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ORIGINAL RESEARCH

Association of *Per3* length polymorphism with bipolar I disorder and schizophrenia

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Background: Sleep–wake disturbances have frequently been reported in bipolar disorder and schizophrenia, and are considered to be caused by an underlying circadian rhythm disorder. The study presented here was designed to investigate the existence of *Per3* polymorphism in bipolar disorder type I (BD-I) and schizophrenic patients in South India.

Methods: Blood samples were collected from 311 BD-I patients, 293 schizophrenia patients, and 346 age- and sex-matched normal controls. *Per3* genotyping was performed on DNA by polymerase chain reaction using specific primers.

Results: An increased prevalence of five repeat homozygotes was seen in BD-I patients as compared with healthy controls (odds ratio =1.72 [95% confidence interval: 1.08–2.76, P=0.02]). In BD-I patients, the frequency of the five repeat allele was higher (allele frequency =0.41), and that of the four repeat allele lower (allele frequency =0.36) (χ^2=4.634; P<0.03) than in the control group. No significant association was observed in the allele frequencies of four and five repeat alleles in schizophrenia patients when compared with controls.

Conclusion: The occurrence of the five repeat allele of *Per3* may be a risk factor for BD-I onset in this ethnic group.

Keywords: circadian rhythms, clock genes, *Per3* polymorphism, bipolar disorder, schizophrenia

Introduction

Most organisms have evolved an internal timekeeping system as a temporal program for adapting to the rhythmic environmental fluctuations which accrue from the earth's rotation (eg, light–dark and temperature cycles). In humans this system involves, numerous behavioral and physiological parameters, including sleep–wake behavior and emotional and cognitive functioning, all of which oscillate rhythmically with a circadian pattern.[1,2] There is a close interdependency between the circadian system and the body's homeostatic mechanisms. There is evidence suggesting that circadian processes, involving 24-hour oscillations in sleep propensity, interact with homeostatic mechanisms such as increases in sleep drive during wakefulness and reductions in drive during sleep.[3,4] Consequently, the cross talk between these two systems is a reliable predictor of the sleep onset and offset of individuals.[5,6]

In certain psychiatric conditions, however, these rhythmicities may be disrupted. Sleep perturbations, reductions in sleep onset latency, early morning awakenings, and specifically disturbed rapid eye movement sleep have been observed in patients with the diagnosis of bipolar disorder (BD).[7,8] Indeed, several reports have provided evidence suggesting that the desynchronization of the internal timekeeping system can be an important contributing factor in the onset of BD.[9,10]

Abnormal sleep–wake cycles, including changes in circadian phase (both advances and delays), discontinuous and compartmentalized sleep episodes, and disassembly

in rest–activity cycles, have also been observed in patients suffering from schizophrenia.[11–13]

A variable tandem repeat polymorphism coding for 18 amino acids has been identified in exon 18 of *Per3,* a circadian clock gene involved in the regulation of the transcription–translation feedback loop generating 24-hour periodicity.[14] This polymorphism has been linked to a variety of circadian and pathological phenomena. For example, there is evidence that *Per3* length polymorphism is associated with sleep homeostasis and cognition,[15] diurnal preference,[16–18] and delayed sleep-phase syndrome.[16,18]

In one study, five repeat homozygotes of *Per3* genotype were found to be associated with the onset of BD.[19] By contrast, the postpartum onset of BD correlated with four repeat homozygotes.[20] Nievergelt et al[21] reported evidence for the association of *Per3* haplotypes with BD, whereas in their replicative study they did not find any association between manic-depressive illness and *Per3* repeat polymorphism.[22] In schizophrenia, two single nucleotide polymorphisms were reported to be associated with the disorder[9] but no association of a *Per3* repeat polymorphism was found in 148 ethnically Han Chinese schizophrenics.[23] In view of these conflicting reports, and to further clarify the role of *Per3* in the etiology of BD and schizophrenia, we sought to investigate whether an association existed between *Per3* length polymorphism and bipolar disorder type I (BD-I) or schizophrenia in a group of South Indian patients. To the best of our knowledge, this is the first study on this matter to have been carried out in an Indian population.

Methods

Ethics statement

All information with regard to the protocol was explained to the participants, and their written consent was obtained. The guidelines for the ethical treatment of patients in investigational studies were followed[24] and the experimental protocol was reviewed and approved by the Institutional Ethical Committee of the Madurai Kamaraj University.

Sample collection

Blood samples (2 mL) were collected from patients suffering from BD-I (n=311, male =48.9%, female =51.1%) and schizophrenia (n=293, male =61.4%, female =38.6%), and normal controls (n=346, male =53.2%, female =46.8%). The mean ages of participants in the three groups were 37.8±10.6 years (± standard deviation; BD-I patients), 36.4±10.1 years (schizophrenia patients), and 35.1±8.8 years (normal controls). All BD-I and schizophrenia patients included in the study were either in- or outpatients of the MS Chellamuthu Trust and Research Foundation, located in Madurai city. Patient and control information was collected over 3 years. Clinical interviews of the patients were undertaken using the Mini-International Neuropsychiatric Interview[25] and clinical histories of longstanding symptoms were taken. Diagnoses of patients were made by a trained psychiatrist and confirmed by a senior psychiatrist. More details about the patients, including symptoms and mood profile, were obtained from family members and/or a close relative.

Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) diagnostic criteria for schizophrenia included all subtypes (paranoid, disorganized, catatonic, undifferentiated, and residual); BD-I diagnostic criteria also included all six DSM-IV subtypes.[26]

All BD-I and schizophrenia patients were undergoing routine treatment for their respective disorders. Control subjects were initially screened and excluded from participation if they exhibited any symptoms of schizophrenia or mood disorders, or if they reported that any of their first-degree relatives had such symptoms. Using unstructured interviews, healthy control subjects were selected after a psychiatric screening. No prescribed questionnaire was used to determine any type of symptom in the controls. Hospital staff, students, and staff of the university were selected as controls. Selected controls did not have any history of head injury, sleep disorders, or any other major disorder which could cause a bias. All the healthy controls and patients confirmed that they were of South Indian ancestry.

Per3 genotyping

DNA was isolated from participants' blood samples using the phenol–chloroform method. A polymerase chain reaction (PCR) was performed using specific primers (5′-TGTCTTTTCATGTGCCCTTACTT-3′ and 5′-TGTCTGGCATTGGAGTTTGA-3′) as described elsewhere.[27] The PCR reaction was carried out in 10 µL tubes containing 1 µL of 1× PCR reaction buffer, 0.5 µL (0.5 mM) of deoxynucleotide triphosphates (dNTPs) mix, 1.5 µL (0.4 mM) forward/reverse primer mix, 0.05 µL (0.025 U) *Taq* polymerase enzyme (Genet Bio Co Ltd, Daejeon, South Korea), 2 µL (100 ng) of genomic DNA and 4.95 µL of Milli-Q® water. The PCR amplification conditions included an initial step of 94°C for 5 minutes, 40 cycles of amplification (94°C for 30 seconds, 60.6°C for 30 seconds, 72°C for 30 seconds), and a final extension at 72°C for 5 minutes. The resultant PCR products showed three genotypes based on their DNA size, characterized as 1) $Per3^{5/5}$ homozygous allele (401 bp), 2) $Per3^{4/4}$ homozygous allele (347 bp), and

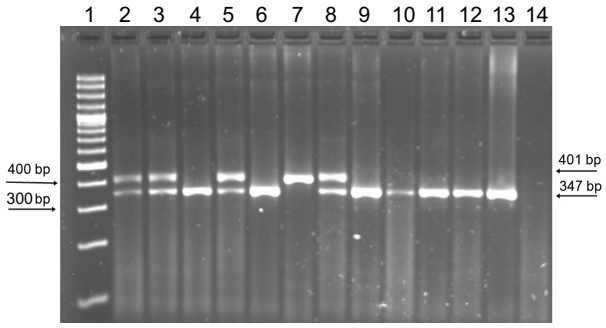

Figure 1 Agarose gel showing *Per3* alleles.
Notes: Lane 1: 100 bp marker; lanes 2, 3, 5, and 8: 4/5 heterozygous allele (401 and 347 bp); lanes 2–5: control samples; lanes 4, 6, and 9–13: 4/4 homozygous allele (347 bp); lanes 5–13: bipolar disorder patient samples; lane 7: 5/5 homozygous allele (401 bp); lane 14: no template control.

3) *Per3*$^{4/5}$ heterozygous allele (347 and 401 bp) (Figure 1). Subsequently, the amplified DNA sequences were sequenced for further confirmation.

Statistical analysis

The distributions of four and five repeat allele frequencies in the population were determined using the Hardy–Weinberg exact test.[28] Deviations from Hardy–Weinberg equilibrium and the association of allele frequencies of four and five repeat alleles with BD-I, schizophrenia, and control subjects were analyzed by chi-square testing using SPSS software (v 20; IBM, Armonk, NY, USA). *P*-values of less than 0.05 were taken as evidence of statistical significance.

Results

Biallelic repeat variants of *Per3* in BD-I, schizophrenia, and control subjects are summarized in Table 1. The distribution of *Per3* genotypes in BD-I, schizophrenia, and normal healthy individuals were within the limits of the Hardy–Weinberg equilibrium. The observed and expected allele frequencies of both alleles in the three groups were similar and no group varied from the limit. The observed genotype frequencies of the controls were 4/4=141, 4/5=163, and 5/5=42, and expected genotype frequencies were 4/4=143.1, 4/5=158.8, and 5/5=44.1, and they were similar and did not show any statistical significance ($P>0.05$). Similarly, the observed genotype frequencies of BD-I patients were 4/4=109, 4/5=146, and 5/5=56 and their expected genotype frequencies did not have any major difference (4/4=106.5, 4/5=150.9, and 5/5=53.5) and did not exhibit statistical significance ($P>0.05$). Likewise, the observed (4/4=115, 4/5=137, and 5/5=41) and expected genotype frequencies (4/4=114.9, 4/5=137.2, and 5/5=40.9) of the schizophrenia patients were similar and did not possess any significant difference ($P>0.05$).

An increased prevalence of five repeat homozygotes was seen in BD-I patients (odds ratio =1.72 [95% confidence interval: 1.08–2.76, $P=0.02$]). The frequency of five repeat allele (allele frequency [AF] =0.41) was higher, and that of four repeat allele lower (AF =0.36), in BD-I patients ($\chi^2=4.634$, $P<0.03$) than in the control cohort (Table 1). The analysis of allele frequencies of four and five repeats did not reveal any statistically significant differences between patients with schizophrenia and the control group ($\chi^2=0.385$; $P=0.53$).

There were differences in the allele frequencies of *Per3* and this observation was particularly notable among female patients with BD-I ($\chi^2=4.532$; $P<0.05$) (Table 2). No significant differences were found in the allele frequencies of four and five repeat alleles in male and female schizophrenia patients when compared with male and female control groups (Table 3).

Discussion

The foregoing results support the conclusion that *Per3* polymorphism is associated with BD-I but not with schizophrenia. Benedetti et al[19] reported an association between earlier onset of BD with five repeat allele in the homozygous state. Another study suggested an influence of *Per3* polymorphism in the postpartum onset of BD;[20] interestingly, in the latter investigation,[20] four repeat homozygotes have been associated with bipolar episodes. However, no significant difference was observed in the onset and number of episodes between *Per3* repeat alleles and BD.[27]

In a previous study, which used the Temperament and Character Inventory,[29] five repeat homozygotes were found to be significantly linked with certain mood behaviors

Table 1 Genotypic distribution of the 4-/5-length polymorphism of *Per3* in bipolar disorder type I (BD-I) and schizophrenia patients and controls

Group	N	Genotype, n (%)			Allele, n (%)		χ^2	*P*
		4-/4-	4-/5-	5-/5-	4-	5-		
Control	346	141 (40.8)	163 (47.1)	42 (12.1)	445 (0.64)	247 (0.36)	4.634	0.03*
BD-I	311	109 (35.1)	146 (47.0)	56 (18.0)	364 (0.59)	258 (0.41)		
Schizophrenia	293	115 (39.3)	137 (46.7)	41 (14.0)	367 (0.63)	219 (0.37)	0.385	0.53

Notes: 4-, 4-repeat allele; 5-, 5-repeat allele; *statistically significant.
Abbreviations: N, number of samples; n, number of genotypes and alleles.

Table 2 Genotypic distribution of the 4-/5-length polymorphism of *Per3* in bipolar disorder (BD) type I patients and controls based on sex

Group	N	Genotype, n (%)			Allele, n (%)		χ^2	*P*
		4-/4-	4-/5-	5-/5-	4-	5-		
Male control	184	73 (39.7)	90 (48.9)	21 (11.4)	236 (0.64)	132 (0.36)	0.763	0.38
Male BD	152	60 (39.5)	65 (42.8)	27 (17.8)	185 (0.61)	119 (0.39)		
Female control	162	68 (41.9)	73 (45.1)	21 (12.9)	209 (0.65)	115 (0.35)	4.532	0.03*
Female BD	159	49 (30.8)	81 (50.9)	29 (18.3)	179 (0.56)	139 (0.44)		

Notes: 4-, 4-repeat allele; 5-, 5-repeat allele; *statistically significant.
Abbreviations: N, number of samples; n, number of genotypes and alleles.

including novelty seeking, extravagance, cooperativeness, compassion, and integrated conscience in a subset of bipolar individuals.[27] The genotype frequencies observed in the BD-I group in the present study (*Per3*$^{4/4}$ homozygous allele =35.0%, *Per3*$^{4/5}$ heterozygous allele =46.9%, and *Per3*$^{5/5}$ homozygous allele =18.0%) were closely similar to those reported previously in BD patients (35.3%, 46.5%, and 18.2%, respectively).[19]

Control allele frequencies of four repeat (AF =0.64) was slightly higher than the allele frequency found in Hindus of Maharashtra, India (AF =0.57) (M Thomas, Department of Biology, University College London, personal communication, March, 2012) and slightly less than the mean of the global population (AF =0.68).[30] As indicated by post-Hardy–Weinberg equilibrium testing, the genotype and allele frequencies in the three groups presumably remained undisturbed by selection force. Furthermore, no selective advantage was observed over any particular *Per3* allele globally.[30] In sex-based analysis, differences in the allele frequencies of both male and female BD-I patients were observed when compared with control subjects. Although the sample size was small, the differences between the observed and expected allele frequencies of the female BD-I group were found to be statistically significant, whereas these differences did not reach significance in the male BD-I group.

Since the frequency of occurrence of the five repeat allele was significantly greater in BD-I patients than in control individuals, the high occurrence of five repeat allele frequency may be correlated with the behavioral phenotype of the corresponding allele. For instance, patients with five repeat homozygotes showed short sleep latencies and spent more time in slow wave sleep.[15] Five repeat allele individuals also exhibited more depressive symptoms and a lower motivational level than those with a four repeat allele.[31] Sleep–wake behaviors such as a short sleep duration, early bedtime and sleep onset, spending more time in bed, hypervigilance, and reduced daytime sleep durations have been observed more frequently in subjects with five repeat homozygotes than in those with four repeat homozygotes or heterozygotes.[32]

How the *Per3* gene contributes to the pathogenesis of psychiatric disorders is still unclear. A possible explanation for the occurrence of mood disorders is that in affected individuals, the circadian clock may show poor adaptability to different seasons.[33,34] Another possible way in which a polymorphism of *Per3* could influence mood is by affecting sleep.[33,35] No previous study has shown evidence specifically linking the *Per3* genotype to any mental health outcome measures.[32] The evidence presented in the current study is consistent with the hypothesis that *Per3* alleles could influence the mental health status of individuals by modulating the sleep phase. Relevant to this suggestion has been the finding of an association between a single nucleotide polymorphism of *Per3* and poor sleep quality in BD patients.[36] Morningness and eveningness preferences, which are strongly correlated with *Per3*, could modify the phase of the sleep and could conceivably result in abnormal mood profiles. In addition, *Per3* polymorphism might perturb the delicate balance between mood and sleep and the balance toward episodes of mania or depression in individuals who have a high genetic

Table 3 Genotypic distribution of the 4-/5-length polymorphism of *Per3* in schizophrenia patients and controls based on sex

Group	N	Genotype, n (%)			Allele, n (%)		χ^2	*P*
		4-/4-	4-/5-	5-/5-	4-	5-		
Male control	184	73 (39.7)	90 (49)	21 (11.3)	236 (0.64)	132 (0.36)	0.373	0.54
Male schizophrenia	180	68 (37.8)	87 (48.3)	25 (13.9)	223 (0.62)	137 (0.38)		
Female control	162	68 (42.0)	73 (45)	21 (13.0)	209 (0.65)	115 (0.35)	0.036	0.84
Female schizophrenia	113	47 (41.6)	50 (44.3)	16 (14.1)	144 (0.64)	82 (0.36)		

Notes: 4-, 4-repeat allele; 5-, 5-repeat allele.
Abbreviations: N, number of samples; n, number of genotypes and alleles.

loading toward BD. This suggestion is consistent with previous findings that certain genotypes and alleles are more vulnerable to schedule changes and that this interaction can have behavioral and health-related consequences.[37]

The foregoing results in schizophrenic patients are in accordance with an earlier report that showed no correlation between *Per3* repeat polymorphism and schizophrenia in a Chinese sample.[23] Indeed, the onset of schizophrenia was not correlated with *Per3* or the *Clock* gene variant, although a significant association of *Clock* allele frequency was found in a schizophrenic sample.[23] It should be noted that elevated levels of interleukin-6 have been observed in individuals affected with schizophrenia and a correlation of *Per3* repeat alleles with interleukin-6 concentration was reported.[38] These observations underscore the necessity for additional studies in larger samples and of different ethnic composition to further substantiate the conclusion that a lack of association between *Per3* repeat alleles and schizophrenia truly exists.

Prevalence of *Per3* repeat variants is hypothesized to augment phosphorylation of PER (PERIOD) proteins. Polymorphic repeats consist of sites for phosphorylation and a differential level of phosphorylation is caused by the insertion of an additional repeat.[16] The present findings of an association of *Per3* polymorphism with BD-I, but not with schizophrenia, are consistent with models suggesting that these represent different genres of psychiatric disorders. The findings further suggest that clock genes are complexly involved in either the expression and/or maintenance of these disorders. Furthermore, changes observed in the circadian phase of schizophrenia are quite distinct from the phase pattern of BD.[9] This implies that the symptoms and pathogenesis of schizophrenia and BD are different and have a specific relationship with the circadian clock.

The study presented here suffered from several limitations. India comprises multi-ethnic groups and the possible impact of population stratification in genetic association studies must be taken into consideration.[39] Population stratification raises the necessity for its confirmation in larger samples of patients and in different ethnic populations of the Indian subcontinent. Indeed, major genetic studies in the world have been carried out without considering the large Indian population and its different ethnicities.[40] Since the controls were recruited from among hospital staff and students, an important bias could be that these subjects had higher levels of education and cognitive performance than patients. In addition, the present study gives no information on the association of the onset of BD-I among patients with the genotypic variants of *Per3*. Such a study is needed to support a direct link of the polymorphic variants observed with BD. Another disadvantage of the study is that it does not analyze the association between *Per3* polymorphism and specific sleep behavior in the same population.

In any event, the data presented are compatible with the hypothesis that a length polymorphism in the circadian clock gene *Per3* influences the pathogenesis of BD-I but not of schizophrenia.

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Disclosure

All authors declare that they have no proprietary, financial, professional, nor any other personal interest of any nature or kind in any product or services and/or company that could be construed or considered to be a potential conflict of interest that might have influenced the views expressed in this paper.

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