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Original Article

Chronotype and a *PERIOD3* variable number tandem repeat polymorphism in Han Chinese pilots

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Abstract: An association has been determined between variable number tandem-repeat (VNTR) polymorphisms in the *PERIOD3* gene (*PER3*, rs57875989) and chronotype. An association has been found in which the longer *PER3(5)* allele is correlated with diurnal preference and shorter *PER3(4)* allele is linked with preference for evening, respectively. In this study, we explored the genotype frequency and relationship to the chronotype of a *PER3* VNTR polymorphism in Han Chinese pilots compared to other populations to further develop aviation safety research. DNA samples were genotyped with respect to the 4-repeat and 5-repeat alleles of the *PER3* VNTR polymorphism. We compared and analyzed *PER3* VNTR genotype frequencies of a general Han Chinese population and Han Chinese pilots. The chronotypes of our subjects were evaluated by the morningness-eveningness questionnaire (MEQ). The distribution of *PER3* VNTR genotype frequencies from 240 Han Chinese was determined (*PER3(4/4)*, 78.3%; *PER3(4/5)*, 20.0%; *PER3(5/5)*, 1.7%) and compared to the genotype frequencies of 126 Han Chinese pilots (*PER3(4/4)*, 71.4%; *PER3(4/5)*, 26.1%; *PER3(5/5)*, 2.4%). Statistical analysis revealed no significant difference between the general Han Chinese population and Han Chinese pilots regarding the *PER3* VNTR genotype and allele frequencies ($x^2 = 2.170$, $p > 0.05$). Furthermore, MEQ results showed no association between the *PER3* VTNR polymorphism and chronotype. However, *PER3* VNTR genotype frequencies differed significantly between Han Chinese and other ethnic groups previously reported, such as Caucasians, African Americans and Italians. These data indicate that the proposed role of the *PER3* VNTR needs further clarification and the role of *PER3(5)* allele in sleep regulation needs to be investigated in more detail. In particular, a study of *PER3* polymorphisms with a larger sample size of Han Chinese individuals and Han Chinese pilots may be required.

Keywords: *PER3* VNTR, chronotype, circadian rhythm, sleep deprivation, diurnal preference, gene frequency, Han Chinese

Introduction

Pilot fatigue is one of the biggest threats to aviation safety due to impairments in alertness and performance. However, fatigue is a normal response to many aspects of flight operations, such as sleep loss, shift work, and long-haul flights. Its negative impact on flight crew performance can be significant. All pilots must remain alert and contribute to flight safety by their actions, observations and communications. There are two major physiological phenomena that have been established in creating fatigue: sleep loss and circadian rhythm disruption [1]. Circadian rhythm is a daily periodicity, with an approximate 24-hour cycle in the biochemical, physiological or behavioural processes of living beings. Although it is endogenously generated, it may be modified or entrained by external environmental cues, called Zeitgebers [2-5]. In humans, the most obvious circadian rhythm is the daily pattern of sleep and wakefulness [3, 4]. According to the most widely accepted model of sleep regulation, the timing of sleep and wakefulness is controlled by two processes: a sleep homeostatic process that underlies

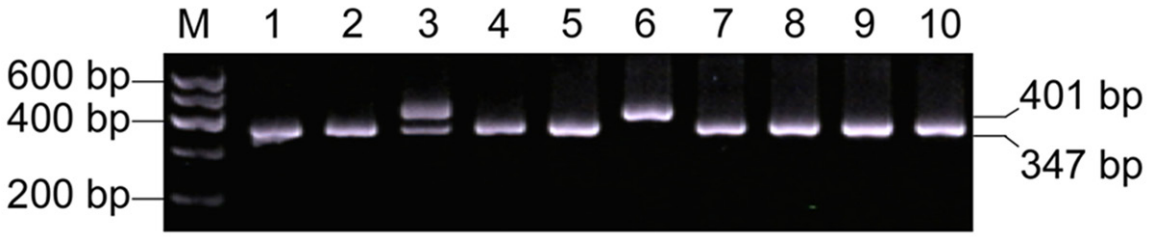

Figure 1. *PER3* VNTR polymorphisms. The *PER3* VNTR genotypes were typically shown by 2.5% of agarose gel electrophoresis from PCR products. Lane 6 is a homozygote of the longer allele (*PER3(5/5)*); lanes 1, 2, 4, 5, 7, 8, 9 and 10 are homozygotes of the shorter allele (*PER3(4/4)*); and lane 3 is the heterozygote (*PER3(4/5)*) respectively.

the rise of sleep propensity during wakefulness and its dissipation during sleep, and a circadian process that determines the thresholds for switching between sleep and wakefulness [5]. The accepted model for the molecular machinery that generates circadian rhythms involves a number of clock genes and their products [6].

The primate-specific, variable number tandem-repeat (VNTR) polymorphism in the coding region (18th exon) of the clock gene, *PERIOD3* (*PER3*), contains a 54-nucleotide (rs57875989) that is repeated four (*PER3(4)* allele) or five (*PER3(5)* allele) times [7, 8]. The *PER3* VNTR polymorphism confers vulnerability to sleep loss and circadian misalignment through its effects on sleep homeostasis [6, 13]. Differences in sleep-wake cycle, sleep propensity and cognitive performance during sleep loss were noted between individuals who are homozygous for the shorter or longer allele in the general population living at low latitudes in the southern hemisphere [9]. Under conditions of total sleep deprivation, cognitive deficits of *PER3(5/5)* individuals appeared during the 2-4 hours interval after the midpoint of the melatonin rhythm. Executive functions of *PER3(5/5)* homozygotes greatly declined at approximately 6-8 a.m. [10]. The *PER3* VNTR polymorphism has also been reported to be strongly associated with delayed sleep phase syndrome (DSPS), which is a circadian rhythm sleep disorder [11, 12]. The VNTR polymorphism in the human *PER3* gene exhibits significantly different in shorter allele frequencies between Papua New Guineans (0.19) and East Asians (0.80-0.89), whereas European, American and African populations have intermediate frequencies (0.6-0.7) [13]. To date, the *PER3* VNTR allele frequency of a Han Chinese population and Han Chinese pilots have not been reported. Furthermore, the geographical and ethnic differences in allele frequency have yet to be explored for Asians.

The terms “chronotype” or “morningness-eveningness” are used to describe differences in individual sleep-wake patterns. Individuals who go to sleep early, get up early, and feel and perform better in the morning are classified as morning-types. Likewise, individuals who go to bed late, wake up late, and perform better later in the day are classified as evening-types [14]. The *PER3* VNTR polymorphism has been reported to be associated with chronotype, i.e., the preferred timing of waking and sleep.

The Horne-Östberg morningness-eveningness questionnaire (MEQ) is a self-assessment questionnaire developed by Horne and Östberg in 1976 that is frequently used to assess an individual’s circadian rhythm [15]. The MEQ consists of 19 questions, with each question having four response options. Responses to the questions are combined to form a composite score, which indicates the degree to which the respondent favors mornings versus evenings. Based on their score, individuals are divided into one of five chronotype categories: definite evening type (DET), moderate evening type (MET), neither type (NT), moderate morning type (MMT) and definite morning type (DMT) [15]. The longer allele *PER3(5)* has been associated with a morning preference, and the shorter allele *PER3(4)* with an evening preference [7, 11]. A number of studies have evaluated the chronotypes of individuals in different populations, but there have been no comparable studies in a Han Chinese population. Moreover, there are no published data on the chronotypes of Han Chinese pilots. Individual differences in sleep and chronotype may be associated with certain physical and mental

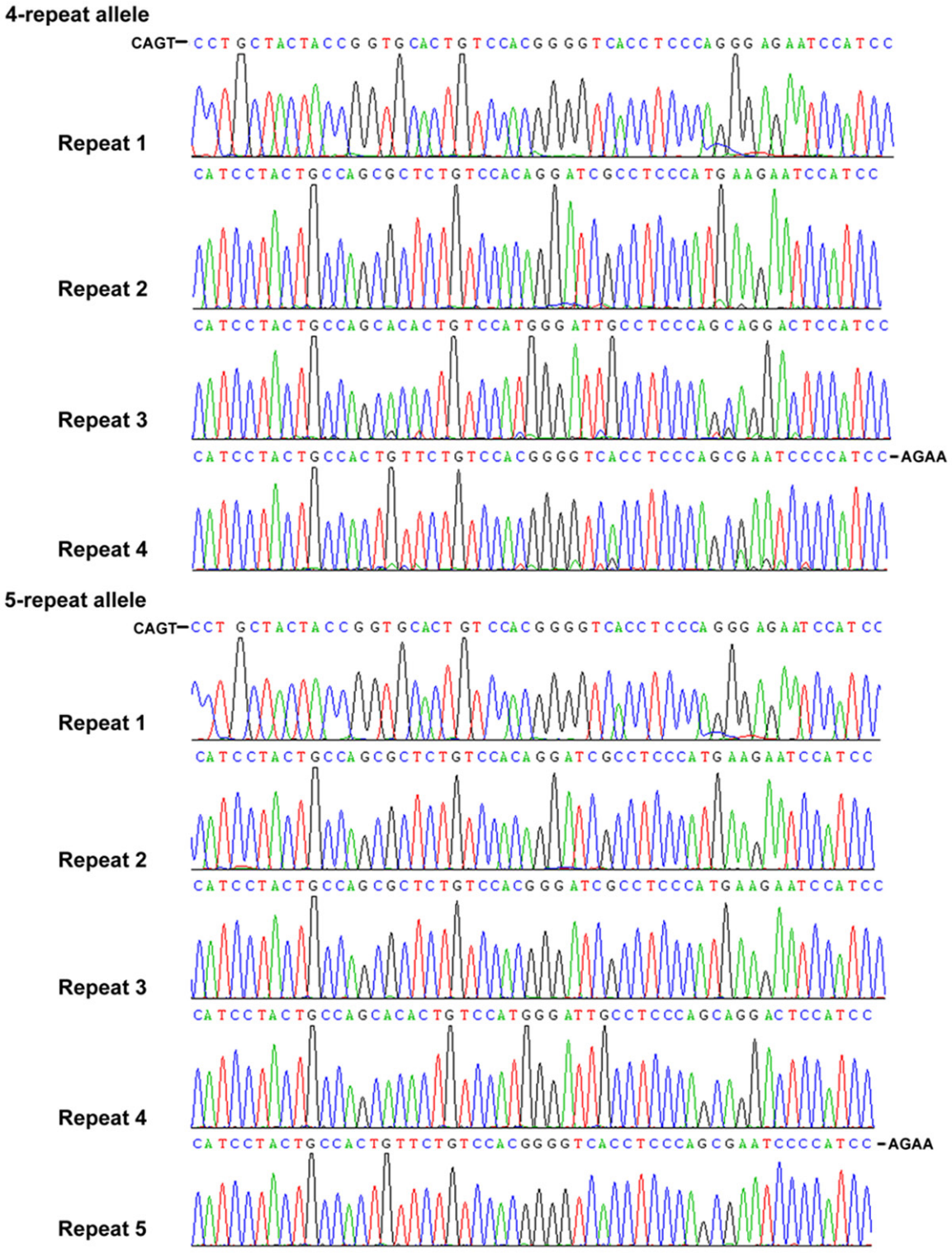

Figure 2. Nucleotide sequences of each allele in the polymorphic repeat region of the *PER3*.

health characteristics, but few genetic determinants of these differences have been identified [16]. As the adequate sleep status of pilots is imperative for aviation safety, it is better to investigate the association between *PER3* VNTR polymorphism and chronotype.

Table 1. Comparison of *PER3* genotype frequency between Han Chinese pilots and Han Chinese [n (%)]

Population	*PER3(4/4)*	*PER3(4/5)*	*PER3(5/5)*	Total	x^2 value	*P* value
General Han Chinese	188 (78.3)	48 (20.0)	4 (1.7)	240	2.170	0.338
Han Chinese Pilots	90 (71.4)	33 (26.1)	3 (2.4)	126		

Materials and methods

Study participants

Study participants were drawn from two sources, the Han Chinese participants consisting of 240 individuals (120 females and 120 males), the mean age (± SD) at recruitment was 49.0 ± 16.0 year and Han Chinese pilots consisting of 126 male participants, the mean age at recruitment was 30.6 ± 6.2 years and their average overall flying experience was 12 years. The geography and ethnicity were matched between pilots and non-pilots to minimize the potential population stratification. Written consents were obtained from all cadets who participated in this study. After obtaining an informed written consent, the study was conducted in accordance with the ethical standards for biological rhythm research on animals and human beings [17], and was approved by the Regional Committee for Medical Research Ethics in the Navel General Hospital of PLA, China.

Sample collection and genomic DNA extraction

A minimum of 2 mL fasting whole blood, containing anticoagulant of K_2EDTA, was obtained and stored at 4°C. Genomic DNA was extracted from whole human blood using the PAXgene Blood DNA Kit (Qiagen, Cat. 761125) according to a manufacture's manual. The concentration of genomic DNA used for PCR amplification was 200-400 ng/μL and the 260/280 optical density (OD) ratio was 1.7-1.9.

PER3 genotyping

PCR primer pairs (forward primer: 5'-TGTCTTTTCATGTGCCCTTACTT-3'; reverse primer: 5'-TGTCTGGCATTGGAGTTTGA-3' [18]) were synthesized and purified by Sangon Inc. (Shanghai, China). Each 10-μL PCR reaction mixture contained 150 ng genomic DNA, 5 pM of each primer and 2× PCR mix buffer (Tiangen). An MJ research PTC-225 thermocycler was used to heat the samples (95°C for 5 minutes; 30 cycles of 40 s at 94°C, 30 s at 60°C, and 40 s at 72°C; and a final elongation step of 72°C for 12 min). The amplified products were analyzed by 2.5% agarose gel electrophoresis. A 401-bp fragment was amplified of the *PER3* VNTR five repeat alleles. A 347-bpfragment was amplified from the *PER3* VNTR four repeat alleles. Two bands of different sizes indicated heterozygote individuals. Genotype results were verified by direct sequencing.

Horne-Östberg morningness-eveningness questionnaire (MEQ)

The MEQ consists of 19 questions that relate to sleep/wake behaviors and yields scores that range from 16 to 86. Based on their scores, individuals were divided into one of five chronotype categories: 16-30 DET; 31-41 MET; 42-58 NT; 59-69 MMT; and 70-86 DMT [15].

Statistical analyses

Distribution differences of *PER3* genotype frequencies between Han Chinese pilots and general Han Chinese were compared. In addition, the differences of *PER3* genotype frequencies among the chronotypes of the pilots were compared. All analyses were executed using Stata 12.0 software (Stata Corporation, College Station, Texas, USA).

Results

PER3 VNTR genotype

The *PER3* VNTR genotype was identified from differing sizes of PCR amplified fragment lengths (**Figure 1**). The direct sequencing analysis for PCR-amplified *PER3* genomic fragments showed the veracity of the genotyping results (**Figure 2**).

Comparison of PER3 VNTR genotype frequencies between Han Chinese pilots and the general Han Chinese population

The *PER3* genotype frequencies of Han Chinese pilots were compared to those from the general Han Chinese population (**Table 1**). Han Chinese pilots had *PER3(4/4)* in 71.4%, *PER3(4/5)* in 26.1% and *PER3(5/5)* in 2.4%. No significant

Table 2. Comparison of *PER3* VNTR genotypic frequencies between Han Chinese, Caucasians, African Americans and Italians [n (%)]

Population	*PER3(4/4)*	*PER3(4/5)*	*PER3(5/5)*	Total	Reference
Han Chinese	188 (78.3)	48 (20.0)	4 (1.7)	240	
Caucasians	20 (40.8)	24 (49.0)	5 (10.2)	49	[19]
African Americans	31 (40.8)	37 (48.7)	8 (10.5)	76	[19]
Italian	35 (35.3)	46 (46.5)	18 (18.2)	99	[19]

Table 3. Results of MEQ and *PER3* VNTR genotypes of Han Chinese pilots (n)

Chronotype	*PER3(4/4)*	*PER3(4/5)*	*PER3(5/5)*	Total	x^2 value	*P* value
DMT	2	2	0	4	3.280	0.773
MMT	24	7	0	31		
NT	59	23	3	85		
MET	5	1	0	6		
DET	0	0	0	0		
Total	90	33	3	126		

differences were found between the *PER3* genotype frequencies of the Han Chinese pilots and the general Han Chinese population (x^2 = 2.170, p = 0.338). In addition, the genotype distributions were found to be in accordance with Hardy-Weinberg equilibrium.

Comparison of PER3 VNTR genotype frequencies between Han Chinese, Caucasians, African Americans and Italians

The results of *PER3* VNTR genotype frequencies of Han Chinese was compared with those from previous studies with populations of Caucasians [19], African Americans [19] and Italians [18]. The study of *PER3* genotype frequencies in a Han Chinese population showed that *PER3(4/4)*, *PER3(4/5)* and *PER3(5/5)* were found in 78.3%, 20.0% and 1.7% respectively. These results demonstrate significant statistical differences when compared to Caucasians (x^2 = 41.33, p < 0.01), African Americans (x^2 = 41.33, p < 0.01) and Italians (x^2 = 66.84, p < 0.001) (**Table 2**).

MEQ chronotype

The MEQ was completed by 126 pilots (100% recovery and effective rates) and correlated with their *PER3* genotype. The chronotype results were divided into five types. Four subjects were described as DET, 31 subjects as MET, 85 subjects as NT, 6 subjects as MLT, and no subjects were described as DLT (**Table 3**). The correlation between chronotype and genotype showed no statistically significant differences (x^2 = 3.280, p = 0.773). We merged definitely early-type and moderately early-type with morning-type individuals, and moderately late-type and definitely late-type were merged with night-type individuals for statistical analysis. The analysis of the correlation between alleles and chronotypes showed that there was no difference in the allele frequency of the three chronotypes in Han Chinese pilots (Fisher's exact test: x^2 = 6.819, p = 0.702) (**Table 4**). The results of the MEQ also revealed that the chronotype of the homozygote individuals of genotype *PER3(5/5)* were NT and thus did not indicate that allele *PER3(5)* was related to morning preference. Furthermore, there were two subjects of DET with the *PER3(4/4)* genotype and the *PER3(4/5)* genotype, which may have been a result of the small sample size.

Discussion

PER3 VNTR genotype and allele frequencies between Han Chinese pilots and a general Han Chinese population

We identified the *PER3* VNTR genotypes of 126 Han Chinese pilots. The genotype frequencies of *PER3(4/4)*, *PER3(4/5)* and *PER3(5/5)* were 71.4% (n = 90), 26.1% (n = 33) and 2.4% (n = 3), among which the *PER3(4)* and *PER3(5)* alleles comprised 84.5% and 15.5%, respectively. Compared with the *PER3* genotype and allele frequencies of a general Han Chinese population, there were no statistically significant differences (x^2 = 2.170, p > 0.05). The genotype distributions were in Hardy-Weinberg equilibrium. These results indicate that the *PER3(5/5)* genotype need not be the sifting factor during pilot selection and the training process. Of course, we may need a larger sample size to verify this conclusion.

PER3 VNTR genotype frequency between Chinese, Caucasians, African Americans and Italian populations

Different races and ethnicities have been reported to have different *PER3* VNTR genotype frequencies. We identified the *PER3* VNTR gen-

Table 4. *PER3* VNTR allele frequency and chronotype of Han Chinese pilots (%)

Chronotype	*PER3(4)*	*PER3(5)*	Total	x^2 value	*P* value
Morning Type	87.1	12.9	100	6.819	0.702
Neither Type	88.1	18.1	100		
Evening Type	91.7	8.3	100		

otypes of 240 Chinese and compared them to the genotype frequencies in Caucasians [19], African Americans [19] and Italians [18]. The results revealed that the principal genotype of Chinese was *PER3(4/4)*. Interestingly, the frequency of *PER3(4/4)* was approximately twice as much as the other three populations and accounted for 78.3% of the sample size. However, the *PER3(5/5)* genotype of the Han Chinese population was the least frequency, accounting for just 1.7% of the sample size, which was between 10% to 16.7% as much as the other three populations. Our data indicate significant differences in the genotype frequency distribution between Han Chinese and different geographical/ethnic populations. A larger sample size with clearer chronotype and *PER3* classifications may be needed to verify the conclusions [8, 20].

Chronotype and PER3 VNTR polymorphism

Homozygotes for the longer allele (*PER3(5)*) of the VNTR polymorphism are typically associated with increased morning preference, earlier waking times and bedtimes, and reduced daytime sleepiness [16, 21]. In another study that investigated morning or evening type preference in a student population of a Norwegian University, a 75% correlation for *PER3(4)* was reported. However, the authors did not find any correlation between *PER3* VNTR polymorphisms and the chronotype [20]. Similarly, our results also did not confirm an association between the *PER3* VNTR polymorphism and chronotype. Therefore, diurnal preference does not appear to be associated with the *PER3* VNTR polymorphism in our sample of Han Chinese pilots. Furthermore, the MEQ analysis for the pilots also suggested that there was no correlation between *PER3(5)* and diurnal preference. It may be that the small number of samples involved in the present results, but the distribution of MEQ scores is likely to be biased by several other factors, including gender, age, genetic background, latitude and social habits [22].

Conclusion

The study of the influence of circadian rhythm on the flying ability of pilots has great potential to improve aviation safety. We have identified the *PER3* genotypes in Han Chinese pilots, and Han Chinese general population and analyzed the correlation to population of pilots. Although our results demonstrated no significant differences between the genotype frequency and chronotype, we raise some interesting questions. In particular, is the identification of *PER3(5/5)* genotypes in pilots of significant importance for pilot screening in flight training and mission operations? Furthermore, our results could provide a genetic reference tool for screening pilots in order to prevent in-flight accidents.

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Disclosure of conflict of interest

None.

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