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A Polymorphism in the Human Timeless Gene is not Associated with Diurnal Preferences In Normal Adults

Mario Pedrazzoli[1], Lin Ling[1], Laurel Finn[2], Terry Young[2], Daniel Katzenberg[1] and Emmanuel Mignot[1]

[1]*Center for Narcolepsy, Department of Psychiatry, Stanford University*
[2]*Department of Preventive Medicine, University of Wisconsin-Madison*

The effect of a single nucleotide polymorphism, a glutamine to arginine amino acid substitution in the human *Timeless* gene (Q831R, A2634G), on diurnal preferences was studied in a random sample of normal volunteers enrolled in a population-based epidemiology study of the natural history of sleep disorders. We genotyped 528 subjects for this single nucleotide polymorphism and determined morningness-eveningness tendencies using the Horne-Ostberg questionnaire. Our results indicate that Q831R Timeless has no influence on morningness-eveningness tendencies in humans.

CURRENT CLAIM: The A to G polymorphism in the position 2634 of human timeless gene is not related with diurnal preferences.

Genetic studies in the last years have advanced our understanding of the molecular mechanisms responsible for the control of circadian rhythms. Most of these studies have used model organisms such as *Neurospora, Arabidopis*, *Drosophila* and mouse. These studies have led to the conclusion that circadian rhythmicity is generated at the cellular level by transcription-translation feedback loops. Most interestingly, similar core genetic components were found to generate circadian rhythms in both *Drosophila* and mammals.

In mammals, the hypothesized core components of the cellular clock are *Clock*, *Bmal1*, *Period (Per)* 1, *Per*2, *Per*3, *Timeless* (*Tim*), the *Cryptochromes* (*Cry*) 1, *Cry*2 and *Casein Kinase I epsilon* (*CK*ε). It has been proposed that a heterodimer formed by the products of Clock and Bmal1 promotes the transcription of the Per gene while the Per-Tim, Per-Per, Tim-Cry and Per-Cry dimers block the effect of the Bmal1-Clock heterodimer, forming a transcription-translation feedback loop responsible for circadian rhythm control (Sangoram et al., 1998; Lowrey et al., 2000). The role of *CK*ε would be to regulate *Per* activity by phosphorylation (Lowrey et al., 2000). Mutations within some of these circadian genes have been shown to produce abnormally short or long free running periods (Vitaterna et al., 1994). In mammals, mutations in *Clock*, *Per*2, *Cry*1 and *Cry*2 have been shown to disrupt circadian period and/or entrainment by light (Vitaterna et al., 1994; van der Horst et al., 1999; Zheng et al., 1999).

In spite of this progress, few studies have addressed the issue of circadian control at the molecular level in humans. Mutations at the level of these genes are likely to produce a circadian phenotype in humans. Circadian rhythm disorders such as Delayed or Advanced Sleep Phase syndrome have been reported in families (Fink and Ancoli-Israel, 1997; Jones et al., 1999) and may be due to highly penetrant circadian gene mutations. In animals, mutations such as *Clock* or *Tau* (recently characterized as the CKε gene, Lowrey et al., 2000) are associated not only with a long or short free running period but also with alterations in the phase angle of entrainment under light-dark conditions, a phenomenon equivalent to extreme morningness-eveningness tendencies. Similarly, one subject with Familial Advance Sleep Phase Syndrome has recently been shown to have an abnormally short free running period under constant conditions (Jones et al., 1999).

A similar correspondence between free running period and diurnal preferences may also occur in normal subjects. In a recent study, diurnal preferences were assessed using the Horne-Ostberg questionnaire and found to correlate with individual free running periods measured in the same subjects under constant conditions (Duffy et al., 1999). In normal rodents, interindividual variations in the free running period have been shown to correlate with the phase angle of entrainment, a phenomenon referred to as the Aschoff rule (Aschoff, 1965). It is therefore possible that polymorphisms at the level of some of these circadian genes may have milder phenotypic effects manifesting as variations in diurnal preferences for individual subjects.

To test this hypothesis, a systematic study of all known human circadian genes was initiated and the detected polymorphisms correlated with morningness-eveningness tendencies in a large sample of normal subjects (Katzenberg et al., 1998, 1999). This strategy led to the discovery that a *Clock* polymorphism may influence circadian preference (Katzenberg et al., 1998), whereas a human *Per*1 polymorphism does not have any significant effect (Katzenberg et al., 1999).

First described in *Drosophila,* the *Tim* gene was recently sequenced and mapped in humans in the chromosomal region

Correspondence: Emmanuel Mignot, M.D., Ph.D., 1201 Welch Road P114, Stanford, CA 94305-5102, USA, Tel: 650-725-6517, Fax: 650-498-7761, E-mail: mignot@leland.stanford.edu.

12q12-13 (Koike et al., 1998; Sangoram et al., 1998). In the present study, a polymorphism (an A to G) in position 2634 of *hTim* gene was genotyped in 528 normal subjects, and the correlation of this polymorphism with Horne-Ostberg scores was assessed.

METHODS

Subjects

A population-based random sample of 528 middle-aged men and women enrolled in an epidemiological study of the natural history of sleep disorders was used in this analysis (Young et al., 1993). All subjects had undergone blood draw as part of an overnight sleep protocol and were asked to complete the 19-item Horne-Ostberg questionnaire (Horne and Ostberg, 1976). Mean age ±SD was 50.3±7.8 years. 295 subjects (55.8%) were males. Although most subjects were American born, primary ethnic group was ascertained. Most subjects (89.6%) were Caucasians and the most frequent primary heritage recorded was German (39%).

Timeless Polymorphism Typing

DNA extracted from white blood cells was amplified using TL4F 5'CAGTCGCAGAGCACCTACATG3' and TL4R 5'ACGTCCTTATTGGCGAGGTAC3'primers (2min at 95°C, 30 cycles of 94°C 1min, 60°C 1min and 72 1min, followed by 5min at 72°C). The resulting 33 bp PCR product was dot-blotted onto Nylon membranes and hybridized at 42°C in a standard solution (6X SSPE, Denhart's, 0.1% N-laurylsarcosine and 0.02% SDS) with either of two allele-specific digoxin-11-ddUTP labeled oligonucleotides, 2634A (5'CTCATCTTCAGGAGCTGT3') and 2634G (5'GCT-CATCTTCGGGAGCTGTA3'). The resulting filters were washed at 55°C in TMAC (50mM Tris, pH8, 3M tetramethylamonium chloride, 2mM EDTA, 0.1% SDS) and signals detected by chemiluminescence after application of anti-digoxin antibodies (Boehringer-Mannhein). PCR products from 40 randomly selected subjects were also directly sequenced to confirm the oligotyping data.

Data Analysis

Subjects were categorized into three groups on the basis of their *hTim* genotypes (*hTim* 2634A/A, *hTim* 2634A/G or *hTim* 2634G/G). Allele frequencies were derived as described in Ott (1985). Observed genotype frequencies were compared with expected Hardy-Weinberg equilibrium values using χ^2 analysis. Horne-Ostberg scores were calculated in all subjects using pre-established values for each question as previously described (Horne and Ostberg, 1976). Factor analysis was performed to compare factor-loading in our sample with previously published data obtained from 477 undergraduate students (Smith et al., 1989). Linear regression modeling was used to assess differences between groups for Horne-Ostberg scores unadjusted and adjusted for the potential confounded factors of age, sex, and ethnic heritage, as described in Katzenberg et al. (1998). Interactions between the *hTim* genotype groups and age, sex and ethnic heritage were also investigated. Finally, we also compared genotype distribution in the 5 and 10% higher and lower Horne-Ostberg scores with the rest of the population using χ^2 analysis. The statistical significance of regression coefficients was assessed using *t*-tests and p values <0.05 were considered to indicate statistical significance. The SAS statistical package was used for all analyses (SAS Institute, 1998).

RESULTS

Allele frequencies for *hTim* 2634A and *hTim* 2634G were 0.49 and 0.51 when considering the entire population sample or only considering Caucasians. Observed sample sizes for A/A, A/G and G/G were 124/528, 271/528, and 133/528 respectively. These values did not differ significantly from derived Hardy-Weinberg equilibrium values ($\chi^2=0.175$; $p>0.90$). Mean Horne-Ostberg scores, unadjusted and adjusted were then compared between genotypes (Table 1). The mean Horne-Ostberg scores, unadjusted and adjusted for age, sex and ethnic heritage were not significantly different across the *Tim* genotype groups. We also compared the genotype distribution of *hTim* polymorphism in subjects with highest and lowest Horne-Ostberg scores and did not observe any difference. Distribution values for the 5% lowest and highest scores were 9A/A, 15A/G and 6G/G versus 3A/A, 20A/G and 7G/G respectively.

Table 1. Horne-Ostberg Scores by *hTIM* Genotypes

hTIM Genotypes	*n*	Horne-Ostberg Scores* (unadjusted)	(adjusted)†
2634A/A	124	60.2±0.9	60.4±0.9
2634A/G	271	60.7±0.7	61.4±0.6
2634G/G	133	60.0±0.9	60.2±0.8

*The Horne-Ostberg scores are given as Mean ±SD.
†The scores were adjusted for the possible confounding factors of age, sex and ethnic heritage.

DISCUSSION

In this study, the potential effect of a single nucleotide polymorphism (SNP) (A2364G) in the human *Timeless* gene on morningness-eveningness tendencies was explored in a large sample of normal adults. Our results show that a SNP, an A to G substitution at position 2364 resulting in a glutamine to arginine substitution at amino acid position 831, is not associated with morningness-eveningness tendencies.

A possible explanation for this negative result might be a lack of a functional effect for this polymorphism. In both humans and mice, a glutamine is observed at position 831 (Sangoram et al., 1998). This region is found in a region with similarity to *Drosophila Timeless* (*dTIM*), but the corresponding *dTim* residue does not appear to be conserved between *Drosophila* and mammals when commonly recognized alignment procedures are used (e.g., this residue most likely correspond to Tyrosine 916 in *dTim*). The

glutamine to arginine polymorphism may thus be a completely benign polymorphism. The role of *Tim* gene in generating circadian rhythmicity in mammals is also not yet totally elucidated (Zylka et al., 1998; Sangoram et al., 1998; Tischkau et al., 1999). In *Drosophila*, the *Timeless* gene is a well established molecular partner of *Per* and mutations in these genes have been shown to alter circadian rhythmicity. In mammals however, no published data are available on the effects of *Timeless* mutations. Some authors have also suggested a more minor role of mammalian *Timeless* compared to that in *Drosophila*, based on the observation that *Timeless* mRNA does not cycle and that Tim protein may not bind Per protein effectively in mammals (Zylka et al., 1998). A role for Per-Per protein interactions was suggested in this model (Zylka et al., 1998).

On the other hand, other authors have adopted the view that *Timeless* is still the preferential partner of *Per*1 or *Per*2 in mammals and have observed *Tim* mRNA cycling (Takumi et al., 1999; Tischkau et al., 1999). The *hTim* gene may also be involved in the regulation of light transduction effects on circadian rhythms rather than in regulating morningness-eveningness tendencies; light pulses known to induce phase delays cause significant increases in mouse *Tim* mRNA levels (Tischkau et al., 1999).

The reported association of a polymorphism in the *Clock* gene (Katzenberg et al., 1998), one of the genetic determinants of circadian rhythms, with morningness-eveningness tendencies points at the genetic basis of this behavior and shows the need for more studies on the role of other circadian genes on diurnal preferences. Recent studies have shown familial clustering for Delayed and Advanced Sleep Phase Syndrome (Fink and Ancoli-Israel, 1997; Jones et al., 1999) indicating a possible genetic component in which mutations in the circadian genes, including *hTim*, could be involved. The apparent lack of effect of the *hTim* Q831R substitution on diurnal preferences does not entirely exclude the possibility that other SNPs within the *hTim* gene could influence diurnal preference in the general population. The population studied consists of adults and *hTim* polymorphisms may only express a phenotype early in age, for example during adolescence when delayed sleep phase is more common. Rare *hTim* mutations may also be involved in highly penetrant circadian disorders. In spite of these negative results, this area of investigation has great potential. Further polymorphism and mutation screening studies in *hTim* gene are needed to identify genetic factors underlying normal and pathological circadian behaviors.

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