our results suggest that these *CLOCK* gene variants may also have a protective effect against RLS, leading to decreased susceptibility to RLS.

The characteristic circadian rhythmicity of RLS indicates that RLS may be under the influence of circadian genes, and such circadian control may be modulated through melatonin. The pathophysiology of RLS remains largely unknown. Dopamine dysfunction and iron deficiency in the brain are the most wide-

Table 2. Results of case-control association analysis of SNPs between patients with RLS and non-RLS controls

	RLS cases (N=227)	Non-RLS controls (N=229)	p-value		RLS cases (N=227)	Non-RLS controls (N=229)	p-value
CLOCK rs1801260				CLOCK rs2412646			
Genotype			0.232	Genotype			0.783
A/A	180 (79.29)	167 (72.93)		C/C	123 (54.18)	117 (51.09)	
A/G	44 (19.38)	56 (24.45)		C/T	88 (38.77)	96 (41.92)	
G/G	3 (1.32)	6 (2.62)		T/T	16 (7.05)	16 (6.99)	
Dominant model			0.703	Dominant model			0.883
AA	180 (79.30)	167 (72.93)		CC	123 (54.18)	117 (51.09)	
AG+GG	47 (20.70)	62 (27.07)		TC+TT	104 (45.81)	112 (48.91)	
Recessive model			0.498	Recessive model			1.010
GG	3 (1.32)	6 (2.62)		TT	16 (7.05)	16 (6.99)	
AA+AG	224 (98.68)	223 (97.38)		CC+TC	211 (92.95)	213 (93.01)	
Allele			0.085*	Allele			0.607
A	404 (88.99)	390 (85.15)		C	334 (73.57)	330 (72.05)	
G	50 (11.01)	68 (14.85)		T	120 (26.43)	128 (27.95)	
NPAS2 rs2305160				NPAS2 rs6725296			
Genotype			0.333	Genotype			0.858
A/A	9 (3.96)	12 (5.24)		A/A	7 (3.08)	8 (3.49)	
A/G	72 (31.72)	85 (37.12)		A/G	61 (26.87)	66 (28.82)	
G/G	146 (64.32)	132 (57.64)		G/G	159 (70.04)	155 (67.68)	
Dominant model			0.755	Dominant model			0.896
GG	146 (64.32)	132 (57.64)		GG	159 (70.04)	155 (67.68)	
AA+AG	81 (35.68)	97 (42.36)		AA+AG	68 (29.96)	74 (32.31)	
Recessive model			0.747	Recessive model			0.879
AA	9 (3.96)	12 (5.24)		AA	7 (3.08)	8 (3.49)	
AG+GG	218 (96.03)	217 (94.76)		AG+GG	220 (96.91)	221 (96.51)	
Allele			0.146	Allele			0.580
A	90 (19.82)	109 (23.19)		A	75 (16.52)	82 (17.90)	
G	364 (80.18)	349 (74.25)		G	379 (83.48)	376 (82.10)	

Data are presented as N (%). *0.05<p<0.1. SNP, single nucleotide polymorphism; RLS, restless legs syndrome

Table 3. Haplotype analysis of *CLOCK* rs1801260-rs2412646 and comparison between patients with RLS and non-RLS controls

CLOCK haplotype	Overall	Non-RLS controls	Patients with RLS	Permutation p-value
A-C	0.6126	0.6049	0.6256	0.520
A-T	0.2581	0.2467	0.2643	0.536
G-C	0.1155	0.1157	0.1101	0.795
G-T	0.0139	0.0328	9.94E-09	<0.001
Select locus		Permutation p-value		
CLOCK rs1801260/CLOCK rs2412646		0.013		

RLS, restless legs syndrome

ly accepted pathways for understanding RLS.[35] Michaud et al.[31] found that melatonin secretion increased just before sensory and motor symptoms in patients with RLS. Melatonin, with its known inhibitory effect on dopamine release,[36] may be the key element in disentangling the underlying mechanism of circadian rhythmicity in RLS. Often used as a chronotherapeutic agent for various sleep-wake disorders, melatonin worsened motor symptoms in RLS patients when administered exogenously, possibly by further decreasing dopaminergic function.[37] Exogenous administration of melatonin in the evening also causes phase advance,[38] increasing melatonin levels earlier during the day, potentially leading to earlier manifestation of RLS symptoms. This may uncover previously unrecognized symptoms by introducing symptoms before the initiation of sleep. Therefore, it is of great importance to assess the role of melatonin in the circadian rhythmicity of RLS.

At the genetic level, circadian genes may be one candidate that broadens our understanding of the pathophysiology of RLS. Not only do RLS symptoms have circadian rhythmicity, but melatonin is also under circadian control. Furthermore, melatonin formation is directly affected by the *CLOCK* gene.[25] The *CLOCK* gene, located on chromosome 4q12, is a classic circadian gene in the transcriptional-translational feedback loop of the circadian rhythm. The association between *CLOCK* polymorphisms and melatonin circadian rhythm is yet to be fully investigated. In postmenopausal Caucasian women, the G allele of *CLOCK* rs1801260 was found to be associated with higher nocturnal melatonin levels, lower morning melatonin levels, and the opposite pattern was seen with the A allele.[39] However, in the same study, no significant differences were observed in the Asian population.[39] Our results suggest a protective effect of the G allele of *CLOCK* rs1801260 and especially the G-T haplotype of *CLOCK* (rs1801260-rs2412646), and this effect may be mediated by melatonin circadian rhythmicity. To the best of our knowledge, there is no study on the association between *CLOCK* polymorphisms and melatonin circadian rhythm in the Korean population.

Our study has the following limitations. First, the allele frequencies of *CLOCK* rs1801260 showed marginally significant difference between the patients with RLS and non-RLS controls. Such difference of marginal significance should be carefully considered when interpreting the results. It may be due to a limited sample size, or it may indicate the role of the specific SNP in genetic influence. Since the G-T haplotype of *CLOCK* (rs1801260-rs2412646) resulted in much significant difference, the G allele that showed only marginal significance when examined alone may exert its full effect as a component of a specific haplotype. Second, our sample size may limit the generalization of the study results. We had a power of 0.47 and 0.57 to detect an effect size of 0.10 in genotype and allele carrier frequencies, respectively. Further studies with a larger sample size may be needed for replication of these results. Third, population stratification bias cannot be ruled out. However, this possibility is unlikely due to the relatively highly homogeneous nature of the Korean population.[40] Fourth, a possible mechanism underlying the association between *CLOCK* polymorphisms and RLS symptoms would have been more clearly revealed if melatonin circadian rhythms were also assessed by measuring melatonin levels. Future studies on the role of *CLOCK* polymorphisms and their relationship with melatonin circadian rhythm are warranted to further elucidate the pathophysiology of RLS and the contribution of circadian genes.

In conclusion, *CLOCK* polymorphisms may be associated with RLS in the Korean population. The non-RLS controls had significantly higher frequencies of the G allele of *CLOCK* rs1801260 and the G-T haplotype of *CLOCK* (rs1801260-rs2412646) than those of the patients with RLS. In other words, the G allele of *CLOCK* rs1801260 and especially the G-T haplotype of *CLOCK* (rs1801260-rs2412646) may have protective effects against the development and progression of RLS. We also postulated that this possible preventive effect may be related to the circadian rhythm of melatonin. Further research is needed to fully examine the relationship between circadian rhythm genes and the pathophysiology of RLS.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Conflicts of Interest

Author Contributions

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