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The rs1801260 CLOCK polymorphism, links to depression, insomnia and diurnal preference – preliminary findings from a Romanian sample

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Abstract. Objective: We investigated the possible effect of the T3111C *CLOCK* gene polymorphism on depressive disorder, insomnia and diurnal preference. Material and methods: twenty seven subjects, aged 23-71, self-selected from a larger sample of healthy controls and clinically depressed patients were screened for the presence of the aforementioned SNP. Results: We detected a possible association of the T/T allele with depression. Conclusion: No support was found for an involvement in sleep disturbance or circadian preference, but *CLOCK* may be involved in the pathogenesis of depressive disorders.
Key Words: T3111C *CLOCK* gene polymorphism, depression, insomnia.

Rezumat. Obiectiv : Am investigat posibilul efect al polimorfismului T3111C al genei *CLOCK* faţă de tulburarea depresivă, insomnia şi preferinţa diurnă. Material şi metode: Douăzeci şi şapte de subiecţi, cu vârste cuprinse între 23 şi 71 ani, auto-selecţionaţi dintr-un lot mai mare de subiecţi sănătoşi şi pacienţi suferinzi de depresie, au fost examinaţi dacă prezintă polimorfismul sus menţionat. Rezultate: Am detectat o posibilă asociere a alelei T/T cu depresia. Concluzie: Gena *CLOCK* pare să fie implicată în patogeneza tulburării depresive, dar nu şi în dezvoltarea tulburărilor de somn sau în preferinţa diurnă.
Cuvinte cheie: polimorfismul T3111C al genei *CLOCK*, depresie, insomnie.

Introduction. About a decade ago, the first circadian gene polymorphism was reported to be associated with phenotype in humans (Katzenberg et al 1998). It was a single-nucleotide polymorphism (SNP) in the *CLOCK* gene, also known as T3111C or rs1801260, where the C allele was reported to be linked with an increased evening preference, in a mostly North American population (Katzenberg et al 1998). These findings were later replicated by two other studies both in North-American (Friedman et al 2009) and Japanese populations (Mishima et al 2005). Other studies with British (Robilliard et al 2002), mixed European (Johansson et al 2003), Japanese (Iwase et al 2002), Korean (Lee et al 2007) and Brazilian (Pedrazzoli et al 2007) participants did not find any associations with the diurnal preference.

Further research showed that this SNP is associated with insomnia (Benedetti et al 2007; Serretti et al 2003), impaired cognitive performance (Benedetti et al 2008) and altered response to antidepressant therapy (Serretti et al 2005). These findings in Italian subjects affected by mood disorders were not confirmed in other European (Bailer et al 2005; Johansson et al 2003), North-American (Desan et al 2000; Shi et al 2008), or Asian populations (Paik et al 2007).

Purpose. Our study aims to test the hypothesis whether the *CLOCK* T3111C SNP is associated with certain phenotypes such as diurnal preference, insomnia or depression in a self-selected Romanian sample.

Material and Method. *Participants*. The study was approved by the Ethics Committee of "Iuliu Haţieganu" Medicine and Pharmacy University. Patients were recruited from adults presenting at the GP or psychiatrist at Baia Mare County Hospital, while healthy controls were selected from their acquaintances or students attending the Psychology courses at "Babes-Bolyai" University in Cluj-Napoca. We excluded people suffering from other major conditions that affect sleep, such as heart (severe heart failure, unstable angina), dermatologic (psoriasis), gastrointestinal (inflammatory bowel disease), neurologic (stroke, Parkinson disease, epilepsy, traumatic brain injury), pulmonary (obstructive sleep apnoea, persistent asthma, chronic obstructive pulmonary disease), psychiatric (chronic or acute psychosis, bipolar disorder, dementia, mental retardation), endocrine (hypo- or hyperthyroidism) or rheumatologic disorders.
Our initial sample consisted of 349 subjects, aged over 18. Each participant completed a questionnaire assessing several variables and was further invited to participate in the genetic analysis. From the 154 individuals that agreed to receive details about this latter study, 73 completed the final stage of the research, but only 27 have the genotype analyzed. Mean age was 50.03, with a standard deviation of 12.38. Fifteen were men and twelve women. All were of Romanian ethnicity.

Instruments. A questionnaire containing the Romanian translation of the Composite Scale of Morningness (Smith et al 1989), the Sleep Disturbance Questionnaire (Violani et al 2004) (all in Romanian translation) and demographic data was used. History and current state of depression were also assessed.

The Composite Scale of Morningness (CSM) contains 13 questions, most of them having four choices, with a Likert-type response format. Scores are obtained by summing each item score and can range between 13 (extreme evening) and 55 (extreme morning) (Smith et al 1989). Using the 25/75 percentile and age, we have previously determined the cut-off scores for evening and morning types (Voinescu 2009b).

The Sleep Disorders Questionnaire (SDQ) is a short self-rating questionnaire with 18 questions on different sleep problems. The first group of questions evaluates the presence of any sleep disorder in the last month, from insomnia to excessive sleepiness, sleep apnea or parasomnia[1]. A subsequent set of questions investigates the duration, frequency and consequences of the problem, and it is used for evaluation of the severity of sleep disturbances reported. The SDQ is easy to administer and time efficient. A limitation of the SDQ is that it does not allow for a differentiation between primary insomnia and insomnias due to psychiatric disorders or somatic diseases, nor any other etiological consideration (Violani et al 2004).

Procedure. Participation was voluntary and anonymous. Patients, not meeting the exclusion criteria, received an invitation letter from their GP or specialist. The letter explained the nature of research and ethical requirements for confidentiality. If agreed, they were given the questionnaire to be completed. All the participants were also asked to further recruit adults from their acquaintances using the snowball sampling. Students attending a course of psychology were given a web link where to find details about the study and the questionnaire. Completing the survey was considered implied consent to participate in this study. Each participant was informed that there was a 2nd stage of the research that involved blood analysis and was asked if he wanted to be further informed about this.

After the centralization of the responses to the survey, those, who agreed to be contacted, were rung up or emailed and explained the requirements of second phase. A meeting point was agreed in order to sign another informed consent and collect the blood sample. Participation was unpaid.

DNA analysis. Blood was obtained by finger-sticking with a lancet, dropped and dried on a Whatman FTA card. This method is minimally invasive, quicker and less distressing

[1] Parasomnias are a category of sleep disorders that involve abnormal movements, behaviors, emotions, perceptions, and dreams that occur while falling asleep, sleeping, between sleep stages, or arousal from sleep.

than a venipuncture and allows the storage of blood at room temperature for more than 15 years, without degrading the DNA (according to the manufacturer).

FTA cards were then shipped to the Institute of Life Science, Swansea, UK, where DNA was analyzed. A 2mm punch from a FTA card was enough to determine the SNP of an individual. DNA extraction was performed using the FTA Purification reagent. The obtained DNA was amplified using Qiagen's Taq polymerase and the same primers, quantities and conditions as Kissling (Kissling et al 2008). After the amplicon was visualized as described bellow, we performed the restriction enzyme digestion with 5µl of PCR product in a final volume of 12µl with Bsp1286I at 37°C overnight. Fragments were visualized on 2% agarose gel stained with ethidium bromide, following separation at 100V in tris-borate electrophoresis buffer. The gels were viewed under a transilluminator.

Statistical analysis. To examine the statistical significance of the differences, likelihood ratio was used, with $P<0.05$ considered significant. Data analysis was performed using the SPSS (version 16.0.2). Allele frequencies of the group were calculated by direct counting from the genotypes observed and tested for the Hardy-Weinberg equilibrium (HWE) with HWSIM software (available at http://krunch.med.yale.edu/hwsim/) and 100,000 simulations.

Results. There was no significant deviation from Hardy–Weinberg Equilibrium ($X=0.00$; $p=0.50$). Allele frequencies, as well as statistical tests are summarized in
Table 1. T/T allele might be significantly associated with current or past depression.

Table 1

CLOCK genotype and the observed phenotype by frequency and percentage (in brackets)

		C/C allele	*T/C allele*	*T/T allele*	*Total*	*Likelihood ratio*
Past or current depression	Yes	2 (18.2)	2 (18.2)	7 (63.6)	11	X=5.56
	No	1 (6.2)	10 (62.6)	5 (31.2)	16	**P=0.06**
Diurnal Preference	Evening	0 (0)	2 (40.0)	3 (60.0)	5	X=2.00
	Intermediate	2 (14.4)	7 (50.0)	5 (37.6)	14	P=0.73
	Morning	1 (12.5)	3 (37.5)	4 (50.0)	8	
Initial insomnia	Yes	1 (11.1)	5 (55.6)	3 (33.3)	9	X=0.75
	No	2 (11.1)	7 (38.9)	9 (50.0)	18	P=0.68
Middle insomnia	Yes	1 (10.0)	6 (60.0)	3 (30.0)	10	X=1.64
	No	2 (11.8)	6 (35.3)	9 (52.9)	17	P=0.44
Early insomnia	Yes	1 (10.0)	5 (50.0)	4 (40.0)	10	X=0.19
	No	2 (11.8)	7 (41.1)	8 (47.1)	17	P=0.90
Excessive sleepiness	Yes	1 (11.2)	4 (41.2)	4 (44.4)	9	X=0.026
	No	2 (11.7)	7 (41.2)	8 (47.1)	17	P=0.98

Discussion. We presented preliminary results from a research aiming to assess the association between a particular *CLOCK* polymorphism and phenotype, specifically depression, insomnia and diurnal preference. With the exception of depressive disorders that might be linked with the T/T allele, we found no associations between the three alleles and the analyzed phenotype.

Polymorphisms in clock-related genes may constitute a critical mechanism by which circadian and sleep disturbances predispose individuals to depressive illnesses. There is evidence for a relationship between depressive disorders and mutations in certain circadian genes, namely *Per3* (Artioli et al 2007). The T3111C polymorphism of *Clock* was investigated, based on its association with eveningness, but no association was found with the susceptibility to depression (Bailer et al 2005; Desan et al 2000; Kishi et al 2009). Nevertheless, *Clock* gene polymorphisms have been associated with disease chronicity (Benedetti et al 2003) and age onset in patients with bipolar disorder

(Benedetti et al 2007), and with insomnia(Serretti et al 2003), altered response to antidepressant treatment (Serretti et al 2005) or impaired cognitive performance in depressed patients (Benedetti et al 2008). Our preliminary results, if replicated to a larger sample, suggest that there is support for the involvement of *CLOCK* genotype in the development of depressive disorders. The effect on sleep disturbance, particularly insomnia, is still to be researched.

Recently, new data came out about the involvement of *CLOCK* in disease. Adult ADHD was also found to be associated with T3111C *CLOCK* polymorphism in a German group (Kissling et al 2008). Divergent results came from associations with schizophrenia in Japanese individuals (Kishi et al 2009; Takao et al 2007), while cluster headache, obesity and eating disorders were found not be linked with a *CLOCK* SNP in Italian subjects (Cevoli et al 2008; Monteleone et al 2008; Tortorella et al 2007). Other components of the circadian molecular clock, such as *NPAS2* or *BMAL1,* have also been linked with various pathologies, from metabolic diseases to cancer (for a summary of the associations of the molecular clock and disease, see (Voinescu 2009a)).

Our results support the previous findings that did not show any connection between the rs1801260 and the diurnal preference in European populations (Johansson et al 2003; Robilliard et al 2002). Most of the findings in favour of this association come from North-American studies (Friedman et al 2009; Katzenberg et al 1998), therefore our conclusion is not surprising. Diurnal preference was reported to be linked with mutations in other circadian genes, mainly in the *PERIOD* family. SNPs in *PER1* (Carpen et al 2006)*, PER2* (Carpen et al 2005) or *PER3* (Johansson et al 2003) appear to confer a tendency towards morning preference. In a UK-based population sample, it was found that the shorter allele (*PER3*^{4}) of a variable-number tandem-repeat (VNTR) polymorphism in the *PER3* gene was associated significantly with evening preference, whereas the longer one (*PER3*^{5}) was linked with morning preference (Archer et al 2003; Jones et al 2007).

Among the limitations of our research is the current number of participants, which is particularly small for an association study. Sleep disturbance and diurnal preference were self-assessed with psychological instruments that are not as reliable as objective measurements. Therefore, there could be over- or underestimations of the assessed variables. Moreover, we did not ascertain if insomnia appeared independently or as a consequence of a disease.

Although research linking clock genes and disorders is still in an early stage, the findings to date suggest that this approach may be productive, especially in bipolar disorders or seasonal affective disorder (Lamont et al 2007). Thus, the potential utility of a genetic screening tool for the differential diagnosis should not be underestimated. Clock genes may be a good target for this type of approach and could open up perspectives to new diagnostic and therapeutic approaches.

Conclusions. If replicated on a larger sample, this first study of a circadian gene in Romanian subjects could provide evidence that the T3111C polymorphism in the *CLOCK* gene is linked with depression. No support was found for the hypothesis that *CLOCK* has an effect on diurnal preference or insomnia.

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