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A pilot replication study of two PER3 single nucleotide polymorphisms as potential genetic markers for morning and evening earliness-lateness

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ABSTRACT

Polymorphisms in genes of circadian system family seem to be of most importance for understanding of mechanisms underlying self-assessed individual variation in morning–evening preference. A review of earlier reported positive findings indicated that, at least, four polymorphisms in period circadian clock 3 (PER3) showed significant association with, at least, one of sub-constructs of a morningness–eveningness scale. However, similar to other candidate gene studies, these studies suffer from increased likelihood of false positive findings. We tried to replicate some of the most recently published positive results on associations of sub-traits of morningness–eveningness with two PER3 non-synonymous single nucleotide polymorphisms. DNA from buccal swabs was collected from healthy residents of three Russian cities, Moscow ($N = 149$) and Novosibirsk and Stavropol ($N = 248$). The tested hypotheses were formulated in accord with the earlier reported positive findings: the rare alleles might be linked to a higher score on (i) morning earliness–lateness scale (rs2640909, Moscow data-set) and (ii) both morning and evening earliness–lateness scales (rs228729, Novosibirsk and Stavropol datasets). The results provided support for the former hypothesis. These and earlier reported results highlighted several critical issues that remained to be addressed in future independent replications of positive findings on potential genetic markers for morning and evening earliness–lateness.

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Introduction

Several morningness–eveningness scales were developed for ranking and chronotyping people in accord with their preferred times for physical and mental activity, wakefulness, and sleep (Horne & Östberg 1976; Torsvall & Åkerstedt 1980; Smith et al. 1989; Brown 1993; Bohle et al. 2001; Adan & Almirall 1991). It was shown that, in terms of traditional psychometric measures of reliability, a score on a morningness–eveningness scale that includes seven or more questions might be regarded a reliable estimate of morning–evening preference (Di Milia et al. 2013). However, it was also shown that the scales of such size include more than one factorial dimensions (Torsvall & Åkerstedt 1980; Smith et al. 1989; Neubauer 1992; Caci et al. 2005; Randler 2009; Konttinen et al. 2014). In contrast, several multi-dimensional questionnaires were developed by applying conventional psychometric procedures to allow self-assessments of, at least, two – morning and evening – sub-traits of a broad morningness–eveningness trait (Putilov 1990, 2000, 2007; Roberts 1998; Randler et al. 2016). Alternatively, authors of some of recent publications reported results on differential relationship of scorings on two or more sub-constructs of a morningness–eveningness scale, e.g. with scorings on depression scale (e.g. Konttinen et al. 2014; Jankowski 2016).

It is reasonable to suggest that polymorphism in circadian system genes might be of most importance for explaining mechanisms underlying individual variation in the self-perceived differences between people in morning–evening preference. Of those genes of this circadian system family that were examined so far, positive results were yielded in a search for associations of morning–evening preference with polymorphic variants of PER3, PER2, CLOCK, ARNTL, CSNK1ε, etc. In particular, positive findings were reported for, at least, four polymorphisms of PER3 (period circadian clock 3). These results are illustrated in Table 1. They indicate that, similar to other candidate gene studies (Sullivan 2007), the studies of this gene suffer from increased likelihood of false positive findings, e.g. due to a relatively high number of

Table 1. Positive and negative findings on four PER3 polymorphisms as potential genetic markers for morning–evening preference.

Polymorphism	VNTR	SNP		
Marker name	rs57875989	rs10462020	rs2640909	rs228697
Chromosome	1:7829993	1:7820623	1:7830057	1:7827519
Exon	18	15	18	17
Major/Minor	5-/4-repeat	G/T	T/C	C/G
GMAF	–	0.1206	0.1853	0.0603
MAF	–	–	0.2416	0.1001
Positive finding	Ebisawa et al. (2001) Archer et al. (2003) Pereira et al. (2005) Dijk and Archer (2010)	Ebisawa et al. (2001) Johansson et al. (2003) Parsons et al. (2014)	Ojeda et al. (2013)***	Hida et al. (2014) Kripke et al. (2014)
Negative finding	Jones et al. (2007)* Osland et al. (2011) An et al. (2014) Kripke et al. (2014)	Hida et al. (2014)** Kripke et al. (2014)	Dmitrzak-Węglarz et al. (2016)	–

Notes: VNTR: Variable Number Tandem Repeat; SNP: Non-synonymous Single Nucleotide Polymorphism; Major/Minor: Major/Minor allele; GMAF: Global Minor Allele Frequency (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=8863); MAF: Present study results on Minor Allele Frequency.*Association was confirmed but only for youngest group of study participants; **SNP was excluded due to low MAF (<0.05); ***Association was significant but only with one of sub-scales of morningness–eveningness scale named "morningness".

analyses, a relatively high number of genes contributing to a phenotypical trait, a relatively low number of study participants, etc.

In more detail, the pioneer studies succeeded in relating morning–evening preference to variable number tandem repeat (VNTR) in PER3 gene (marker name: rs57875989). They suggested association between the longer (5-repeat) allele and morningness and between the shorter (4-repeat) allele and eveningness, respectively (Ebisawa et al. 2001; Archer et al. 2003; Dijk & Archer 2010). These earlier results were confirmed in a Brazilian population (Pereira et al. 2005). However, it was later emphasized that the association is significant in young subjects (18–29 years of age) and rapidly attenuates with aging (Jones et al. 2007). Moreover, the association was not confirmed in studies of Norwegian (Osland et al. 2011), Chinese (An et al. 2014), and Californian populations (Kripke et al. 2014). Similarly, the first of the reported associations of morning–evening preference with a non-synonymous single nucleotide polymorphism (SNP; rs10462020) was later supported in some (e.g. Johansson et al. 2003; Parsons et al. 2014), but not all studies (e.g. Kripke et al. 2014). Besides, the rare allele was found to be too rare in one of the studies (Hida et al., 2014). The positive findings on two other SNPs (rs2640909 and rs228697) were reported rather recently (Ojeda et al. 2013; Hida et al., 2014; Kripke et al. 2014), and, therefore, they are still waiting for confirmation (Table 1). Notably, one of these associations (rs2640909) was found to be significant for only one of the sub-traits of morning–evening preference named "morningness" (Ojeda et al. 2013), whereas this association was found to be non-significant in the study examining association with a score on the whole morningness–eveningness scale (Dmitrzak-Węglarz et al. 2016).

Consequently, the aim of this pilot study was to try to replicate the most recent findings suggesting significant associations of two PER3 SNPs either with morning–evening preference (rs228729) or only with "morningness" sub-trait of morning–evening preference (rs2640909). For the first time, the samples for such a replication study were collected in Russia, in the cities of Moscow (55° 45′ N 37° 37′ E), Novosibirsk (55° 03′ N 82° 57′ E), and Stavropol (45° 02′ N 41° 58′ E), and, also for the first time, an emic (Russian-language) multi-scale questionnaire was applied for assessment of morningness–eveningness on two separate, morning and evening, earliness–lateness scales (Putilov 2007, 2010). The tested hypotheses were formulated in accord with the earlier reported positive findings:

(1) The rare allele of rs2640909 might be linked to a higher score on morning earliness–lateness scale (reported by Ojeda et al. 2013, and tested using Moscow dataset, $N = 149$).
(2) The rare allele of rs228729 might be linked to a higher score on both morning and evening earliness–lateness scales (reported by Hida et al., 2014, and Kripke et al. 2014, and tested with Novosibirsk and Stavropol datasets, $N = 248$).

Methods

In total, 397 healthy volunteers participated in this pilot replication study. Each study participant denied a history of mental or sleep disorders, any current health problems, and trans-meridian travels during the preceding month. The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Its protocol was approved

by the Ethics Committees of each research institutes/universities, and informed written consent was obtained from each study participant.

A multi-dimensional 72-item questionnaire named "Sleep-Wake Pattern Assessment Questionnaire" (SWPAQ) includes two 12-item scales for self-assessment of morning–evening preference. They were named "morning and evening lateness" and abbreviated as M and E, respectively. Only these two scales were administered to the residents of Moscow (the first data-set), whereas the residents of two other cities (the second data-set) completed the whole questionnaire consisting of six 12-item scales (Putilov 2007, 2010). Empirical evidence for reliability and validity of the SWPAQ's scales were published earlier (e.g. Putilov 2000) and reviewed elsewhere (Putilov 2000, 2010; Putilov et al. 2017). Score on each scale ranges from −12 to 12, and negative score signifies earliness. We calculated scores on M and E scales, their sum and difference (M + E and E−M, respectively).

A sample of adult males genotyped for rs2640909 ($N = 149$) was randomly selected from a larger sample ($N = 299$) of professional bus drivers who were permanently involved in rotated shift work and volunteered to participate in a study of the effect of chronotype on risk of road accidents. Their mean age ± SD (Standard Deviation) was 45.8 ± 1.9 (range from 21 to 66 years old).

The sample of individuals genotyped for rs228697 ($N = 248$) included three sub-samples. Two of them were students volunteered to participate in a questionnaire study of relationships between individual variation in the domains of chronobiology and personality psychology. In the first sub-sample from the North-Caucasus Federal University (Stavropol), the ages of 10 male students ranged from 19 to 30 (21.0 ± 3.3 years old), and the ages of 123 female students ranged from 18 to 21 (19.4 ± 0.7 years old). Ages in the second student sub-sample from the Novosibirsk State Pedagogical University were in the ranges from 17 to 19 (9 males, 18.7 ± 0.7 years old), and from 17 to 30 (88 females, 19.3 ± 1.6 years old). The third sub-sample was genotyped in the process of selection of participants of sleep deprivation experiments aged between 30 and 45 years old. Mean age ± SD of 9 males was 33.0 ± 2.1, and mean age ± SD of 9 females was 35.4 ± 4.2 (ranges 30–36 and 32–43 years old, respectively).

SNP genotyping of the first and second datasets was performed in the laboratories of the Institute of Molecular Genetics, Moscow, and the Research Institute for Molecular Biology and Biophysics, Novosibirsk, respectively. The method of real-time polymerase chain reaction (PCR) in TaqMan™ technology on a StepOnePlus™ amplifier was applied for the analysis of DNA from buccal epithelium cells in the first data-set. Amplification solution (25 µl) contained 2.5 µl 10×PCR buffer solution, 2.5 µl 25 mM magnesium chloride (MgCl2), 2.5 µl 2.5 mM dNTP, 10 pM of TaqMan™ primer, 4 pM of DNA probe, 1.25 units of Taq polymerase (Fermentas), 0.1–0.2 µg of genomic DNA, and deionized water, up to 25 µl of a total volume. For the analysis of the second data-set, DNA was isolated with the kit «BSD-100» (BioSilica, Russia) according to the protocol of the manufacturer for the following amplification and analysis of the SNP polymorphism. Real-time PCR was performed using «BioMaster HS-qPCR SYBR Blue(2×)» (Biolabmix, Russia) with additional MgCl2 to a final concentration of 4.5 mM, 10–100 ηg of genomic DNA, 4 pM of each primer, and deionized water in a total volume of 25 µl. PCR protocol consisted of a 5-min denaturation step at 95 °C (1 cycle), 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s (45 cycles), with high resolution melting. Amplification was performed with «CFX96 Touch™ Real-Time PCR Detection System» («Bio-Rad», U.S.A). The on-line tool was used to design oligonucleotides (http://eu.idtdna.com/calc/analyzer).

Table 2. Earliness–lateness of three rs2640909 genotypes (the first data-set).

	Mean	C/C	T/C	T/T
Observed and expected N or frequencies				
N observed	149	6	60	83
N expected	149	8.7	54.6	85.7
H–W frequencies	100%	5.84%	36.65%	57.52%
Score (SD) on earliness–lateness scale or combination of two scores				
M score	−1.127 (0.905)	4.333(2.509)	−3.400(0.793)*	−4.313(0.675)**
E score	3.293 (0.749)	4.000 (2.076)	2.867 (0.656)	3.012 (0.558)
M+E score	2.166 (1.341)	8.333 (3.716)	−0.533 (1.175)	−1.301 (0.999)*
E−M score	4.42 (0.982)	−0.333 (2.720)	6.267 (0.860)	7.325 (0.731)*

Notes: H-W frequencies: Frequencies expected from the Hardy–Weinberg equilibrium; Mean: Averaged over three genotypes score; SD: Standard Deviation; M score: Score on 12-item morning earliness–lateness scale; E score: Score on 12-item evening earliness–lateness scale; M + E score and E−M score: Sum and difference between E and M scores. Results of one-way ANOVA with factor "Genotype" (homozygotes on minor allele, C/C, heterozygotes, T/C, and homozygotes on major allele, T/T), and with *post hoc* pairwise comparison (Bonferroni test): $^{**}p < 0.01$ and $^{*}p < 0.05$ indicate significance of difference from homozygotes on minor allele.

Genotyping was based on the appearance in fluorescence of the dyes associated with the specific alleles. The averaged times of appearance of the signal were 25–27 cycles for genotype coincidence with the primer; the difference in 5 or more cycles or a cycle or less of the output signal intensity was regarded as homozygous or heterozygous, respectively.

For statistical analyses, the SPSS statistical software package was used (IBM, Armonk, NY, U.S.A, version 22.0). Independent Student's *t*-test was applied to examine differences between carriers of rare alleles and homozygotes on major allele in scores on earliness–lateness scales. We also performed one-way ANOVAs and ANCOVAs (with age as the covariate) to detect differences between three genotypes (homozygotes on minor allele, heterozygotes, and homozygotes on major allele). Bonferroni multiple comparison test was used in the *post hoc* analysis for examining significance of difference between each pair of genotypes. The statistically significant results of such pairwise comparisons are illustrated in Table 2. In the analysis of the second data-set, two- and three-way ANOVAs and ANCOVAs (with age as the covariate) were additionally run to account for differences in sub-samples and/or sex of study participants. Level of significance was fixed at $p = 0.05$.

Results

Observations of allele frequencies for two potential markers of morningness–eveningness (rs2640909 and rs228697) were consistent with those reported for Caucasian and some other populations (Table 1). Minor alleles were slightly overrepresented in our samples, but the frequencies did not differ much from the expected (Tables 2 and 3). We also tested the Hardy–Weinberg equilibrium for each marker. Genotype frequencies for both rs228697 and rs2640909 (Tables 2 and 3, respectively) were not significantly different from those expected by Hardy–Weinberg equilibrium (*P*-value = 0.2277 and 0.2869, respectively).

Independent Student's *t*-test did not yield statistically significant differences between carriers of rare alleles and homozygotes on major allele in scores on any of two (morning and evening) earliness–lateness scales, as well as in their sum and their difference (M, E, M + E, and E−M, respectively).

Table 3. Earliness–lateness of three rs228697 genotypes (the second data-set).

	Mean	G/G	C/G	C/C
Observed and expected N or frequencies				
N observed	248	1	48	199
N expected	248	2.5	45.0	200.5
H-W frequencies	100%	1.02%	18.13%	80.85%
Score (SD) on a earliness–lateness scale or combination of two scores				
M score	−0.533 (1.809)	−8.000 (5.358)	4.000 (0.782)	2.402 (0.385)
E score	3.095 (2.079)	6.000 (6.155)	1.532 (0.898)	1.753 (0.442)
M+E score	2.562 (3.016)	−2.000 (8.931)	5.532 (1.303)	4.155 (0.641)
E−M score	3.627 (2.468)	14.000 (7.308)	−2.468 (1.066)	−0.649 (0.525)

Notes: Results of one-way ANOVA: Significant main effect of factor "Genotype" (the homozygote on minor allele, G/G, heterozygotes, C/G, and homozygotes on major allele, C/C) in ANOVAs of M score and difference between E and M scores, but *post hoc* comparison was not run because $N < 2$ for one of genotypes (see Results). See also notes to Table 2.

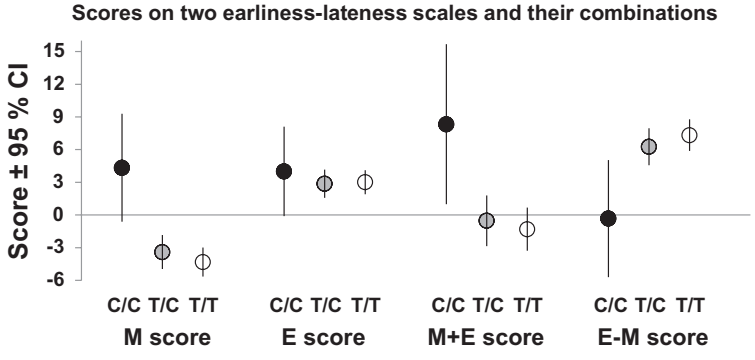

Figure 1. Scores on separate morning and evening earliness–lateness scales and their combinations for three rs2640909 genotypes (the first data-set).

Notes: M score: Score on 12-item morning earliness–lateness scale; E score: Score on 12-item evening earliness–lateness scale; M + E score: Sum of M and E scores; E−M score: Difference between E and M scores. C/C, T/C, and T/T: Homozygotes on minor allele, heterozygotes, and homozygotes on major allele. Vertical bars show ± 95% Confidence Interval (CI) of the genotype-averaged scores.

One-way ANOVA of adult males representing three rs2640909 genotypes revealed statistically significant main effect of genotype on morning earliness–lateness score, M ($F_{2/146} = 5.591$, $p = 0.005$). The difference persisted after controlling for age in the corresponding ANCOVA ($p = 0.005$). Post hoc pairwise comparisons showed that carriers of two copies of minor allele ($N = 6$) significantly differed from any of two other genotypes (see notes to Table 2), whereas the differences between these two other genotypes ($N = 60$ and 89) were not found to be statistically significant (Figure 1). Similar analyses of the main effect of genotype on evening earliness–lateness, E, yielded non-significant results ($F_{2/146} = 0.137$, $p = 0.872$ for ANOVA). The main effect for sum of two earliness–lateness scores, M + E, was still significant ($F_{2/146} = 3.138$, $p = 0.046$ for ANOVA), but *post hoc* pairwise comparisons revealed that only the difference between homozygotes reached a statistically significant level (see notes to Table 2). Moreover, the significant main effect was also yielded by ANOVA and ANCOVA of difference between two earliness–lateness scores, E−M ($F_{2/146} = 3.815$, $p = 0.024$ for ANOVA). Again, *post hoc* pairwise comparisons revealed that only the difference between homozygotes was significant (see notes to Table 2). As can be seen in Figure 1, the

carriers of two copies of rare allele belonged to the evening type. In contrast, the rest of the sample did not show, on average, any shift toward eveningness on sum of two scores, M + E. However, it showed a prominent shift toward morningness on one (morning) scale, M, and it was slightly shifted toward eveningness on another (evening) scale, E.

Table 3 illustrates some results of analysis of the second data-set on association of three rs228729 genotypes with two scales of earliness–lateness and their combinations. In contrast with the hypothesis, significant main effect was yielded in analysis of difference between scores, E–M, rather than sum of scores, M + E (for ANOVA, $F_{2/245} = 3.270$, $p = 0.040$ and $F_{2/245} = 0.707$, $p = 0.494$, respectively). The main effect was also significant for morning earliness–lateness, M (for ANOVA, $F_{2/245} = 3.674$, $p = 0.027$), but the opposing to expected difference between genotypes was reveled (see Table 3). It has to be noted, however, that the group of carriers of two rare alleles of rs228729 was represented by only one study participant, and this might be the major reason for our failure to support the hypothesis.

Discussion

At least four polymorphisms have been investigated so far within the PER3 gene with positive results suggesting that a rare allele is associated with evening preference (Table 1). We tried to replicate the most recent positive findings (on rs2640909 and rs228697) in our pilot study. The administered questionnaire (Putilov 2007, 2010) provided possibility to self-assess individual chronotypological differences between people in preferences scored on two scales named "morning lateness" and "evening lateness" (M and E, respectively). Therefore, the present analysis was the first attempt to examine relationship of PER3 SNPs with each of two separate scales in order to replicate Ojeda et al. (2013) positive findings indicating that the scale-gene association can reach a statistically significant level for only "morningness" subtrait of morningness–eveningness trait. As expected, one of the tested polymorphisms (rs2640909) was successfully linked to one of sub-traits of morningness–eveningness trait, and, as expected, this was the "morningness" sub-trait (Table 2). In both exploration study of Ojeda et al. (2013) and in our confirmation study, a relatively small sample was collected (209 Columbian students in Ojeda et al. 2013; and 149 Moscow bus drivers of older age in our study). Nevertheless, both studies provided the positive results indicating that the rare allele of rs2640909 is associated with lateness in morning behavior and habits (Table 2). We also extended the earlier reported result by a new finding suggesting significant association with difference between the extent of evening and morning lateness (E–M). Earlier, we demonstrated that people with low and high E–M score significantly differed one from another and from two other chronotypes on daily variation in alertness-sleepiness level (Putilov et al. 2015). However, we did not confirm the findings of Hida et al. (2014) and Kripke et al. (2014) on association of the rare allele of rs228697 with lateness in morning and evening behavior and habits (Table 3).

Candidate gene studies suffer from increased likelihood of false positive findings (Sullivan 2007). It is likely that a particular polymorphism of a particular gene from the large family of circadian system genes cannot prominently contribute to the observed individual variation in morningness–eveningness. Moreover, its contribution can vary from one population to another, attenuate with aging or under certain environmental influences, and so on. Therefore, the present positive result on rs2640909 seems to be encouraging and even amusing in the light of such limitations of our pilot replication study as small sample size,

relatively large variation in age of study participants, their engagement in rotated shift work, lack of similarity between this sample participants and participants of the explorative Columbian study, etc. Such kind of limitations can also explain our failure to replicate the earlier reported association of the rare allele of rs228697 with evening preference. Moreover, the extremely low frequency of this allele (just one carrier of its two copies) can serve as an additional explanation for this failure. It made statistically senseless the pairwise comparisons of the homozygote on this allele (when $N < 2$) with other genotypes. As for comparison of homozygotes on major allele with minor allele carriers, neither analysis of rs228697 nor analysis of rs2640909 provided statistically significant findings.

In general, the results of our pilot replication study highlighted, at least, two additional critical issues of a search for potential genetic markers for morningness–eveningness. First, rare frequencies of homozygotes on a minor allele might require genotyping of, at least, a thousand volunteers for selecting just two small groups of participants for future experimental research aimed on comparison of homozygotes on minor and major allele of a particular SNP. Second, it seems to be of importance to account for possible differential relationship of a candidate gene SNP/VNTR to morning and evening earliness–lateness (Figure 1). Therefore, the findings on earlier tested polymorphisms of PER3 and other genes of the circadian system family require additional replication in studies applying a multidimensional questionnaire for self-assessment of morning–evening preference.

Conclusions

Several PER3 polymorphisms have been studied so far as potential genetic markers of self-assessed morningness–eveningness (chronotype). However, in spite of intensive analysis of some of these polymorphisms, it remains unclear which ones are involved in the circadian mechanism underlying the differences between people on preferred times for physical and mental activity, wakefulness, and sleep. Most studied associations have not been replicated in later studies, whereas associations with two more recently reported polymorphisms wait for replication. Our present pilot study provided support for one of these associations. We found that, in accord with the hypothesis based on findings reported by Ojeda et al. (2013), homozygotes on rare variant of rs2640909 had significantly higher score on morning lateness scale, M. Moreover, they had significantly lower score on difference between evening and morning scales, E–M.

Disclosure statement

No potential conflict of interest was reported by the authors.

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