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

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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 (X²=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 haplotype 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.

Keywords CLOCK gene; Circadian rhythm; Restless legs syndrome.
A GWAS of a Korean population reported that the UTRN gene showed a significant association with RLS. 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. 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., CLOCK, TEF, and NR1D1 were found to be associated with periodic limb movement index, number of RLS diagnosis, and Restless Legs Syndrome (RLS) severity. These results suggest that circadian genes could be potential candidate genes for 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. 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.

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 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), genotyping was performed using high-resolution melting (HRM) curve analysis. PCR was performed in a volume of 20 μL per reaction with a 96-well Bio-Rad CFX96 Real Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Reaction mixtures included 1.5 μL of genomic DNA as a template, 1×Sso Fast EvaGreen SuperMix (Bio-Rad), 200 mM of each primer, and sterile H2O. PCR primers for CLOCK and Restless Legs Syndrome

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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. 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. Therefore, we investigated whether there was an association between circadian gene (CLOCK and NPAS2) polymorphisms and primary RLS.
and NPAS2 genes were as follows: CLOCK rs1801260 forward: 5’-GGGAAAGTCCACGATT-3’, reverse: 5’-ATCCAGG CACCTAAAACAC-3’; rs2412646 forward: 5’-TGGAGTAA GTGTTACACAGC-3’, reverse: 5’-TGGGAAATTATAGTC AGAATTGGA-3’; NPAS2 rs2305160 forward: 5’-GATTTG TGGGACCTTCTTGAG-3’, reverse: 5’-ATGAGGACGTGG TGAAGTGTG-3’; and rs2305160 forward: 5’-ACTTCCCGA CCTGTGAT-3’, reverse: 5’-GCTTTACATCCATTTCC 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 χ² test for goodness of fit. To compare the baseline characteristics between the patients with RLS and non-RLS controls, χ² 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 (DYNACOM, 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 (χ²=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 genotype 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 (χ²=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 (permuted 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 genotypes 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.
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(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

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

| 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. Michaud et al. found that melatonin secretion increased just before sensory and motor symptoms in patients with RLS. Melatonin, with its known inhibitory effect on dopamine release, 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. Exogenous administration of melatonin in the evening also causes phase advance, 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. 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. However, in the same study, no significant differences were observed in the Asian population. 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. 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
Heon-Jeong Lee, a contributing editor of the Psychiatry Investigation, was not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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