INTRODUCTION

Seasonal affective disorder (SAD) is characterized by recurrent seasonal mood changes accompanied by a craving for carbohydrates, hypersomnia, and prominent fatigue [1,2]. The pathophysiology of SAD has been reported to be associated with circadian rhythm disruption, including abnormalities in core body temperature, cortisol, and melatonin levels [3,4]. SAD is also known to have a strong genetic component [5]. In addition to seasonality, the pathological form of which is conceptualized as SAD, the diurnal preference is the preferred time of daily activities for an individual. The diurnal preference is a circadian phenotype that can be divided into three chronotypes based on the concept of circadian typology: morning, neither, and evening [6]. Morning types have an earlier sleep-wake cycle and perform best physically and mentally early in the day, while evening types have a later sleep-wake cycle and perform best toward the end of the day [6]. Several studies have shown that evening types may be associated with numerous neuropsychiatric symptoms and disorders, such as depression, anxiety, bipolar disorder, impulsivity, suicidal behavior, autism, and adult-type attention deficit/hyperactivity disorder [7-10]. The chronotype is also considered related to seasonality; evening types are associated with a higher prevalence of SAD and a higher susceptibility to SAD [11-14].

Circadian rhythms, the common mechanisms underlying seasonality and chronotype, are endogenous rhythms with a period of approximately 24 h. The molecular mechanisms of the suprachiasmatic nucleus, consisting of networks of transcriptional-translational feedback loops of the core circadian clock genes, regulate and maintain the inherent circadian rhythmicity [15,16].
Neuronal PAS family member 2 (NPAS2) is the largest clock gene (176.68 kb) located on chromosome 2p11.2–2q13 [17] and is a homologous gene of the circadian locomotor output cycles kaput (CLOCK) that functionally substitutes for CLOCK to regulate circadian rhythmicity. As a member of the bHLH-PAS family of transcription factors [18], NPAS2 forms heterodimers with other circadian proteins, such as BMAL1. NPAS2-BMAL1 heterodimers transcriptionally target Period genes (PER1, PER2, and PER3) and cryptochrome genes (CRY1 and CRY2) and participate in a feedback loop by binding to E-box (CACGTC) enhancer elements [19,20]. To date, NPAS2 polymorphisms have been reported to be associated with disorders or physiological functions that involve abnormalities in the regulation of circadian rhythms, such as breast cancer, prostate cancer, fertility, seasonality, mood disorders, eating disorders, and chronic fatigue syndrome [21-23]. Particularly, findings that several NPAS2 polymorphisms are related to seasonal variation or SAD are accumulating: NPAS2 rs6725296 [24], and NPAS2 rs2305160 [24,25], Hcv2153849 (Ser471Leu) [1], and NPAS2 rs11541353 [26,27].

As aforementioned, the association between the NPAS2 rs6725296 polymorphism and seasonality has been investigated, and the A allele carriers of NPAS2 rs6725296 were associated with higher seasonality scores on the subscales of weight and appetite [24]. Despite this association, the relationship between NPAS2 rs6725296 polymorphism and diurnal preference remains unclear.

In this study, we examined the NPAS2 rs6725296 polymorphism as a potential marker of seasonality and diurnal preference, considering the relationship between seasonality and chronotype in a young healthy Korean population. We hypothesized that carriers of the A allele at NPAS2 rs6725296 are associated with higher seasonality, as indicated by higher mean total and subscale scores of the Global Seasonality Score (GSS) of the Seasonal Pattern Assessment Questionnaire (SPAQ), and more eveningness, as indicated by lower mean total and subscale scores of the Composite Scale of Morningness (CSM).

**METHODS**

**Subjects**

Participants in this study included 510 healthy Korean adults (male=303, female=207), with a mean age of 23.4 years (SD=2.8, age range=18–35 years). The participants were recruited through an Internet advertisement. All study participants resided in Seoul, and well-trained psychiatrists confirmed that none of the participants had lifetime or current psychiatric disorders using the Mini-International Neuropsychiatric Interview. Subjects diagnosed with major medical or psychiatric disorders and those with a family history of major psychiatric disorders (e.g., schizophrenia or mood disorders) were excluded.

This study was approved by the Ethics Committee of Korea University (IFC No. 1067), and written informed consent was obtained from each participant. This study was conducted following the Declaration of Helsinki. Other findings involving these study subjects have been previously reported [25,28-33].

**Assessment of seasonality**

A sample of 510 Korean adults completed the Korean version of the SPAQ, a self-report questionnaire that is used to assess seasonal variations in sleep, appetite, mood, energy level, weight, and social behavior [34]. The GSS is the sum of the scores of the six subscales mentioned above and can range from 0 to 24, with higher scores indicating higher degrees of seasonal variation. Participants also rated their degree of difficulty in adjusting to seasonal changes on a scale from 0 to 5 (none, mild, moderate, marked, severe, and disabling). According to Kasper’s criteria [35], SAD, subsyndromal SAD, and non-seasonal can be classified using the SPAQ as follows: SAD is identified with a GSS greater than 11 and at least moderate difficulty in seasonal changes; subsyndromal SAD, with a GSS of 9 or 10 and at least mild difficulty or GSS greater than 11 and no or mild difficulty; and non-seasonal, all other cases.

**Assessment of diurnal preference**

The same sample of 510 Korean adults completed the Korean version of the CSM, which was verified by Yoon et al. [36]. The CSM is a self-report scale that is used to assess lifestyle, performance, and sleep-wake patterns. The CSM consists of 13 items, with scores ranging from 13 to 55, with higher scores indicating morning preference and lower scores indicating evening preference. The CSM contains three subscales: subgroup 1 for “morningness” (items 1, 3, 6, 8, 10, and 11), subgroup 2 for “activity planning” (items 2, 7, 9, and 13), and subgroup 3 for “morning alertness” (items 4, 5, and 12).

**Genotyping**

Genomic DNA samples were prepared from blood leukocytes using a QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany). We performed genotyping using a high-resolution melting curve analysis [37]. Polymerase chain reaction (PCR) was performed in a 20-μL reaction mixture and a 96-well CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). The reaction mixture included 2 μL of genomic DNA and 200 mM of primer NPAS2 rs6725296, forward: 5’-ACTTCCCAGACCTGTGAT-3’, reverse: 5’-GCTTTACATCCATTCATTCC TTC-3’. SsoFast EvaGreen SuperMix (1Xfinal concentration; Bio-Rad Laboratories, Inc.), and sterile H2O. The standard protocol for PCR amplification was as follows: an initial denaturation step at 98°C for 3 min, followed by 39 cycles of denaturation at 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. The melting profiles were analyzed using the Bio-Rad Precision Melt software.

**Statistical analyses**

We tested the goodness-of-fit of the Hardy–Weinberg equilibrium using the chi-square (χ²) test. Due to the small number of A/A
genotypes (n=9) in this sample, a Kruskal–Wallis test was performed to examine the association between genotypes (A/A, A/G, and G/G), seasonality, and diurnal preference. For seasonality, the mean total GSS and mean scores of the six GSS subscales were used for the analysis. For diurnal preference, the mean total CSM scores and the mean scores of the three subscales (morningness, morning alertness, and activity planning) were used. In addition, Student’s t-test was performed to investigate the effects of A allele carrier status on the mean total and mean subscale scores of GSS for seasonality and the mean total and mean subscale scores of CSM for diurnal preference. A two-tailed alpha of 0.05 was chosen for the analysis. All statistical analyses were performed with SPSS version 22.0 for Windows (IBM Corp., Armonk, NY, USA).

RESULTS

Genotypic distributions
The genotypic distributions of the NPAS2 rs6725296 polymorphisms in the enrolled participants were in the Hardy–Weinberg equilibrium, and no significant differences were found between the observed and expected genotype counts (χ² =0.7, p=0.4). In addition to genotype comparisons, study subjects were compared according to their allele carrier status. The frequency of the G allele of NPAS2 rs6725296 in our sample population was 88.2%.

Association between NPAS2 rs6725296 polymorphism and seasonality
Using Kasper’s criteria, 61 (12.0%) of the 510 participants were classified as having SAD, and 52 (10.2%) of the 510 participants had subsyndromal SAD. In total, 22.2% of the participants were considered seasonal, including SAD and subsyndromal SAD. No significant differences were found in the GSS between male and female participants.

Table 1 shows the mean total GSS and mean GSS subscale scores according to the genotype and allele carrier status of the NPAS2 rs6725296 polymorphism. A Kruskal–Wallis test was performed for genotypic comparison, and there were no statistically significant differences in the mean total and subscale scores of GSS between genotypes (p=0.55 for total GSS; p=0.31 for sleep duration; p=0.12 for social behavior; p=0.57 for mood; p=0.43 for body weight; p=0.41 for energy level; and p=0.79 for appetite). A Student’s t-test was used to compare allele carrier status, and there were no differences in the mean total and subscale scores of GSS between the A allele carriers and the A allele non-carriers (t=0.68, p=0.50 for total GSS; t=1.23, p=0.22 for sleep duration; t=0.61, p=0.54 for social behavior; t=0.90, p=0.37 for mood; t=0.90, p=0.93 for body weight; t=0.33, p=0.74 for energy level; and t=0.26, p=0.79 for appetite). Although not statistically significant, the A allele non-carriers had higher mean total and subscale scores of the GSS, except for energy level, than the A allele carriers.

Association between the NPAS2 rs6725296 polymorphism and diurnal preference
For the participants, the total CSM score ranged from 14 to 51 (mean=32.1, SD=6.4). There were no significant differences in the total CSM score between male and female participants, although females had higher scores than males (32.0±6.2 for males and 32.3±6.3 for females).

Table 2 shows the mean total CSM scores and mean CSM subscale scores in subject groups according to the NPAS2 rs6725296 polymorphism. No significant differences were found in the CSM total and subscale scores between the A allele carriers and the A allele non-carriers (t=0.37, p=0.74 for morningness; t=0.51, p=0.61 for morning alertness; t=0.19, p=0.82 for activity planning). Although not statistically significant, the A allele non-carriers had higher mean total and subscale scores of the CSM, except for energy level, than the A allele carriers.
scale scores according to the genotype and allele carrier status. A Kruskal–Wallis test was used for genotypic comparison, and there were no statistically significant differences in the mean total and CSM subscale scores between the genotypes (p=0.29 for total CSM, p=0.13 for morningness, p=0.57 for alertness, and p=0.61 for activity planning). A Student’s t-test was performed to compare allele carrier status, and there were no differences in the mean total and CSM subscale scores between the A allele carriers and the A allele non-carriers (t=1.06, p=0.29 for total CSM; t=1.13, p=0.26 for morningness; t=1.01, p=0.31 for morning alertness; and t=0.23, p=0.82 for activity planning). Although not statistically significant, the A allele non-carriers had higher mean total and subscale scores for CSM than the A allele carriers.

DISCUSSION

This study investigated the association between the NPAS2 rs6725296 polymorphism and seasonality and diurnal preference; however, no statistically significant associations were found.

To our knowledge, this is the first study to examine the association between the NPAS2 rs6725296 polymorphism and seasonality and diurnal preference in an Asian population. The finding that there was no significant association between the NPAS2 rs6725296 polymorphism and seasonality and diurnal preference suggests that the NPAS2 rs6725296 polymorphism may not contribute to seasonality and diurnal preference. However, the NPAS2 rs6725296 polymorphism may affect seasonality and diurnal preference in combination with other core clock genes, and this possibility cannot be ruled out. Such a possible contribution of this polymorphism may be too small to be discovered in our study.

Our study has some limitations. The lack of association of the NPAS2 rs6725296 polymorphism with seasonality and diurnal preference in this study may be explained by the following limitations. First, our sample size (n=510) may not be sufficient to test the effect of a single-nucleotide polymorphism underlying the complexity of seasonality and diurnal preferences. Furthermore, our sample showed a low frequency (n=9) of the A/A genotype. The number of A/A genotypes was small and a nonparametric statistical method was used for the analysis. Second, covariates, such as age and sex, were not included in the analysis, which may have affected the results. Furthermore, the possible effects of epigenetic factors, gene-environmental factors, and gene-gene interactions on seasonality and diurnal preference were not considered. Third, a population stratification bias cannot be ruled out. Finally, there may be self-reporting bias. Previous genome-wide association studies used objective assessments, such as actigraphy, to monitor activity, sleep time, and sleep duration, in addition to self-reported measures [38]. This study may have limitations, as it was based only on self-reported measures to classify seasonality and diurnal preferences.

In conclusion, this study did not find significant associations between the NPAS2 rs6725296 polymorphism and seasonality and diurnal preference in healthy Korean adults, suggesting that this polymorphism may not contribute to seasonality and diurnal preference. More studies with a larger number of subjects, inclusion of different ethnic groups, and complementation with objective assessment, such as wrist actigraphy, are necessary to elucidate the genetic influence on seasonality and phenotypic expression of circadian rhythmicity in the form of diurnal preference. As the association with circadian rhythms is emerging in numerous psychiatric disorders, future studies are warranted to better understand the genetic effect on circadian phenotypes and personalized treatment strategies based on circadian rhythms.

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Conflicts of Interest
The authors have no potential conflicts of interest to disclose.

Availability of Data and Material
The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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