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Forum Minireview

Circadian Rhythms in the CNS and Peripheral Clock Disorders: Human Sleep Disorders and Clock Genes

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Abstract. Genetic analyses of circadian rhythm sleep disorders (CRSD), such as familial advanced sleep phase syndrome (ASPS) and delayed sleep phase syndrome (DSPS), and morningness-eveningness revealed the relationship between variations in clock genes and diurnal change in human behaviors. Variations such as T3111C in the *Clock* gene are reportedly associated with morningness-eveningness. Two of the pedigrees of familial ASPS (FASPS) are caused by mutations in clock genes: the S662G mutation in the *Per2* gene or the T44A mutation in the *casein kinase 1 delta* (*CK1δ*) gene, although these mutations are not found in other pedigrees of FASPS. As for DSPS, a missense variation in the *Per3* gene is identified as a risk factor, while the one in the *CK1ε* gene is thought to be protective. These findings suggest that further, as yet unidentified, gene variations are involved in human circadian activity. Many of the CRSD-relevant variations reported to date seem to affect the phosphorylation status of the clock proteins. A recent study using mathematical models of circadian rhythm generation has provided a new insight into the role of phosphorylation in the molecular mechanisms of these disorders.

Keywords: circadian rhythm sleep disorder, morningness-eveningness, gene variation, phosphorylation, clock gene

Introduction

With the advent of the 24-h society and associated modern lifestyles, the prevalence of sleep disturbance appears to be increasing. In Japan, around one fifth of adults experience such disorders. As the cost of sleep-related problems is a considerable burden to society, research into sleep disorders is a matter of priority.

The sleep-wake cycle is generated through both circadian and homeostatic processes (1, 2) and altered circadian rhythmicity induces circadian rhythm sleep disorders (CRSD). The prevalence of delayed sleep phase syndrome (DSPS), in which sleep onset and offset are persistently delayed, is around 0.1% – 0.7% (3 – 5). In contrast, advanced sleep phase syndrome (ASPS), in which sleep onset and offset are persistently advanced, and non-24-h sleep wake syndrome, in which sleep hours are progressively delayed, appear to be more rare (6) (Fig. 1).

Genetic analysis has shown that clock gene variations are involved in the development of certain types of CRSD. Functional alterations in the clock genes lead to mal-adaptation of the sleep-wake cycle to the environmental light-dark cycle.

Molecular mechanisms of the biological clock

The core of the biological clock (central generator of the circadian rhythm) is thought to consist of interactions of approximately ten "clock genes", including *Per1/2/3*, *Cry 1/2*, *Bmal1*, *Clock*, and *casein kinase 1 delta/epsilon* (*CK1δ/ε*) (7). *Per1/2/3*, *Cry 1/2*, *Bmal1*, and *Clock* code for transcriptional factors, while *CK1δ/ε* code for kinases that phosphorylate these transcriptional factors. Functional abnormalities of these clock genes affect the circadian phenotype in various species from insects, mice, and hamsters to humans. As the molecular clock mechanism is well conserved among species, it is possible to confirm functional changes of the clock genes found in the human rhythm disorders by in vitro

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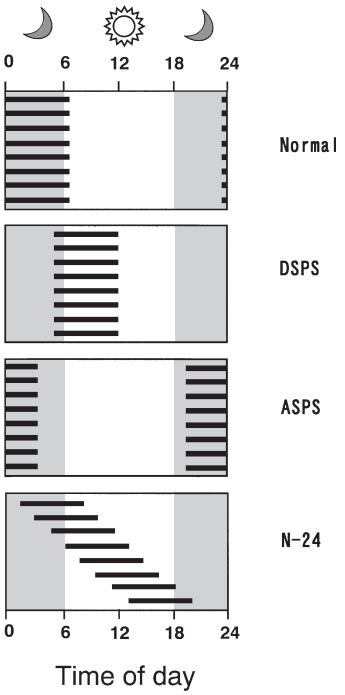

Fig. 1. Schematic diagrams showing sleep patterns in circadian rhythm sleep disorders. Thick lines indicate sleeping hours.

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                              *  *  *   *  *
Human           PER1  LANKA ESVVSVTSQC SFSSTIVHVG DKKPP
Human           PER2  LPGKA ESVASLTSQC SYSSTIVHVG DKKPQ
                            ↓
                            G (FASPS)
Human           PER3  LSTAM LSLGSGISQC GYSSTIVHVP PPETA
                                                ↓
                                                G (DSPS)
Mouse           PER1  LANKA ESVVSVTSQC SFSSTIVHVG DKKPP
Mouse           PER2  LPGKA ESVVSLTSQC SYSSTIVHVG DKKPQ
Mouse           PER3  LSTAA LSVASGISQC SCSSTSGHAP PLQSE
Rat             PER1  LANKA ESVVSVTSQC SFSSTIVHVG DKKPP
Rat             PER2  LPGKA ESVVSLTSQC SYSSTIVHVG DKKPQ
Rat             PER3  LSTAA LSMASGVSQC SCSSTTDHVP PLQSE
Japanese quail  PER2  LPGKP ESVVSLTSQC SYSSTIVHVG DKKPQ
Japanese quail  PER3  LSNKS PSVVSATSQC SYSSTIVHVP HPESE
Xenopus         PER1  PPSKA ESVVSITSQG SFSSTIVHVG DKKPP
Xenopus         PER2  LGAKA ESVVSFTSHC SYSSTIVHVG DKKLQ
Zebrafish       PER1  LHCKA ESVVSATSQC SFSSTIVHVG DKKPP
Zebrafish       PER3  ISTEA MSVVSVTSQC SYSSTIVHVP QPESE
Zebrafish       PER4  LHNKA ESVASVTSQC SFSSTIVHVG DKKPP
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Fig. 2. Circadian rhythm sleep disorders and *Per* gene variations. * denotes the target residues for CK1ε-induced phosphorylation (ref. 25). Arrows indicate an S662G mutation of the *Per2* gene, which causes FASPS (ref. 25), and a V647G variation of the *Per3* gene, which confers susceptibility to DSPS (ref. 15).

and in vivo experiments. This is an advantage in circadian rhythm sleep disorder research.

Morningness-eveningness and clock genes

The T3111C variation of the *Clock* gene was the first human clock-gene variation reported to affect human circadian rhythmicity (8). Katzenberg et al. discovered this variation by comparing the sequence alignment of expressed sequence tags of the *Clock* gene recorded in the public database. They also administered the Horne-Ostberg (H-O) questionnaire, with which the diurnal preference for various activities can be estimated, to normal volunteers and genotyped them for the T3111C variation. Those who carried the C3111 allele demonstrated significantly lower H-O scores, indicating that the allele is associated with a preference for evening. This finding was confirmed by one research group (9), but not by another (10). The T3111C variation has also been putatively associated with DSPS (11). The T3111C variation occurs in the 3' non-translated region and does not show a functional difference in the reporter gene assay (10). It is possible that another variation, which induces functional change, is in linkage disequilibrium with the T3111C variation and is causal for this evening preference. The *Per2* gene C111G variation in the 5' non-translating region (12, 13) and the *Per3* gene V647G variation (14) have also been associated with diurnal preference of activity.

DSPS/non-24-h sleep wake syndrome (N-24) and clock genes

Several researchers have reported associations between clock gene variations and DSPS/N-24. We have reported that the allele frequency of V647G variation in the human *Per3* gene was significantly higher in DSPS patients compared with normal controls, indicating that the variation acts as a risk factor for DSPS (15). Although the role of the PER3 protein in circadian rhythm generation has yet to be elucidated, PER3 protein is known to form a complex with PER1/2 and CRY1/2 proteins, enter the nucleus, and suppress the transcription induced by the BMAL1/CLOCK complex (16, 17). Ablation of the mouse *Per3* gene shortens the circadian rhythm period but does not abolish it (18). The V647 amino acid is well conserved among the many *Per* genes in vertebrates and is localized close to the target residues of CK1ε-induced phosphorylation (Fig. 2) (19). It is therefore likely that the V647G variation alters CK1ε-induced phosphorylation of PER3 protein and leads to an abnormal circadian rhythm phenotype. We have also identified 4-repeat/5-repeat variation of 54 nucleotide pairs, which code for 18 amino acid residues, in the human *Per3* gene (15). While one study found DSPS and diurnal preference to be positively associated with the 4-repeat allele (20), other studies have not confirmed this (15, 21). Further research is warranted to clarify the functional significance of the variable number of tandem repeats.

When we screened for variations of the *CK1ε* gene,

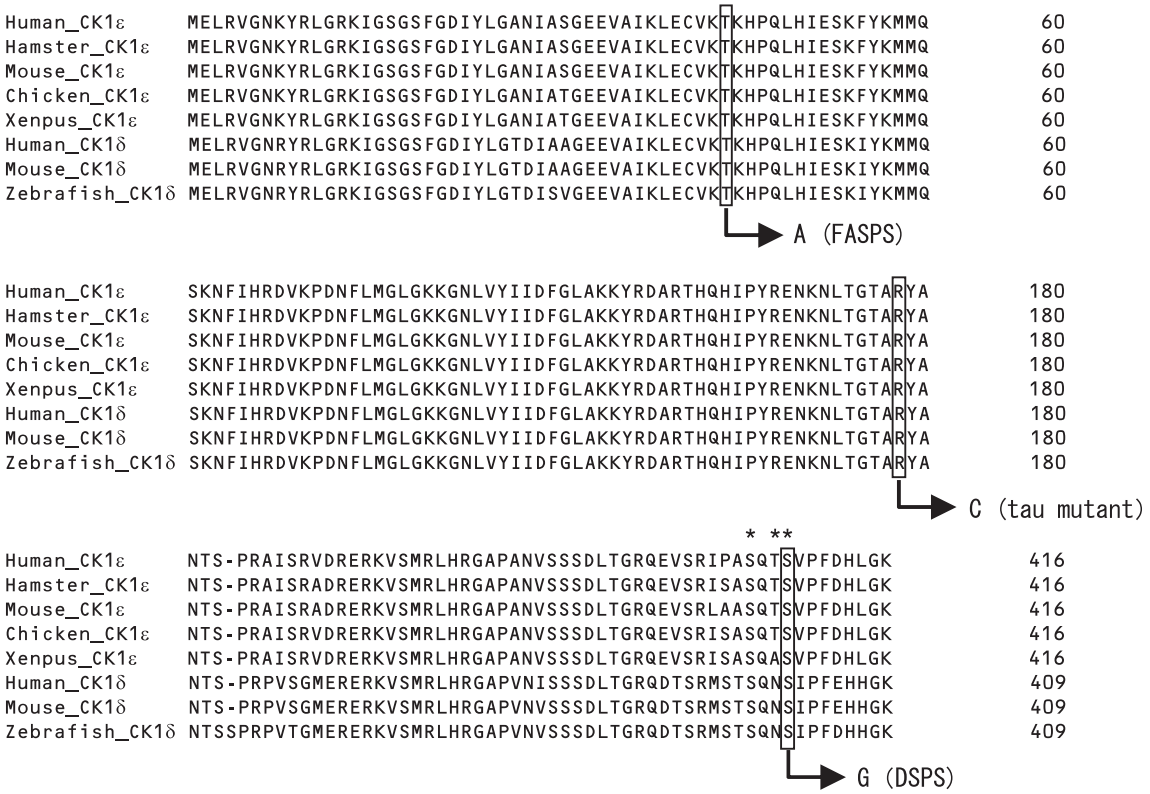

Fig. 3. Circadian rhythm sleep disorders and *CK1δ/ε* gene variations. Arrows indicate a *CK1δ* T44A mutation found in FASPS (ref. 26), a *CK1ε* S408N variation relevant to DSPS (ref. 22), and a *CK1ε* R178C mutation in a hamster *tau* mutant (ref. 31).

which phosphorylates clock proteins and modulates the circadian rhythm in DSPS/N-24 patients, we detected a missense variation, S408N. This variation occurred significantly more often in controls than in DSPS/N-24 subjects, indicating that the variation protects against developing the disorders (22). S408 is conserved among CK1*δ/ε* genes of many vertebrates and is one of the putative target residues for autophosphorylation (Fig. 3) (23). As the kinase activity of CK1*δ/ε* is reduced by autophosphorylation, it seems likely that the abolition of one of the autophosphorylation sites by the S408N variation would increase the enzyme activity. Indeed, an in vitro kinase assay using CK1*ε* protein purified from *E. coli* confirmed that CK1*ε* with the S408N substitution exhibited higher enzyme activity compared to wild-type CK1*ε*. Interestingly, CK1*ε* with the S408N variation also showed higher enzyme activity against PER3 compared to wild-type CK1*ε*. As described above, the V647G variation of the *Per3* gene is associated with DSPS and seems to alter phosphorylation of the PER3 protein. CK1*ε*-induced phosphorylation of PER3 protein might play an important role in adjustment of circadian rhythm to the environmental light-dark cycle.

ASPS and clock genes

Several pedigrees have exhibited a familial preponderance for ASPS (6, 24); this disorder is termed familial ASPS (FASPS). ASPS is thought to be rare; however, its frequency may have been underestimated because it is not well known among either medical staff or patients. So far, causative mutations of clock genes have been found in two of the FASPS pedigrees. The S662G mutation of the *Per2* gene cosegregated with affected patients in one of the FASPS pedigrees (25). S662G mutation abolishes one of the phosphorylation target sites for CK1*ε* (Fig. 2), and an in vitro experiment showed that the mutation reduces CK1*ε*-induced phosphorylation of PER2 protein. One of the patients in the pedigree exhibited a significantly shorter circadian period compared with the average among normal age- and sex-matched controls (23.3 h *vs* 24.2 h). This finding indicates that the S662G mutation in the *Per2* gene shortened the circadian rhythm cycle, resulting in the development of ASPS. In another pedigree of FASPS, a T44A mutation of the *CK1δ* gene reportedly caused the disorder (Fig. 3) (26). Moreover, an in vitro assay demonstrated that this mutation reduced the enzyme activity of CK1*δ*. In that study, when human *CK1δ* genes

with or without the T44A mutation were introduced into mice and flies, the mutation shortened the circadian period in mice but elongated it in flies. Hence, although clock components are highly conserved between flies and mice, the functional contribution of each component may differ between these species.

CRSD and altered phosphorylation of clock components

As described above, many of the reported clock gene variations involved in the development of CRSD seem to alter the CK1δ/ε-induced phosphorylation of the clock proteins. How does the altered phosphorylation of clock proteins lead to these behavioral differences? Both of the *Per2* gene S662G mutation and the *CK1*δ gene T44A mutation reduce the phosphorylation of PER proteins in vitro.

In addition, the *tau* mutation in the hamster *CK1*ε gene reduces its kinase activity in vitro and shortens the circadian period. We can speculate that lowered phosphorylation of PER proteins would induce delayed deterioration and early accumulation of these proteins, leading to acceleration of the circadian cycle. If reduced phosphorylation of PER proteins by CK1δ/ε shortens the circadian period, it seems likely that increased kinase activity induced by the S408N variation in the CK1ε gene might elongate the circadian period and confer susceptibility to DSPS/N-24. However, our study demonstrated that S408N protects against DSPS/N-24 (22).

Surprisingly, a recent study showed that a *tau* mutation in the *CK1*ε gene and a T44A mutation in the *CK1*δ gene increased kinase activity in vivo; this is opposite to the results obtained in vitro (27). These in vivo results were first indicated by mathematical modeling showing that the circadian period is shortened by increased phosphorylation of clock proteins.

In order to elucidate the effects of the S408N mutation, the kinase activity of CK1ε with S408N variation should be further investigated in vivo. Alternatively, mice with the variation should be studied in order to observe the phenotypic change.

Interindividual differences in human circadian rhythmicity and clock genes

Cells in peripheral tissues can generate circadian rhythm in the same way as can neuronal cells in the suprachiasmatic nucleus (SCN). When peripheral cells are cultured in vitro and stimulated by high concentrations of serum or dexamethasone, the amount of clock gene mRNA expressed fluctuates for several days, showing circadian rhythms with a period reflecting the central clock in the SCN.

Brown et al. performed punch biopsies of human skin, cultured the fibroblasts, and stimulated them with dexamethasone to determine the circadian period of the fibroblasts and compare this period among individuals (28). They found interindividual differences of circadian period that appeared to be based on genetic factors. The functional clock gene variations detected are likely to contribute to individual differences of circadian period as well as to CRSD.

Conclusions

The reported associations of the variations in the clock genes and their effects on human behavior are summarized in Table 1.

The molecular mechanisms of the circadian clock are well-conserved characteristics among various species, and the circadian machinery has relatively few components. This means that the circadian clock system is a valuable model system for analyzing complex human behaviors through genetic, biochemical, and physiological analysis and mathematical modeling.

Because clock genes play a role in various physiological phenomena as tissue regeneration, metabolism, bone formation, fat generation, and cancer, in addition to sleep-wake cycles and the circadian rhythms of hormone production, the variations involved in the alteration of human circadian rhythmicity might affect these phenomena. Psychoses as mood disorders, in which circadian rhythm disruption plays some role, may be

Table 1. Reported relationships between the clock-relevant gene variations and human circadian rhythm phenotypes

Clock gene	Circadian phenotype	Association	References
CLOCK	M-E	+	8
Per1	M-E	–	29
Timeless	M-E	–	30
Per2	FASPS	+	25
Per3	DSPS	+	15
CLOCK	DSPS	+?	11
CLOCK	M-E	–	10
NPAS2, Per3	Seasonal affective disorder, M-E	+	14
Per2	FASPS	–	12
Per3	DSPS, M-E	+	20
CK1ε	DSPS	+	22
CK1δ	FASPS	+	26
Per2	M-E	+	13

M-E: Morningness-eveningness.

also associated with clock gene variations.

Further analysis of clock machinery will clarify the contribution of the circadian rhythm to the maintenance of physical and mental health.

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