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Original Article

Influence of *Per3* genotypes on circadian rhythmicity in flight cadets after militarized management

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Abstract: Objective: The purpose of this study was to explore the effect of *PERIOD3* (*PER3*) genotypes on circadian rhythmicity in flight cadets after militarized management. Methods: We performed a preliminary study in 146 newly enrolled male flight cadets. Venous blood samples were collected, and genotyping of *PER3* (4/5) was determined by using PCR. The morningness-eveningness questionnaire (MEQ) survey was given to flight cadets upon enrollment and after militarized management for 24 months respectively. Comparison of frequency distribution of *PER3* genotypes between cases and controls (120 well-matched civilians) was performed using the X^2 test. We also compared the circadian rhythmicity upon enrollment and 24 months after enrollment in flight cadets, and analyzed the connection of changes in circadian clock with *PER3* genotypes. Results: The frequency distribution of *PER3* genotypes in flight cadets was not significantly different from that in controls subjects. MEQ survey results showed chronotype within flight cadet group varied widely at the two time-points: the moderately morning type (50%) and the neither type (41.1%) upon enrollment; the neither type (76.7%) and the moderately morning type (21.2%) 24 months after enrollment. The circadian rhythm of individuals with the *PER3 (5/5)* genotype showed no significant difference before and after 24 months of militarized management, whereas notable changes were found in individuals with the *PER3 (4/4)* genotype (n=116, X^2=37.26, $P < 0.001$). Conclusion: In conclusion, we provide some evidence that circadian rhythm of flight cadets with the *PER3 (5)* allele are less likely to be affected compared to those with the *PER3 (4)* allele.

Keywords: Circadian rhythmicity, *PER3* gene, flight cadet, militarized management

Introduction

A prominent circadian rhythm in human is the sleep-wake daily routine [1, 2]. Sleep is reportedly to affect physiological and psychological status such as quality of life [3-5], learning [6], and work [7]. Recent studies have shown that a number of circadian genes play an important role in regulating circadian rhythmicity of the general population [3], including *PERIOD3* (*PER3*), one of the core circadian clock genes. There is a 54-bp non-direct repeat variable number tandem repeat (VNTR) in exon 18 of the primate *PER3* gene [8], which harbors four or five copies of tandem repeated 54 bp sequence encoding 18 amino acids. Existing data have shown that the *PER3 (5)* allele with long VNTRs is associated with morning preferences (morningness), whereas the *PER3 (4)* allele with short VNTRs is linked to night preferences (eveningness) [3, 9].

Relationship between the *PER3* gene and circadian rhythmicity had been studied using subjects at one certain period, thus the results may be biased due to various confounding factors such as long-term unhealthy sleeping habits ascribed to study, work or illness. As yet, there have been no previous reports on circadian rhythmicity via comparisons at different time points.

In the present study, we selected a group of newly enrolled flight cadets to investigate the

Table 1. Comparison of the *PER3* genotypic frequency in the flight cadets and controls [n (%)]

PER3 genotypes	Flight cadets	Controls	X^2 value	*P* value
PER3 (4/4)	116 (79.5%)	96 (80.0%)	0.7110	0.70
PER3 (4/5)	27 (18.5%)	23 (19.2%)		
PER3 (5/5)	3 (2.0%)	1 (0.8%)		
Total	146 (100%)	120 (100%)		

Table 2. Distribution of the *PER3* allelic frequency in the flight cadets and controls

PER3 allele	Flight cadets	Controls	X^2 value	*P* value
PER3 (4)	259 (88.7%)	215 (89.6%)	0.1061	0.7446
PER3 (5)	33 (11.3%)	25 (10.4%)		
Total	292 (100%)	240 (100%)		

influence of *PER3* genotypes on circadian rhythmicity.

Materials and methods

Subjects

This study was approved by the institutional review board (ethics committee of PLA Navy General Hospital). One hundred and forty-six new male flight cadets were recruited. All cases were Han Chinese (mean age 18.8±0.7 years) and had went through a rigorous physical examination and screening for familial disease history before enrollment. The individuals with acute or chronic diseases that might affect sleeping status were excluded. 120 sex-, age- and ethnicity-matched controls were randomly selected from a healthy civilian population who visited our hospital to have a routine physical examination. Patients and control subjects were genetically unrelated and provided written informed consent to participate.

Morningness-eveningness questionnaire (MEQ)

The HO MEQ comprising 19 physiological clock-related questions is widely used to identify human circadian rhythms [10]. The survey was conducted at two time points: on enrollment and 24 months after enrollment. According to the questionnaire score, the chronotypes of subjects were grouped into 5 categories: definitely morning, moderately morning, neither, moderately evening, and definitely evening.

Genotyping

Blood collection tubes (K_2EDTA, Cat. 367-861, BD Vacutainer) were obtained from BD (Becton, Dickinson and Company), and Taq PCR MasterMix was obtained from TIANGEN BIOTECH (Cat. KT201). Two millimeters of venous blood was collected, and genomic DNA was extracted using PAXgene Blood DNA Kit. Genotypes for *PER3 4/5* repeats were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as previously described [11]. In brief, PCR amplifications were performed using the primers 5'-TGTCTTTTCATGT-GCCCTTACTT-3' (forward) and 5'-TGT-CTGGCATTGGAGTTTGA-3' (reverse) under the following conditions: 10 microl mixture containing 1×PCR Hot Start buffer, 150 ng genomic DNA, 10 pM primers (forward: 5 pM, reverse: 5 pM), 200 mM dNTP, 0.025 U/ml of Hotmaster Taq DNA polymorphism (Eppendorf, Italy). The reactions were carried out according to the following protocal: 6 min at 94°C, followed by 35 cycles of 94°C for 40 s, 60°C for 30 s, 70°C for 40 s, with the final step of 70°C for 12 min. The amplified products were analyzed on ethidium bromide stained agarose gel electrophoresis. A 401 bp fragment was amplified by 5 repeat allele. The fragment of 4 repeat allele was 347 bp. The two bands of different sizes could be amplified by heterozygous individuals.

Statistical analysis

Statistical data were performed using SSPS 16.0 software (StataCorp LP, College Station, TX, USA). Hardy-Weinberg equivalence (HWE) was checked by two-tailed X^2 test, which was also used to detect the frequency difference between groups. $P < 0.05$ was taken as the significance level.

Results

Comparison between PER3 genotypic distribution of the flight cadets and controls

As shown in **Table 1** and **Figure 1**, the genotypes of *PER3 4/5* repeats between flight cadets [116 homozygous wild type (79.5%), 27 heterozygous mutated (18.5%), and 3 homozygous mutated (2.0%)] and controls [96 homozygous wild type (80.0%), 23 heterozygous mutated (19.2%), and 1 homozygous mutated (0.8%)]

Table 3. Comparison of chronotype in flight cadets at the time of enrollment and 24 months after enrollment

Sleep type	0 month (number of people (%))	24 months (number of people (%))	Z	*P* value
Definitely morning type	12 (8.2%)	0 (0%)	6.6591	<0.0001
Moderately morning type	73 (50.0%)	31 (21.2%)		
Neither type	60 (41.1%)	112 (76.7%)		
Moderately evening type	1 (0.7%)	3 (2.1%)		
Definitely evening type	0 (0.0%)	0 (0%)		
Total	146 (100%)	146 (100%)		

Table 4. Distribution of the MEQ scores in flight cadets upon enrollment and 24 months after enrollment

PER3 genotype	MEQ scores of flight cadet at enrollment	MEQ score of flight cadets 24 months after enrollment	*t*	*P* value
PER3 (4/4)	60.1±7.2	53.9±6.1	14.55	<0.001
PER3 (4/5)	59.6±6.6	51.6±5.1	5.68	<0.001
PER3 (5/5)	50.7±8.0	48.0±8.2	1.91	0.1281

Table 5. *PER3* genotypes and chronotype of flight cadets based on the MEQ

Type	*PER3 (4/4)*		*PER3 (4/5)*		*PER3 (5/5)*	
	Flight cadets at enrollment	Flight cadets after 24 months	Flight cadets at enrollment	Flight cadets after 24 months	Flight cadets at enrollment	Flight cadets after 24 months
Definitely morning type	9	0	3	0	0	0
Moderately morning type	62	27	10	4	1	0
Neither type	44	87	14	23	2	2
Moderately evening type	1	2	0	0	0	1
Definitely evening type	0	0	0	0	0	0
Total	116	116	27	27	3	3
X^2 value	37.26		7.70		2.0	
P value	< 0.001		0.016		0.367	

were similarly distributed and no significant difference was revealed (Fisher's exact test: X^2=0.7110, *P*=0.70, *P* > 0.05). The observed genotype frequencies in cases and controls were in accord with HWE (*P* > 0.05).

Also, cases were not significantly different from the controls with respect to the allelic distributions (chi-square test: X^2=0.1061, *P*=0.7446, *P* > 0.05) (**Table 2**). These results showed that genetic background of the flight cadets was comparable to the controls, and this implicated the representiveness of our samples.

Comparison of circadian rhythmicity in flight cadets on enrollment and 24 months after enrollment

The chronotype of flights cadets at the two time points was classified according to the typing method of MEQ (**Table 3**). We found the circadian rhythmicity in flight cadets at the time of enrollment and 24 months after enrollment was significantly different (Wilcoxon Score: Z=6.6591, *P* < 0.0001), suggesting militarized management may have major effects on chronotype.

Analysis of MEQ scores of PER3 genotypes in flight cadets (at enrollment and 24 months after enrollment)

The average MEQ score for individuals who harbored the *PER3 (4/4)* was the highest at enrollment with ~10 points difference compared with the individuals with the *PER3 (5/5)* genotype (**Table 4**). The score difference was reduced to ~5 points after 24 months of the militarized management. The MEQ scores of *PER3 (5/5*) individuals were not significantly different

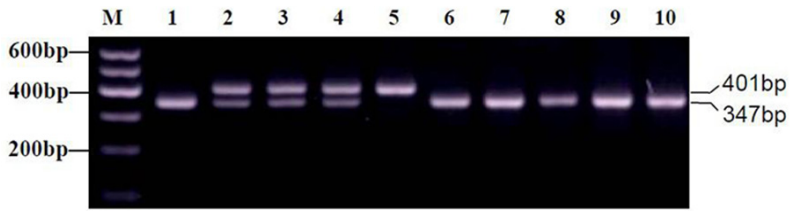

Figure 1. Identification of the *PER3* genotypes in 10 flight cadets. 2.5% Agarose gel electrophoresis of PCR amplification products of the 18th exon of PER3 in 1×TAE buffer. Lanes 1, 6-10 show individuals with homozygous *PER3 (4/4)*, lanes 2-4 show individuals with heterozygous *PER3 (4/5)*, lane 5 is individual with homozygous *PER3 (5/5)*.

(*t*=1.91, *P*=0.1281) before and after militarized management, suggesting that the chronotype of *PER3 (5/5)* individuals is stable and resistant to external changes, whereas *PER3 (4/4)* individuals are prone to be influenced by environmental factors.

Analysis of the relationship between chronotype and PER3 genotypes

Analysis of chronotype and *PER3* genotype distribution in flight cadets uncovered a couple of trends: (1) individuals with *PER3* genotypes were mainly definitely morning type, moderately morning type, and neither type (one person at enrollment and 3 persons after 24 months were moderately evening type); (2) no individuals were categorized as the definitely evening type (**Table 5**).

Statistical analysis showed significant differences in circadian rhythm associated with the *PER3 (4/4*) genotype but not the *PER3 (4/5)* or *PER3 (5/5)* genotype. The results indicated that the *PER3 (4*) allele may serve as a circadian rhythm modifier and the *PER3 (5)* allele maintains circadian rhythm in a stable condition.

Discussion

There has been much speculation on the role of *PER3* gene in human circadian rhythmicity regulation. Herein, we reported for the first time changes of chronotype in a Han Chinese population comprising 146 flight cadets at different time points. The purpose of our study was to explore the influence of militarized management on chronotype in flight cadets with PER3 4/5 genotypes. We observed significant difference in circadian rhythmicity between upon enrollment and 24 months after enrollment, and this suggested that the chronotype of flight cadets would be altered as environment changes. Seve ral lines of evidence have shown the ability of MEQ to detect chronotype is associated with age. For example, Jones *et al.* [10] investigated the association of circadian rhythmicity using MEQ with age and found a significant association, with the youngest age group (18-29 years) having the highest MEQ scores. In this work, we enrolled a group of young subjects (age range 18.8-20.8) and therefore infer that changes in chronotype of flight cadets is associated with militarized management, and life under militarized management constitutes a cause of these changes.

The role of *PER3* in morningness or eveningness preference remains largely unknown. Although many groups have set out to determine the relation of *PER3* and circadian rhythmicity, the previous studies have produced mixed results. Archer *et al.* [3] investigated the linkage between *PER3* and Delayed Sleep Phase Syndrome (DSPS), showing *PER3 (4)* is linked with eveningness preference. However, the results of a Brazilian study in DSPS patients by Pereira *et al.* [12] showed a clear association of *PER3 (5)* with eveningness preference. A circadian rhythm phenotype-related study by Viola *et al.* [14] showed that the homozygous *PER3 (5/5)* had significant effects on sleep structure including sleep homeostasis and shorter waiting time before sleep compared with the homozygous *PER3 (4/4)*. In addition, a circadian rhythm phenotype-related study by Viola *et al.* [14] showed that the homozygous *PER3 (5/5)* had significant effects on sleep structure including sleep homeostasis and shorter waiting time before sleep compared with the homozygous *PER3 (4/4)*. Conversely, the study by Osland *et al.* [13] with a fixed 75% power to detect an association of the *PER3 (4)* with preference for morningness or eveningness suggested no association of the *PER3* clock gene and chronotype in a Norway population. In this investigation, we selected a repre-

sentative Han Chinese population, and found no extreme diurnal preference associated with *PER3 (4*) allele or the *PER3 (5)* allele. The mixed findings may be caused by sampling variances, study design and different ethnic groups. Therefore, the exact role of *PER3 (4/5)* genotypes in circadian rhythmicity merits further investigation.

In conclusion, our study indicated the *PER3 (4*) allele, rather than the *PER3 (5)* allele, represented a potent modifier of circadian rhythmicity. Further well-designed studies are warranted to validate the association between *PER3 (4/5)* genotypes and chronotype and to identify the population more adaptable to environmental changes.

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Disclosure of conflict of interest

None.

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