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Screening of Clock Gene Polymorphisms Demonstrates Association of a *PER3* Polymorphism with Morningness–Eveningness Preference and Circadian Rhythm Sleep Disorder

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A system of self-sustained biological clocks controls the 24-h rhythms of behavioral and physiological processes such as the sleep–wake cycle. The circadian clock system is regulated by transcriptional and translational negative feedback loops of multiple clock genes. Polymorphisms in circadian clock genes have been associated with morningness–eveningness (diurnal) preference, familial advanced sleep phase type (ASPT), and delayed sleep phase type (DSPT). We genotyped single-nucleotide polymorphisms in circadian clock genes in 182 DSPT individuals, 67 free-running type (FRT) individuals, and 925 controls. The clock gene polymorphisms were tested for associations with diurnal preference and circadian rhythm sleep disorder (CRSD) phenotypes. The *PER3* polymorphism (rs228697) was significantly associated with diurnal preference and the FRT phenotype. The minor allele of rs228697 was more prevalent in evening types than in morning types (sex-adjusted odds ratio (OR), 2.483, Bonferroni-corrected $P = 0.012$) and in FRT individuals compared with the controls (age- and sex-adjusted OR, 2.021, permutated $P = 0.017$). Our findings support the notion that *PER3* polymorphisms could be a potential genetic marker for an individual's circadian and sleep phenotypes.

Sleep–wake cycles are regulated by two components, homeostatic drive and circadian drive[1]. Sleep and wakefulness occur sequentially, and sleep propensity increases gradually with extended wakefulness and decreases rapidly during sleep. Sleep propensity is under the control of sleep homeostasis, and sleep timing is under the control of circadian clocks. The circadian clock system regulates daily behavioral and physiological rhythms such as body temperature, hormone secretion, blood pressure, metabolism, and cognitive performance besides sleep/wakefulness. These rhythms are generated by the central circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus and are entrained by environmental cues (e.g., light–dark cycles)[2,3]. The molecular mechanism of the circadian clock system involves transcription-translation negative feedback loops of multiple clock genes and post-transcriptional and post-translational modification and degradation of clock proteins[4,5]. The transcription factors BMAL1 and CLOCK form heterodimers, which activate transcription of *Cryptochrome* (*Cry*) and *Period* (*Per*) by binding to E-box motifs in their promoter regions. CRY and PER proteins gradually accumulate in the cytoplasm. Phosphorylation of CRY and PER is regulated by casein kinase I (CKI). CRY, PER, and CKI proteins form complexes that translocate to the nucleus and interact with the BMAL1–CLOCK heterodimers, thereby inhibiting transcription of the *Cry* and *Per* genes. Although the circadian

function of *Timeless* (*Tim*) remains to be determined, *Tim* is known to modulate neuronal firing rhythms in the SCN[6].

Circadian rhythm sleep disorders (CRSDs) are defined by persistent or recurrent disturbed sleep–wake cycles and comprise several subtypes: advanced sleep phase type (ASPT), delayed sleep phase type (DSPT), and free-running type (FRT). ASPT is characterized by extremely early involuntary sleep timing, DSPT by significantly delayed sleep timing, and FRT by sleep timing that occurs with a 30-min to 1-h delay each day. CRSD is thought to result from impairment of the circadian clock system[7–9]. Also, circadian characteristics vary greatly among individuals[10,11]. The inter-individual differences in daily activity/sleep time are known as morningness–eveningness (diurnal) preference. The morning type manifests earlier timings for sleep and physiological rhythms than the intermediate type, and still earlier than the evening type[12,13]. Individual diurnal preference is morning type during childhood, which subsequently switches to evening type during adolescence before starting to return to morning type in adulthood[14,15]. Males show evening preference while females show more morning preference, but this gender-related difference disappears in the elderly[11,16]. The diurnal preference is commonly assessed by self-reported questionnaires, the Horne–Östberg Morningness–Eveningness Questionnaire (MEQ)[10] and the recently developed Munich ChronoType Questionnaire (MCTQ)[11]. The diurnal preference and CRSD phenotypes are thought to be influenced by genetic factors. For example, polymorphisms in the *CLOCK*, *NPAS2*, *PER2*, and *TIM* genes are associated with diurnal preference[17,18], sleep timing[19], and sleep disorders[20–22]. Furthermore, the *PER3* gene has polymorphisms in the promoter region[23], missense polymorphisms that result in amino acid substitution and a variable number tandem repeat (VNTR) consisting of either 4 or 5 repeated 54-bp sequences encoding 18 amino acids (*PER3*^{4} or *PER3*^{5})[24]. These *PER3* polymorphisms are associated with diurnal preference and/or DSPT[23–25]. Although the results of some other studies are inconsistent with the associations[26,27], a number of genetic studies have shown that genetic factors significantly contribute to individual differences in circadian and sleep phenotypes[28–30].

Missense mutations in the *PER2* and *CKIδ* genes have been found in large pedigrees with familial ASPT[31,32]. These amino acid substitutions in PER2 and CKIδ reduce the phosphorylation level of PER2, thereby shortening the intrinsic circadian period (τ) and giving rise to the familial ASPT phenotype. These findings indicate that clock gene polymorphisms, especially missense polymorphisms, may alter the function of these genes, thereby modifying diurnal preference and sleep–wake patterns. To investigate how genetic variations impact circadian and sleep phenotypes, we genotyped single-nucleotide polymorphisms (SNPs) in a number of circadian clock genes in controls and DSPT and FRT patients and tested these SNPs for associations with diurnal preference in controls and in patients with the CRSD phenotype. Our control subjects' diurnal preference was assessed by the Horne–Östberg MEQ[10]. Age-adjusted MEQ scores of 16–41 denote evening types, 42–58 denote intermediate types, and 59–86 denote morning types. The DSPT and FRT patients were diagnosed according to the International Classification of Sleep Disorders 2 (ICSD-2)[33].

Results

Association between *PER3* and diurnal preference. The 925 controls consisted of 245 morning types (79 men and 166 women; mean ± SD age-adjusted MEQ score: 62.81 ± 3.77), 594 intermediate types (163 men and 431 women; mean ± SD age-adjusted MEQ score: 51.13 ± 4.31), and 86 evening types (32 men and 54 women; mean ± SD age-adjusted MEQ score: 38.23 ± 3.55). Men showed more evening preference than women ($\chi^2 = 9.077$, $P = 0.011$; adjusted residual = ±2.3). The allele frequency of the 9 SNPs in 6 genes was compared among morning, intermediate, and evening types (Table 1). As shown in Table 1, only the SNP rs228697 in *PER3*

Table 1 | Genotype and minor allele frequencies of 9 SNPs in 6 genes in morning, intermediate, and evening types

Gene	SNP	Allele (A/a)	Variation (amino acid)	M (N = 245)			I (N = 594)			E (N = 86)			M	I	E	
				AA	Aa	aa	AA	Aa	aa	AA	Aa	aa	MAF			*P* (*χ2*)
CLOCK	rs1801260	T/C		0.714	0.269	0.016	0.714	0.263	0.024	0.64	0.302	0.058	0.151	0.155	0.209	0.162
NPAS2	rs2305160	G/A	A/T	0.629	0.322	0.049	0.677	0.288	0.035	0.628	0.291	0.081	0.21	0.179	0.227	0.163
PER1	rs2585405	C/G	P/A	0.339	0.502	0.159	0.347	0.461	0.192	0.349	0.512	0.14	0.41	0.423	0.395	0.75
PER2	rs2304672	C/G		0.873	0.127	0	0.904	0.094	0.002	0.872	0.128	0	0.063	0.049	0.064	0.411
	rs934945	G/A	G/E	0.478	0.396	0.127	0.476	0.441	0.082	0.465	0.43	0.105	0.324	0.303	0.32	0.662
PER3	rs228697	C/G	P/A	0.898	0.102	0	0.833	0.163	0.003	0.791	0.186	0.023	0.051	0.085	0.116	0.01*
	rs2640909	T/C	M/T	0.812	0.184	0.004	0.771	0.214	0.015	0.767	0.198	0.035	0.096	0.122	0.134	0.238
TIM	rs774047	A/G	Q/R	0.363	0.449	0.188	0.347	0.463	0.19	0.372	0.43	0.198	0.412	0.422	0.413	0.927
	rs2291739	C/T	P/L	0.347	0.445	0.208	0.301	0.49	0.209	0.326	0.442	0.233	0.431	0.454	0.453	0.679

A, major allele; a, minor allele; M, morning type; I, intermediate type; E, evening type; N, number; *P*, *P* value for the difference in MAF among M, I and E;
*, $P < 0.05$.

was significantly associated with diurnal preference ($\chi^2 = 9.157$, $P = 0.010$). The major allele C of rs228697 was more common in morning types than in evening types, and the minor allele G of rs228697 was more common in evening types than in morning types (sex-adjusted odds ratio (OR), 2.483; 95% confidence interval (CI), 1.339–4.603; Bonferroni-corrected $P = 0.012$; crude $P = 0.004$). Subjects with the G-positive genotype (CG, GG) for rs228697 showed lower age-adjusted MEQ scores than those with the G-negative genotype (CC) for rs228697 (mean ± SEM age-adjusted MEQ score: 51.70 ± 0.68 vs 53.26 ± 0.29; F(1, 922) = 4.561; $P = 0.033$).

Association between *PER3* and FRT. We then assessed the probability of the 9 SNPs producing DSPT and FRT phenotypes (Table 2). The SNP distributions did not differ between the controls and DSPT individuals. However, the rs228697 distribution significantly differed between controls and FRT individuals. The frequency of the G allele for rs228697 was significantly increased in FRT individuals compared with controls (age- and sex-adjusted OR, 2.021; 95% CI, 1.160–3.524; permutated $P = 0.017$; crude $P = 0.011$). The G-positive genotype (CG, GG) for rs228697 was more prevalent in FRT individuals (CG, GG, 0.284) than in controls (CG, GG, 0.154) with an age- and sex-adjusted OR of 2.253 (95% CI, 1.233–4.118; $P = 0.008$). Therefore, the *PER3* SNP rs228697 was significantly associated with the FRT phenotype in this cohort.

Discussion

We found that the G allele of rs228697 in *PER3* was more common in evening types than in morning types and in FRT individuals than in controls using a very large sample of control individuals and CRSD patients. These findings are in accordance with previous reports[23–25] suggesting genetic associations between *PER3* polymorphisms and diurnal preference and/or CRSD phenotypes. A *PER3* haplotype defined by the G allele of rs10462020 and the C allele of rs10462021 has been shown to be related to DSPT[24]. Furthermore, the 4-repeat allele of *PER3* VNTR (*PER3*^{4}) has been associated with extreme evening preference and DSPT, whereas the 5-repeat allele of *PER3* VNTR (*PER3*^{5}) has been associated with extreme morning preference[25]. In addition, the polymorphisms in the *PER3* promoter have been associated with DSPT[23]. Although the SNPs rs10462020 and rs10462021 were excluded from further analysis due to the low minor allele frequency (MAF) < 0.05, and the *PER3*^{4} and *PER3*^{5} alleles as well as the previously described polymorphisms in the *PER3* promoter, were not directly investigated in this study, the present and previous findings strongly suggest that *PER3* polymorphisms can provide potential biomarkers for estimating individual diurnal preference and CRSD phenotypes.

Morning types and evening types have been reported to differ in homeostatic sleep regulation and neurobehavioral functions in response to sleep fragmentation and sleep deprivation[29]. Mongrain et al. have demonstrated that the morning type exhibits a higher initial level and faster dissipation rate of sleep pressure than the evening type[34]. These results suggest that diurnal preference may reflect individual differences in both homeostatic and circadian regulation. Moreover, there are inter-individual differences in the impairment of neurobehavioral functions (attention, decision making, etc.) in response to sleep deprivation and sleep restriction[35–37]. A number of studies indicate that genetic traits contribute to individual differences in sleep homeostasis, circadian rhythms, and cognitive performance[28,38]. Individuals homozygous for *PER3*^{5} demonstrate more morning preference, greater sleep propensity at baseline and after sleep deprivation, and a lower level of cognitive performance than those homozygous for *PER3*^{4} [39,40]. The results of our previous study have implied that an altered expression profile of *PER3* may reflect deteriorated homeostatic sleep drive in the elderly[41]. Additionally, Archer et al. have reported that the polymorphisms in the *PER3*

Table 2 | Genotype and minor allele frequencies of 9 SNPs in 6 genes in controls, DSPT, and FRT individuals

Gene	SNP	Allele (A/a)	Variation (amino acid)	Control (N = 925)			DSPT (N = 182)			FRT (N = 67)			Control	DSPT		FRT	
				AA	Aa	aa	AA	Aa	aa	AA	Aa	aa	MAF	MAF	permutated P ($\chi 2$)	MAF	permutated P ($\chi 2$)
CLOCK	rs1801260	T/C		0.707	0.268	0.025	0.665	0.297	0.038	0.687	0.284	0.03	0.159	0.187	0.218	0.172	0.715
NPAS2	rs2305160	G/A	A/T	0.659	0.297	0.043	0.698	0.264	0.038	0.612	0.343	0.045	0.192	0.17	0.355	0.216	0.502
PER1	rs2585405	C/G	P/A	0.345	0.477	0.178	0.335	0.538	0.126	0.403	0.418	0.179	0.417	0.396	0.489	0.388	0.535
PER2	rs2304672	C/G		0.893	0.106	0.001	0.918	0.077	0.005	0.866	0.119	0.015	0.054	0.044	0.666	0.075	0.539
	rs934945	G/A	G/E	0.476	0.428	0.096	0.516	0.401	0.082	0.448	0.507	0.045	0.31	0.283	0.518	0.299	0.950
PER3	rs228697	C/G	P/A	0.846	0.149	0.004	0.846	0.143	0.011	0.716	0.284	0	0.079	0.082	0.941	0.142	0.017*
	rs2640909	T/C	M/T	0.782	0.204	0.014	0.775	0.198	0.027	0.657	0.343	0	0.116	0.126	0.765	0.172	0.088
TIM	rs774047	A/G	Q/R	0.354	0.456	0.19	0.407	0.467	0.126	0.284	0.522	0.194	0.418	0.36	0.058	0.455	0.527
	rs2291739	C/T	P/L	0.316	0.474	0.211	0.363	0.489	0.148	0.313	0.478	0.209	0.448	0.393	0.082	0.448	1

A, major allele; a, minor allele; N, number; MAF, minor allele frequency; *P*, *P* value for the difference in MAF between DSPT or FRT patients and controls;
*, $P < 0.05$.

Table 3 | Genotype and minor allele frequencies of 30 SNPs in 7 genes in 925 controls

Gene	SNP	Allele (A/a)	Variation (amino acid)	AA	Aa	aa	MAF	*P* (HWE)	Genotyping Assay ID
CLOCK	rs34897046	C/G	S/C	1	0	0	0	1	C__25595098_10
	rs6855837	C/A	L/I	1	0	0	0	1	C__29101689_10
	rs3762836	A/G	H/R	0.981	0.019	0	0.01	1	C__27479322_10
	rs1801260	T/C		0.707	0.268	0.025	0.159	1	C___8746719_20
CRY2	rs2863712	T/G	W/G	1	0	0	0	1	C__16079564_10
NPAS2	rs34628006	A/G	T/A	1	0	0	0	1	C__25757964_10
	rs2305160	G/A	A/T	0.659	0.297	0.043	0.192	0.243	C__15976652_10
	rs11541353	C/T	S/L	1	0	0	0	1	C___2153849_10
	rs58728948	G/A	A/T	1	0	0	0	1	C__25757546_10
PER1	rs2585405	C/G	P/A	0.345	0.477	0.178	0.417	0.592	C__16260899_10
	rs3027193	G/A	R/H	1	0	0	0	1	C__15770159_10
PER2	rs2304672	C/G		0.893	0.106	0.001	0.054	0.472	C___2129919_1_
	rs35572922	G/T	A/S	1	0	0	0	1	C__25973284_20
	rs4429421	G/A	V/I	0.991	0.009	0	0.004	1	C__27970170_10
	rs35333999	G/A	V/I	1	0	0	0	1	C__25992030_10
	rs35998480	T/A	F/Y	1	0	0	0	1	C__25958587_10
	rs934945	G/A	G/E	0.476	0.428	0.096	0.31	1	C___8740718_20
PER3	rs10462020	T/G	V/G	0.928	0.07	0.002	0.037	0.731	C__25956444_10
	rs35687686	C/T	R/W	1	0	0	0	1	C__25970031_10
	rs228696	C/T	L/P	1	0	0	0	1	C_____10225_10
	rs35899625	T/G	L/W	1	0	0	0	1	C__25967284_10
	rs228697	C/G	P/A	0.846	0.149	0.004	0.079	0.606	C_____10224_10
	rs2640909	T/C	M/T	0.782	0.204	0.014	0.116	0.96	
	rs2640905	C/G	S/C	1	0	0	0	1	C__16268919_10
	rs35802556	C/T	T/I	1	0	0	0	1	
	rs10462021	A/G	H/R	0.928	0.07	0.002	0.037	0.731	
	rs35072750	A/G	T/A	1	0	0	0	1	C__25963142_20
TIM	rs774027	T/A	L/I	0.359	0.453	0.188	0.415	0.048	C___8340562_10
	rs774047	A/G	Q/R	0.354	0.456	0.19	0.418	0.064	C___3134218_10
	rs2291739	C/T	P/L	0.316	0.474	0.211	0.448	0.215	C__15966257_10

A, major allele; a, minor allele; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; *P*, *P* value for HWE.

promoter are associated with DSPT and that these polymorphisms have an effect on its expression level[23]. The data reported here showed that the *PER3* SNP rs228697 was associated with diurnal preference and FRT phenotype in our large sample of CRSD patients and controls. These findings suggest that the *PER3* gene may play a functional role in homeostatic sleep and/or circadian clock systems. Furthermore, *PER3* polymorphisms may predict individual differences in vulnerability to sleep deprivation and restriction.

The SNP rs228697 (C/G) corresponds to the SNP in exon 17 of the *PER3* gene and causes the amino acid substitution (P864A)[24]. Although the amino acid substitution of proline (P) to alanine (A) does not make a significant difference to polarity or hydrophobicity, the amino acids P and A differ in their hydropathy index. Accordingly, the P/A substitution could alter the secondary structure and/or phosphorylation status of *PER3* leading to dysregulation of the homeostatic sleep and circadian clock systems. It is intriguing that P864A is located in potential Src homology (SH)3 domains in PER3. SH3 domains are found in many proteins in an array of signaling pathways that regulate the cytoskeleton, the Ras gene family, and the Src kinase family, as well as many other signaling cascades[42,43]. The proline-rich motif X-P-X-X-P is defined as the minimal consensus sequence of the SH3-binding sites. X is often an aliphatic amino acid[44]. The P864A substitution would disrupt two potential SH3-binding motif domains in PER3 (LPDPP(864) and PP(864)VCP), which could alter the binding interaction between PER3 and its partner protein(s), thereby disturbing the function of PER3 in the homeostatic and circadian regulation of sleep. CKI interacts with the scaffolding protein NCK that consists of SH2 and SH3 domains[45]. As CKI is known to regulate PER protein stability[46,47], it is interesting to speculate that the interaction between CKI and PER3 may be partially mediated via the scaffolding afforded by the interactions between the SH3 domain in NCK and the putative SH3 ligand in PER3. However, how this allelic variation of *PER3* modifies sleep and circadian phenotypes remains to be determined.

FRT is defined by sleep timing that occurs with a 30-min to 1-h delay each day. Therefore, prolongation of τ has been considered a critical factor for determining the FRT phenotype[7–9]. Free-running patterns in sleep–wake cycles are often observed in blind individuals[48]. Because totally blind individuals are not capable of perceiving photic signals, some may show free-running sleep patterns as a consequence of the loss of photic entrainment. Thus, the pathophysiology of sighted individuals with FRT might also be associated with an impaired photic entrainment mechanism as well as with prolonged τ. We recently reported that sighted FRT patients have a longer τ than intermediate types, but not when compared with evening types[49]. Moreover, the results of the present study demonstrate that extreme evening preference and FRT are associated with the same polymorphism in the *PER3* gene. Based upon these findings, there may be a genetic trait or traits shared by individuals with extreme evening preference and FRT phenotypes. In contrast, DSPT was not associated with any polymorphisms in the *PER3* gene or other clock genes investigated in this study. It appears likely that this results from the heterogeneity of the DSPT population. Not only circadian clock defects, but also psychiatric problems and/or consequent reduction of social (non-photic) entrainment are thought to be causative factors for the development of DSPT phenotypes[50].

There are some limitations to this study. It would be difficult to conduct a replication study using another sample of patients due to the extremely low prevalence of CRSDs in the Japanese population (0.13%)[51]. Other groups have shown associations of *PER3* and diurnal preference and/or CRSD phenotypes using their samples[23–25]. We did not control for medical treatments in our subjects, such as medication and light therapy, although these treatments would only alter the medical condition of patients and not their diagnoses.

Table 4 | Sequences for primers and TaqMan probes in *PER3*

Gene	SNP	Allele (A/a)	Forward primer	Reverse primer	TaqMan probes	
PER3	rs2640909	T/C	5′- CGCCTCCCATGAA GAATCCA-3′	5′-TGGCAGTAGGATG GGATGGA-3′	5′-FAM-CAATCCCATG GACAGTG-NFQ-3′	5′-VIC-AATCCCGTGG ACAGTG-NFQ-3′
	rs35802556	C/T	5′-GAAGAGCCCATCT GGAGAATGAT-3′	5′-GGTACCTGGTATGT CATGAGAATGC-3′	5′-FAM-CTCAGGTGTC TGCCG-NFQ-3′	5′-VIC-CTCAGGTATCT GCCG-NFQ-3′
	rs10462021	A/G	5′-GAAGACCTGGAAAAG CTAGAAAGTATGA-3′	5′-ACCTTAGCCAGCT CCTCCTTT-3′	5′-FAM-CCAGTTTTCTC ATGGGCA-NFQ-3′	5′-VIC-CCAGTTTTCTCG TGGGCA-NFQ-3′

A, major allele; a, minor allele.

In conclusion, our findings corroborate the involvement of *PER3* in regulating the homeostatic sleep and/or circadian systems. The *PER3* gene has the potential to serve as a biomarker for evaluating genetic traits of sleep and circadian phenotypes and as a research target to understand the mechanism underling the pathophysiology of FRT. Life styles have dramatically changed during the past century. Shift work and jet lag induce acute sleep deprivation and chronic sleep restriction, which is associated with deteriorated neurobehavioral performance[35,52]. Furthermore, a misalignment between endogenous circadian rhythms and sleep–wake cycles known as internal desynchronization is thought to cause sleep shortage and circadian rhythm disturbance, leading to elevated risks for autonomic disorders, cardiovascular diseases, metabolic diseases, and mood disorders[53–55]. Therefore, predicting sleep and circadian phenotypes may help develop interventions to improve quality of life and potentially prevent various diseases.

Methods

Subjects. The study population consisted of 182 DSPT individuals (111 men and 71 women; mean ± SD age: 26.68 ± 9.25 years), 67 FRT individuals (48 men and 19 women; mean ± SD age: 26.72 ± 9.79 years), and 925 controls (274 men and 651 women; mean ± SD age: 36.45 ± 12.10 years). Patient subjects and controls were all unrelated, sighted Japanese men and women who were recruited at medical and research institutes on mainland Japan. None of the controls had a history of sleep disorders or psychosis. The DSPT and FRT patients were diagnosed by trained psychiatrists according to the International Classification of Sleep Disorders II (ICSD-II, 1990, 1997)[33]. The diagnostic criteria for DSPT are (1) inability to fall asleep and wake up spontaneously at the desired time; (2) persistent delayed phase of the major sleep episode in relation to the desired time for sleep; (3) symptoms present for at least 1 month; and (4) sleep of normal quality and duration when not required to maintain a conventional sleep–wake schedule. The criteria for FRT are (1) insomnia or excessive sleepiness related to misalignment between the endogenous circadian rhythm and the 24-h light–dark cycle; (2) chronic sleep–wake cycle with a longer period than 24 h; and (3) symptoms present for at least 1 month. Ten DSPT patients had comorbidities as follows: bipolar, one; major depression, three; pervasive developmental disorder, two; seasonal affective disorder, two; sleep apnea syndrome, two. Seventeen FRT patients had comorbidities as follows: bipolar, one; major depression, one; seasonal affective disorder, one. The protocol was approved by the respective institutional ethical review boards. All subjects provided written informed consents. The present study was conducted according to the principles of the Declaration of Helsinki.

Markers and genotyping. A total of 30 SNPs in 7 genes, *CLOCK*, *CRY2*, *NPAS2*, *PER1*, *PER2*, *PER3*, and *TIM*, were selected (Table 3). These genes have been previously validated in some populations (http://www.ncbi.nlm.nih.gov/snp/). Overall, 28 SNPs resulted in amino acid substitutions, and 2 SNPs, rs1801260 and rs2304672, were in the 5′ UTR of *CLOCK* and *PER2*, respectively. The SNP rs1801260 was associated with evening preference in a Japanese population[18] and rs2304672 was found in a Japanese pedigree of familial ASPT[21]. Thirty SNPs including rs1801260 and rs2304672 were genotyped in 925 Japanese controls. The Hardy–Weinberg equilibrium (HWE) was tested for each marker. The SNPs showing monomorphism with low frequency (MAF < 0.05) or not in HWE ($P < 0.05$) were excluded from further analysis. Therefore, 9 SNPs in 6 genes, *CLOCK*, *NPAS2*, *PER1*, *PER2*, *PER3*, and *TIM*, were genotyped in 249 CRSD individuals.

Blood samples were collected from all subjects and used for DNA isolation. Genomic DNA was extracted from leukocytes with the QIAamp DNA Blood Midi Kit (QIAGEN K.K., Tokyo, Japan). Genotyping was performed by a TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The genotyping assay IDs for 27 SNPs are listed in Table 3 and the primers and TaqMan probes for rs2640909, rs35802556, and rs10462021 are listed in Table 4.

Self-assessment. The Japanese version of the Horne–Östberg MEQ[10] was administered to assess subjects' diurnal preference; this tool has been validated in a Japanese population[56]. Because diurnal preference changes with age, MEQ scores were adjusted by age (age-adjusted MEQ score, MEQ score + 0.3512 × (39.212 − age))[57]. Age-adjusted MEQ scores of 16–41 denote evening types, 42–58 denote intermediate types, and 59–86 denote morning types. Thus, lower MEQ scores indicate evening preference.

Statistical analysis. HWE was estimated using Haploview 4.1 (http://www.broad.mit.edu/mpg/haploview/)[58]. The chi-squared test was performed to compare the allele frequency and genotype distribution for each SNP marker among diurnal preference groups (morning, intermediate, and evening types). The sex-adjusted ORs and 95% CIs with Bonferroni correction were calculated to evaluate the rs228697 frequency across the three diurnal preference groups. One-way analysis of variance adjusted for sex was performed to compare age-adjusted MEQ scores between subjects with genotype CG or GG for rs228697 and those with genotype CC for rs228697. The chi-squared and permutation tests (N = 10,000) were performed to evaluate the difference in allele frequency between patients (DSPT or FRT) and controls using Haploview 4.1. The age- and sex-adjusted ORs and 95% CIs were calculated to evaluate the rs228697 frequency between FRT patients and controls. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS ver.11.5.1J (SPSS Japan Inc., Tokyo, Japan), unless otherwise stated.

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Author contributions

A.H. and K.M. designed the research. A.H., S.K., Y.K., M.K., H.O., H.K., M.U., T.E., Y.I., Y.K., M.O., K.T. and K.M. performed the research. A.H., S.K., M.K. and H.O. analyzed the data. A.H. and K.M. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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