

CIRCADIAN RHYTHMS

Association of the Length Polymorphism in the Human *Per3* Gene with the Delayed Sleep-Phase Syndrome: Does Latitude Have an Influence Upon It?

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Study Objectives: The objective of this study is to analyze the influence of a previously reported *hPer3* gene-length polymorphism in the delayed sleep-phase syndrome and in morningness-eveningness tendencies at low latitudes in the southern hemisphere.

Design: We have genotyped a length polymorphism in the *hPer3* gene characterized by a short repeat allele (4-repeat) and a long repeat allele (5-repeat).

Participants: Seventeen patients with delayed sleep-phase syndrome; 156 volunteers chosen according to Horne-Östberg questionnaire to have morning, intermediate, or evening preference; and 110 volunteers with no Horne-Östberg score as a sample of the general population.

Results: We have found a higher frequency of 5-repeat allele in the

delayed sleep-phase syndrome group and an association of this polymorphism with diurnal preference.

Conclusion: Our results suggest that latitude has a role in the influence of *hPer3* gene polymorphism on delayed sleep-phase syndrome and confirm previous data showing its association with morningness-eveningness tendencies.

Key words: *Per3* gene, circadian rhythm, morningness-eveningness, molecular clock, sleep disorders, delayed sleep phase syndrome.

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INTRODUCTION

IN RECENT YEARS, THERE HAS BEEN A RAPID AND SIGNIFICANT PROGRESS IN UNDERSTANDING THE MOLECULAR BASIS OF THE CIRCADIAN RHYTHMICITY, AS SEVERAL CLOCK GENES HAVE BEEN CLONED IN MAMMALS. Studies with these genes focusing on loss of function have demonstrated abnormal sleep/wake length periods, disturbed entrainment, and loss of persistence of circadian rhythms. Current findings clearly indicate that the core components of the pacemaker work on the basis of feedback loops of gene expression and repression.¹⁻⁵

The same genes found in rodents remain well preserved in humans and the same mechanisms that underlie endogenous circadian rhythmicity in these animals are likely to play an important role in regulating human circadian rhythms.⁶ The wake/sleep cycle is regulated in a circadian fashion, in addition to its homeostatic regulation. Since mutations in rodent clock genes are related to circadian abnormal regulation of the rest/activity cycle, it is likely that mutations in the same genes may give rise to circadian rhythm-related syndromes in humans. Alternatively, less drastic changes in these genes, such as natural polymorphisms, could be related to subtle differences in circadian phenotypes, as seen in the so-called “morning” and “evening” persons, ie, individuals who reportedly function bet-

ter during specific parts of the day.

Since the first publication⁷ reporting an association of a polymorphism in the *Clock* gene with diurnal preference, there has been a steady increase in the number of studies assessing possible correlations between polymorphisms or mutations and circadian phenotypes in humans. Katzenberg et al⁸ and Pedrazzoli et al⁹ did not find any association of the human *Per1* and *Timeless* polymorphisms with diurnal preference. Toh et al¹⁰ reported a familial case of advanced sleep phase syndrome with a mutation in the *hPer2* gene. Ebisawa et al¹¹ reported an association of polymorphisms in the human *Per3* gene (*hPer3*) with the delayed sleep phase syndrome (DSPS) in Japan, and Archer et al¹² reported an association of a length polymorphism in the same *hPer3* gene with evening preference and DSPS in England.

These last results were obtained in developed countries located in the northern hemisphere, where there is considerable seasonal variation in day length along the year. A pertinent question is whether these effects of the *hPer3* length polymorphism would also be observed in populations living in the southern hemisphere. In order to verify this possibility, we have genotyped this polymorphism in a population selected for diurnal preference in the cities of São Paulo and Curitiba, Brazil, and also in a group of patients with DSPS living in the city of São Paulo.

MATERIAL AND METHODS

Subjects

Volunteers

A total of 1089 volunteers answered the Horne-Östberg (HO)¹³ questionnaire for determination of diurnal preference. Based on their HO scores, 58 volunteers in the category of morning preference, 40 under the category of evening preference, and 58 in the category of intermediate (mean age 25.85 ± 7.1 years, 66.6%

Disclosure Statement

This is not an industry supported study. Drs. Pereira, Tufik, Louzada, Benedito-Silva, Lopez, Lemos, Korczak, D’Almeida, and Pedrazzoli have indicated no financial conflicts of interest.

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women, 77% Caucasian) were selected. One hundred and ten volunteers (mean age 48.91 ± 17.35 years, 60% women, 76.36% Caucasian), for whom HO scores were not obtained, participated in the study as a sample of the general population.

Patients

The study included 17 patients (mean age 28.88 ± 11.5 years, 47 % women, 77% Caucasian) with a diagnosis of DSPS, according to the *International Classification of Sleep Disorders*,¹⁴ in the Department of the Psychobiology/Sleep Institute, São Paulo, Brazil. Twelve of the patients agreed to undergo sleep recording and had their circadian profiles of rectal temperature and melatonin secretion assessed; they also wore an actigraph for 2 weeks, which allowed us to have a physiologic confirmation of their condition. The study was approved by the Committee on Ethics of the Federal University of São Paulo, and the patients gave informed consent with acknowledgement of their awareness of the theme of the study. Case and control groups shared the same sociodemographic characteristics and ethnic background.

Genotyping

Blood samples were collected from all participants in the study, and DNA was extracted from white cells.¹⁵ The *hPer3* length polymorphism was genotyped as in Ebisawa et al¹¹ and Archer et al¹² studies.

Statistical Analysis

The allelic frequencies in the groups were compared using the χ^2 test with the Yates correction; for comparison among chronotype groups, we used χ^2 for trend. The *P* value was set at .05.

RESULTS

Table 1 shows time of sleep onset, time of melatonin-secretion onset, time of minimum body temperature, and genotype for the patients with DSPS. The mean sleep-onset time for the patients was $2:27$ AM ± 72.9 minutes, the mean time of melatonin-secretion onset was $11:25$ PM ± 140.49 minutes, and the mean temper-

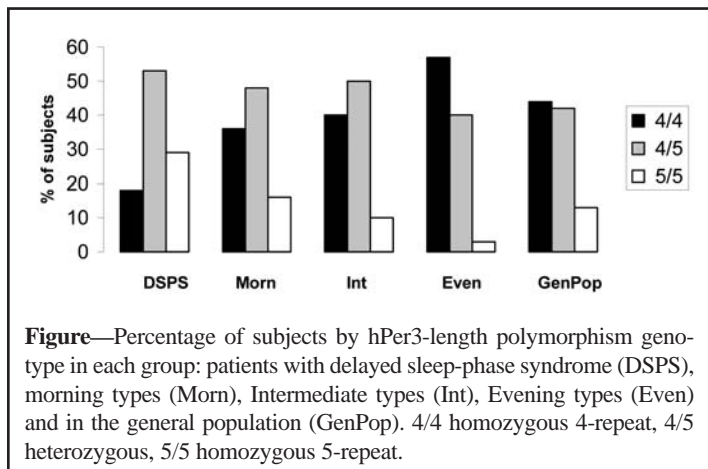
ature nadir was $6:53$ AM ± 126.14 minutes.

Our genotyping data showed a significantly higher 5-repeat and lower 4-repeat allele frequencies in the DSPS group (5-repeat = 0.56, 4-repeat = 0.44) when compared with the sample from the general population (5-repeat = 0.34, 4-repeat = 0.66; $\chi^2 = 4.85$, *P* = .028, odds ratio, 95% CI: 2.4 [1.1-5.3]) or when compared with evening group (5-repeat = 0.23; 4-repeat = 0.77; $\chi^2 = 5.82$, *P* = .016). Interestingly, we did not observe any difference when comparing the frequencies of the patients with DSPS with those from the morning group (5-repeat = 0.40; 4-repeat = 0.60; $\chi^2 = 2.95$, *P* = .086). A significant trend was observed among morning, intermediate, and evening groups (χ^2 test for trend = 5.91, *P* = .015), with the frequency of 5-repeat allele significantly higher in the morning group ($\chi^2 = 6.33$, *P* = .01, odds ratio 95% CI: 2.23[1.1-4.5]) as compared to the evening group. The intermediate group is different from neither the evening nor the morning groups (*P* > .05). Table 2 shows the *hPer3* allele frequencies in the studied groups. The figure shows the genotypic frequencies for each group.

DISCUSSION

Our data show that the 5-repeat allele is significantly higher in the group of patients with DSPS than in the general population and suggest a role for this allele in the pathophysiology of the syndrome. Our results thus confirm the findings of Archer et al,¹² showing an association of the *hPer3* length polymorphism in the exon 18 with diurnal preference in humans. Surprisingly, however, our results show an effect of the opposite allele on DSPS; while Archer et al¹² found no 5-repeat allele homozygous for the DSPS individuals, the present study found nearly 30% of the patients with DSPS to be homozygous for the 5-repeat allele.

A possible reason for the difference between our results and



Figure—Percentage of subjects by *hPer3*-length polymorphism genotype in each group: patients with delayed sleep-phase syndrome (DSPS), morning types (Morn), Intermediate types (Int), Evening types (Even) and in the general population (GenPop). 4/4 homozygous 4-repeat, 4/5 heterozygous, 5/5 homozygous 5-repeat.

Table 1—Sleep-Onset Time, Melatonin-Secretion Onset, Temperature Nadir, PER3 genotype for the Patients with Delayed Sleep-Phase Syndrome

DSPS Patients	SOT	MSO	TN	PER3 genotype
1	3:12 AM	10:30 PM	4:12 AM	4/5
2	3:47 AM	2:01 AM	9:41 AM	4/5
3	1:34 AM	7:07 PM	4:01 AM	4/4
4	1:03 AM	9:50 PM	6:52 AM	4/5
5	2:19 AM	00:58 AM	_____	5/5
6	2:24 AM	00:38 AM	7:24 AM	4/5
7	11:53 PM	7:45 PM	4:30 AM	4/5
8	2:52 AM	00:12 AM	8:36 AM	4/4
9	3:46 AM	00:09 AM	6:26 AM	5/5
10	1:43 AM	00:27 AM	7:51 AM	5/5
11	3:05 AM	_____	6:08 AM	5/5
12	3:46 AM	2:02 AM	10:06 AM	4/5

DSPS refers to delayed sleep-phase syndrome; SOT, sleep-onset time; MSO, melatonin-secretion onset; TN, temperature nadir.

Table 2—*hPer3* Length Polymorphism Allele Frequencies in Patients with Delayed Sleep-Phase Syndrome, Morning Types, Intermediate Types, and Evening Types and in the General Population.

Group	4-repeat allele frequency	5-repeat allele frequency
General population	0.66	0.34
DSPS	0.44	0.56
Morning	0.60	0.40
Intermediate	0.65	0.35
Evening	0.77	0.23

DSPS refers to delayed sleep-phase syndrome.

those of Archer et al¹² is the ethnic background of the studied populations. The Brazilian population is formed mainly of a European/Portuguese-Brazilian/Indian background^{16,17} mixed with a variety of African groups and later, in the beginning of 20th century, with a variety of European ethnicities (mainly Italian and Spanish) and Asiatic ethnicities (mainly Japanese).¹⁶ Because the control and DSPS patient groups are formed mainly by the same background with a major proportion of European Caucasians, we believe that there is no reason for ethnic bias in our sample. Besides, the *hPer3* allele frequencies in our population sample are very similar to those described in Archer et al's¹² study, suggesting similarity between both populations.

Another possible reason for the difference between our results and those of Archer et al¹² is the latitude of the cities where the studies were carried out. London is located at 51°30'N, whereas São Paulo is located at 23°32'S. There is a considerable difference between these cities in terms of the length of days throughout the year. In São Paulo, the average day length during the winter months is about 10.5 hours and the longest day length, about 13.5 hours during the summer months (3-hour difference), whereas, in London, the shortest days last about 8.0 hours and the longest about 16.0 hours (8-hour difference). In addition, the climate is also very different, including temperature variations throughout the year (such variations are less drastic in São Paulo) and sun brightness. Under such extremely different conditions, organisms may have to adopt different strategies to entrain, sometimes losing track of the day length, which may be, at least in part, dependent on the *Per3* genotype and latitude. Recent studies in *Drosophila melanogaster* have demonstrated that variations in the *dPer* gene are associated with responses to temperature and day length—variables related to latitude.^{18,19}

It is well known that the entrainment by light depends on the time when the circadian system is exposed to light. The phase of entrainment depends on the strength of the zeitgeber and on the individual's free-running period. Circadian systems are influenced by intensity and duration of the light signal, responding more or less strongly, leading to steeper or flatter phase-response curves.²⁰ At different latitudes, such as those found in London and São Paulo, the same genotypes exposed to different zeitgeber strengths could result in different phenotypes. However, at the present time, it is difficult to elucidate the exact mechanism by which the *hPer3* gene could influence the phase of entrainment and/or the intrinsic free-running period in different latitudes.

Our data also reveal an unexpected association between DSPS and morningness in the Southern hemisphere, as we have not found any difference in the frequency of this polymorphism among these groups. The apparent contradiction of these findings may reflect the presence of common mechanisms underlying the DSPS and morning-preference phenotypes,²¹ since similar features in the phase relationship between endogenous circadian rhythms and the wake/sleep cycle, as a longer interval between the circadian phase of body temperature and the sleep offset, have been shown.^{22, 23}

Assuming that the Archer et al¹² data and the data we have collected hold true, we may infer that patients with DSPS who move to latitudes different from their original latitude would experience an improvement in their condition. In agreement with this proposition, Gottesmann²⁴ reported a DSPS case where the patient experienced an amelioration in the symptoms after moving from Paris (latitude 48°52N) to Rio de Janeiro (22°54S).

Tournier et al²⁵ studied the clock genes' mRNA transcription under short and long photoperiods and reported that the expression pattern of all clock genes is affected by photoperiodic changes, mainly the *mPer3* gene in which the mRNA expression shows marked changes in amplitude and a large phase advance exposed to short photoperiod.

The importance of the *Per3* gene in the operation of the molecular clock system has been debated. The first *Per3* gene descriptions in the literature^{26,27} reported it as unresponsive to light pulses throughout the day. Animals with disruption in the *Per3* gene do not appear to show any major variation in the circadian phenotype, except for a minor reduction in the circadian period length, suggesting a nonessential role of this gene in the molecular clock system. However, the present results, in combination with the data collected by Archer et al¹² and Ebisawa et al,¹¹ indicate that the *hPer3* gene may play an important role in the pathogenesis of DSPS, and in diurnal preference, and may be involved in tracking the day length in the course of the year, instead of having a direct effect on the maintenance of the circadian period.

It has been proposed that the circadian pacemaker consists of a morning oscillator locking at dawn (M) and an evening oscillator locking at dusk (E), these 2 oscillators being composed at the molecular level by the *Per1/Cry1* and the *Per2/Cry2* genes, respectively.²⁸ A role for the *Per3* gene has not been included in this mechanism. Instead, *Per3* has been assigned a side role, maybe as a clock-controlled gene. Based on this hypothesis, Daan et al²⁸ suggested that there should be a coupling force in the oscillating system that would maintain the phase relationship of both oscillators (M and E) under free-running conditions. Two reports published lately indicate that PER3 can have this function; Takumi et al²⁷ have reported that *mPer3* has a broader peak of expression that reaches the peak of expression of both *mPer1* and *mPer2*, and Yagita et al²⁹ reported that the nuclear entry of *mPer1* and *mPer2* involves physical interactions with *mPer3*. Thus, it is possible that the PER3 protein could have this coupling role proposed by Daan.²⁸

These clock proteins, mainly the PER proteins, are quite complex. They comprise PAS domains associated with a cytoplasmic localization domain, casein-kinase-binding sites, phosphorylation sites, and a nuclear localization domain. They have a very dynamic kinetics, including nuclear import and export that are not yet completely understood.

The results obtained here were unexpected, and attempts should be made to replicate our findings in equivalent latitudes. Additional data on the function of this length polymorphism in the physiology of the protein and animal models exploring its effect during long and short photoperiods are also needed.

The discovery of genetic variations regulating circadian phenotypes may have a significant therapeutic impact on preventive medicine. It may prove helpful in furthering understanding and treatment of sleep and circadian rhythm disturbances, as well as in the prevention of health hazards caused by night and shift work.

REFERENCES

1. Ralph, MR, Menaker, M. A mutation of the circadian system in golden hamsters. *Science* 1998;241:1225-7.
2. Vitaterna, MH, King, DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science* 1994;264:719-25.
3. van der Horst, GT; Muijtjens, M; Kobayashi, K, et al. Mammalian

- Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 1999;398:627-30.
4. Zheng B, Larkin DW, Albrecht U, et al. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* 1999;400:169-73.
 5. Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR. Target disruption of the mPer3 gene: subtle effects on circadian clock function. *Mol Cell Biol* 2000;20:6269-75.
 6. Cermakian N, Boivin DB. A molecular perspective of human circadian rhythm disorders. *Brain Res Rev* 2003;42:204-20.
 7. Katzenberg D, Young T, Finn L, et al. A CLOCK polymorphism associated with human diurnal preference. *Sleep* 1998;21:569-76.
 8. Katzenberg D, Young T, Lin L, Finn L, Mignot E. A human period gene (hPer1) polymorphism is not associated with diurnal preference in normal adults. *Psychiatr Genet* 1999;9:107-9.
 9. Pedrazzoli M, Ling L, Finn L, Young T, Katzenberg D, Mignot E. A polymorphism in the human timeless gene is not associated with diurnal preferences in normal adults. *Sleep* 2000;3:73-6.
 10. Toh KL, Jones CR, He Y, et al. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 2001;291:1040-3.
 11. Ebisawa T, Ushiyama M, Kajimura N, et al. Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. *EMBO Rep* 2001;21:342-6.
 12. Archer SN, Robilliard DL, Skene DJ, et al. A length polymorphism in the circadian clock gene per3 is linked to delayed sleep-phase syndrome and extreme diurnal preference. *Sleep* 2003;26:413-5.
 13. Horne JA, Östberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* 1976;4:97-110.
 14. *The International Classification of Sleep Disorders, Revised: Diagnostic and Coding Manual*. Rochester, MN: American Sleep Disorders Association; 1997.
 15. Miller AS, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 1988;16:1215.
 16. Ribeiro, D. *The Brazilian People: The Formation and the Meaning of Brazil*. Gainesville: University Press of Florida; 2000:19-45.
 17. Carvalho-Silva DR, Santos FR, Rocha J, Pena SD. The phylogeography of Brazilian Y-chromosome lineages. *Am J Hum Genet* 2001;68:281-6.
 18. Rogers AS, Rosato E, Costa R, Kyriacou CP. Molecular analysis of circadian clocks in *Drosophila simulans*. *Genetica* 2004;120:213-22.
 19. Majercak J, Chen WF, Edery I. Splicing of the period gene 3'-terminal intron is regulated by light, circadian clock factors, and phospholipase C. *Mol Cell Biol* 2004;24:3359-72.
 20. Roenneberg T, Wirz-Justice A, Mrosovsky M. Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms* 2003;18:80-90.
 21. Iwase T, Kajimura N, Uchiyama, M, et al. Mutation screening of the human Clock gene in circadian rhythm sleep disorders. *Psychiatry Res* 2002;109:121-8.
 22. Ozaki S, Uchiyama M, Shirakawa S, Okawa M. Prolonged interval from body temperature nadir to sleep offset in patients with delayed sleep phase syndrome. *Sleep* 1996;19:36-40.
 23. Duffy JF, Rimmer DW, Czeisler CA. Association of intrinsic circadian period with morningness-eveningness, usual wake time, and circadian phase. *Behav Neurosci* 2001;115:895-9.
 24. Gottesmann C. Is the delayed sleep phase syndrome a physical or psychological disease? A case report of disappearance following a change of latitude. *Psychiatry Clin Neurosci* 2000;54:543-6.
 25. Tournier BB, Menet JS, Dardente H, et al. Photoperiod differentially regulates clock genes expression in the suprachiasmatic nucleus of Syrian hamster. *Neuroscience* 2003;118:317-22.
 26. Zylka MJ, Shearman LP, Weaver DR, Reppert SM. Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* 1998;20:1103-10.
 27. Takumi T, Taguchi K, Miyake S, et al. A light-independent oscillatory gene mPer3 in mouse SCN and OVLN. *EMBO J* 1998;17:4753-9.
 28. Daan S, Albrecht U, van der Horst GT, et al. Assembling a clock for all seasons: are there M and E oscillators in the genes? *J Biol Rhythms* 2001;16:105-16.
 29. Yagita K, Yamaguchi S, Tamanini F, et al. Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes Dev* 2000;14:1353-63.