Human Molecular Genetics, 2010, Vol. 19, No. 20 ***4017–4025***
doi:10.1093/hmg/ddq316
Advance Access published on July 23, 2010

Genetic variants and abnormal processing of pre-miR-182, a circadian clock modulator, in major depression patients with late insomnia

Ester Saus[1], Virginia Soria[2], Geòrgia Escaramís[1], Francesca Vivarelli[1], José M. Crespo[2,3], Birgit Kagerbauer[1], José Manuel Menchón[2,3], Mikel Urretavizcaya[2,3], Mònica Gratacòs[1,*] and Xavier Estivill[1,4]

[1]Genes and Disease Program, Center for Genomic Regulation (CRG-UPF), and CIBER en Epidemiología y Salud Pública (CIBERESP), Barcelona 08003, Catalonia, Spain, [2]CIBER en Salud Mental (CIBERSAM), and Psychiatry Department, Bellvitge University Hospital, Idibell, Barcelona 08907, Catalonia, Spain, [3]Department of Clinical Sciences, Bellvitge Campus, Barcelona University, Barcelona 08907, Catalonia, Spain and [4]Experimental and Health Sciences Department, Pompeu Fabra University, Barcelona 08003, Catalonia, Spain

Received May 14, 2010; Revised and Accepted July 20, 2010

Previous studies in mice have reported five different microRNAs (miRNAs; miR-219-1/132/183/96/182) to be modulators of the endogenous circadian clock and have presented experimental evidence for some of the genes involved in the molecular clock machinery as target sites. Moreover, disruption of circadian rhythms has long been implicated in the pathophysiology of major depression (MD). We investigated these miRNAs and some of their target sites at the sequence and functional levels as possible predisposing factors for susceptibility to MD and related chronobiological subphenotypes. Mutational screening was performed in a sample of 359 MD patients and 341 control individuals. We found a significant association between the T allele of the rs76481776 polymorphism in the pre-miR-182 and late insomnia in MD patients. Previous studies have reported an association between insomnia and *CLOCK* gene, a predicted miR-182 target site. A significant overexpression of miR-182 was detected by quantitative real-time polymerase chain reaction in cells transfected with the mutated form of the pre-miR-182 when compared with wild-type form. Moreover, a significant reduction in luciferase activity of plasmids with 3′ UTR of *ADCY6*, *CLOCK* and *DSIP* genes was shown when transfecting cells with the mutated form of pre-miR-182 compared with cells that did not express miR-182. These data indicate that abnormal processing of pre-miR-182 in patients carrying the T allele of the rs76481776 polymorphism may contribute to the dysregulation of circadian rhythms in MD patients with insomnia, which could influence expression levels of the mature form of miR-182 and might increase downregulation in some of its target genes.

INTRODUCTION

Circadian rhythms are 24 h biological oscillations, which, in mammals, are controlled by a master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The master clock, in turn, synchronizes circadian oscillators in peripheral tissues and adjusts the rhythmic fluctuation of a broad range of cellular and physiological functions such as body temperature, hormone release, metabolic rate or the sleep/wake cycle (1). These circadian rhythms of hundreds of clock-controlled genes allow organisms to anticipate daily changes in the environment, and appear to depend on transcriptional–translational feedback loops of activating and repressing elements that produce oscillations in gene activation (2).

MicroRNAs (miRNAs) are small, non-coding, regulatory transcripts of ∼22 nucleotides in length that influence trans-

*To whom correspondence should be addressed at: Center for Genomic Regulation (CRG), PRBB Building, Room 521, Charles Darwin s/n (Dr Aiguader 88), Barcelona 08003, Catalonia, Spain. Tel: +34 933160177; Fax: +34 933160099; Email: monica.gratacos@crg.cat

lation and stability of mRNAs, thus contributing to the regulation of gene expression affecting a variety of biological processes (3). Owing to their ability to modulate expression at the transcriptional and posttranscriptional level (mechanisms known to be involved in the regulation of circadian oscillation) miRNAs have been suggested as potential players in the SCN clock (4). The first evidence of miRNA cycling in mammals came from the recent identification of two brain-specific miRNAs (miR-132 and miR-219-1) reported to be modulators of endogenous circadian clock in the SCN in mice (5). Those authors showed that miR-132 plays a role in the photic entrainment of circadian rhythms, the process by which the SCN is synchronized to a 24 h period by the daily light–dark cycle. miR-219-1 modulates period length, which naturally occurs as a light–dark cycle of ~24 h. Moreover, the authors functionally characterized miR-132 and miR-219-1 within the context of the circadian clock and presented experimental evidence of genes *Rfx4* and *Phlpp* as respective targets (5). In another study, several miRNAs specifically expressed in the mouse retina exhibited daily oscillations in their transcription levels (6). Among this subgroup of miRNAs were members of the miR-183/96/182 cluster, with predicted targets known to be important in the regulation of the circadian rhythm such as *Adcy6* or *Clock*. The authors validated *Adcy6* as a target site for miR-182 and miR-96, and they also showed that the expression of these miRNAs was in antiphase to the *Adcy6* transcript (6).

Dysfunction of circadian rhythms is hypothesized to play a role in the pathophysiology, disease recurrence and the circadian phenotypes typically observed in major depression (MD; reviewed in 7). A high proportion of MD patients, for example, present abnormal timing of sleep and wakefulness, appetite and social rhythms or daily mood fluctuations. Interestingly, these manifestations of abnormal circadian function return to normality with antidepressant or mood stabilizer treatment and patient recovery (8). Following this hypothesis, several authors have explored the possible link between variants in genes pertaining to the molecular clock and the susceptibility to develop major mood disorders (MMD), including unipolar and bipolar patients, finding strong evidence of this relationship in different studies (9–14). As well as disease susceptibility, circadian phenotypes have also been investigated in MMD patients. *CLOCK* is associated with early, middle and late insomnia, diurnal activity pattern and insomnia evolution during antidepressant treatment (15–17), and *PER3* is associated with worse mood in the evening, diurnal preference and delayed sleep phase syndrome (9,14,18).

We therefore hypothesized that naturally occurring variations in the sequences of miR-132, miR-219-1 or the miR-183/96/182 cluster (or in their precursor forms), which act as modulators of the endogenous clocks, or changes in their target binding sites in the *RFX4*, *PHLPP*, *ADCY6* and *CLOCK* genes known to be involved in circadian clock period and entrainment could be related to a higher susceptibility to MD and, more specifically, to related chronobiological subphenotypes frequently observed among these patients. We tested this possibility by targeted resequencing of the genomic regions containing these miRNAs and the above-mentioned corresponding target sites in a sample of 359 unrelated subjects with MD and 341 control individuals, and then performed association analyses comparing the distribution of the genetic variation in relation to MD diagnosis and circadian rhythm-related subphenotypes.

Table 1. Demographic and clinical characteristics of the patients with major depression

	Major depression ($n = 359$)	Controls ($n = 341$)
Sex, *n* (%)		
Male	126 (35.10)	201 (58.94)
Female	233 (64.90)	140 (41.06)
Age at collection, mean ± SD (years)	57.65 ± 15.33	39.76 ± 11.92
Age at onset of illness, mean ± SD (years)	39.21 ± 15.42	—
Depression subtype, *n* (%)		
Unipolar	217 (60.45)	—
Bipolar	142 (39.55)	—
HAM-D[a] score, mean ± SD	28 ± 7.15	
Circadian rhythms-related subphenotypes		
Seasonality[b]		—
SPAQ score, mean ± SD	10 ± 6.16	
SPAQ >10, *n* (%)	182 (50.7)	
Diurnal preference[c]		—
MEQ score, mean ± SD	16.69 ± 4.10	
Morningness (MEQ >17), *n* (%)	164 (45.68)	
Eveningness (MEQ <12), *n* (%)	41 (11.42)	—
Insomnia[d], *n* (%)		
Early insomnia	253 (70.47)	—
Middle insomnia	236 (65.74)	—
Late insomnia	249 (69.36)	—

[a]Severity of index depressive episode for the study measured by the Hamilton Rating Scale for Depression (HAM-D).
[b]Seasonality measured with the Seasonal Pattern Assessment Questionnaire (SPAQ).
[c]Diurnal preference (chronotype) assessed by the Horne and Östberg Morningness–Eveningness Questionnaire (MEQ).
[d]Insomnia measured with items 4, 5 and 6 of the Hamilton Depression Rating Scale (HAM-D).

RESULTS

Resequencing of pre-miRs and targets sites in genes involved in circadian rhythm

First, using sequence alignments, we confirmed that the five different pre-miRNAs (miR-132, miR-219-1 and the miR-183/96/182 cluster) and their mature forms are conserved between mice and humans (Supplementary Material, Fig. S1A). Furthermore, we verified that *RFX4* and *PHLPP* (target sites of miR-132 and miR-219-1, respectively), and *CLOCK* and *ADCY6* (target sites for the miR-183/96/182 cluster) are also conserved predicted targets in humans (Supplementary Material, Fig. S1B).

Afterwards, we performed a targeted mutational screening in a sample of 359 MD patients looking for sequence variations in the genomic regions containing the precursor forms of the cited miRNAs and also in the corresponding target sites of the above-mentioned genes. Demographics and clinical characteristics of the sample are provided in Table 1.

We did not find sequence changes in the mature or the precursor forms of miR-132, miR-219-1 and miR-183 in the MD samples. Nor did we find changes with respect to their predicted targets, *RFX4* and *PHLPP* (respective targets for

Table 2. Description of sequence variants found at pre-miR-182 and pre-miR-96 in patients with major depression

dbSNP ID[a]	Contig position[b]	New variants	Human genome localization[c]	Position regarding miRNA	Alelles[d]	MAF (%) Cases	MAF (%) Controls	Genotyping rate (%)	HWE *p*
miR-182									
rs76481776	NT_007933.14:g.54593803	no	chr7:129197463	Within pre-miRNA-182	C > T	7.5	6.7	93	1.00
rs77586312	NT_007933.14:g.54593804	yes	chr7:129197464	Within pre-miRNA-182	G > A	0.1	0	93	—
rs75953509	NT_007933.14:g.54593811	yes	chr7:129197471	Within pre-miRNA-182	T > C	0.1	0	93	—
rs80041074	NT_007933.14:g.54593815	yes	chr7:129197475	Within pre-miRNA-182	C > T	0.1	0.3	93	—
miR-96									
rs73159662	NT_007933.14:g.54598144	no	chr7:129201804	Within pre-miRNA-96	G > A	1	0.5	92	—
rs41274239	NT_007933.14:g.54598150	no	chr7:129201810	Within pre-miRNA-96	A > G	0.6	0	92	—

HWE *p*, Hardy-Weinberg equilibrium *P*-value; MAF, minor allele frequency.
[a]Based on NCBI dbSNP Build 129 release.
[b]Contig position of reference assembly regarding NCBI database (http://www.ncbi.nlm.nih.gov/).
[c]Nucleotide position regarding human genome assembly hg18.
[d]Corresponds to RefSNP allele regarding dbSNP build 129 if previously described.

Table 3. Association analysis of rs76481776 in pre-miR-182 with late insomnia in major depression patients

Model	MD patients, *n* (%) No late insomnia	Late insomnia	Odds ratio	CI (95%)	*P*-value[a]	AIC[b]
Codominant						
C/C	57 (96.6)	202 (82.8)	1		0.007285	281.1
C/T	2 (3.4)	40 (16.4)	5.56	(1.27–24.23)		
T/T	0 (0)	2 (0.8)	0			
Dominant						
C/C	57 (96.6)	202 (82.8)	1		0.002177*	279.5
C/T-T/T	2 (3.4)	42 (17.2)	6.21	(1.42–27.09)		
Recessive						
C/C-C/T	59 (100)	242 (99.2)	1		0.18107	287.1
T/T	0 (0)	2 (0.8)	0			
Additive						
			5.82	(1.38–24.49)	0.001788*	279.2

MD, major depression.
[a]*P*-value adjusted by sex, age at collection and polarity of depression (unipolar/bipolar) and considered statistically significant after Bonferroni correction for multiple testing when <0.00625 (*).
[b]AIC means Akaike information criterion, which attempts to find the minimal model that correctly explains the data.

miR-132 and miR-219-1), and *ADCY6* and *CLOCK* (targets for the cluster of miR-183/96/182). Nevertheless, we found four sequence variants within the precursor form of miR-182 (rs76481776, already described, and rs77586312, rs75953509 and rs80041074, newly discovered) and two changes in the pre-miR-96 (rs41274239 and rs73159662, already described) (Supplementary Material, Fig. S2). Regarding pre-miR-182, each of the two new variants rs77586312 and rs75953509 were present in only one out of 359 MD patients and in none of the controls, whereas rs76481776 and rs80041074 were encountered in both patients and control individuals. Regarding variations in pre-miR-96, rs73159662 was present in both groups studied, whereas rs41274239 was found only in MD patients (Table 2). All sequence variants found in the different amplicons studied, although not in our regions of interest, are summarized in Supplementary Material, Table S1.

Association analyses of rs76481776 in pre-mir-182

Only the rs76481776 polymorphism was tested in association analyses, as it was the only variant being a common SNP at both groups studied [minor allele frequency (MAF) >5%]. Power calculations determined that, under a log-additive model, our sample (359 cases and 341 controls) had 80% power to detect a genotype relative risk of 1.7 for a variant with an MAF of 7.1%.

Performing case–control analysis testing for diagnosis, we found no statistically significant association either when considering all MD patients as a single group or when stratifying the sample by depression polarity (unipolar or bipolar; Supplementary Material, Table S2). In the MD patients group, we further explored the clinical variables related to circadian rhythms, namely seasonality, diurnal preference and early, middle and late insomnia. We found that MD patients carrying the T allele have a higher risk of presenting late insomnia ($P = 0.0018$; OR = 5.82; CI 95% = 1.38–24.49; Table 3).

Expression analysis of miR-182 and miR-182* by qRT-PCR

To test for a possible functional role of variant rs76481776 in the regulation of mature miRNA processing, we

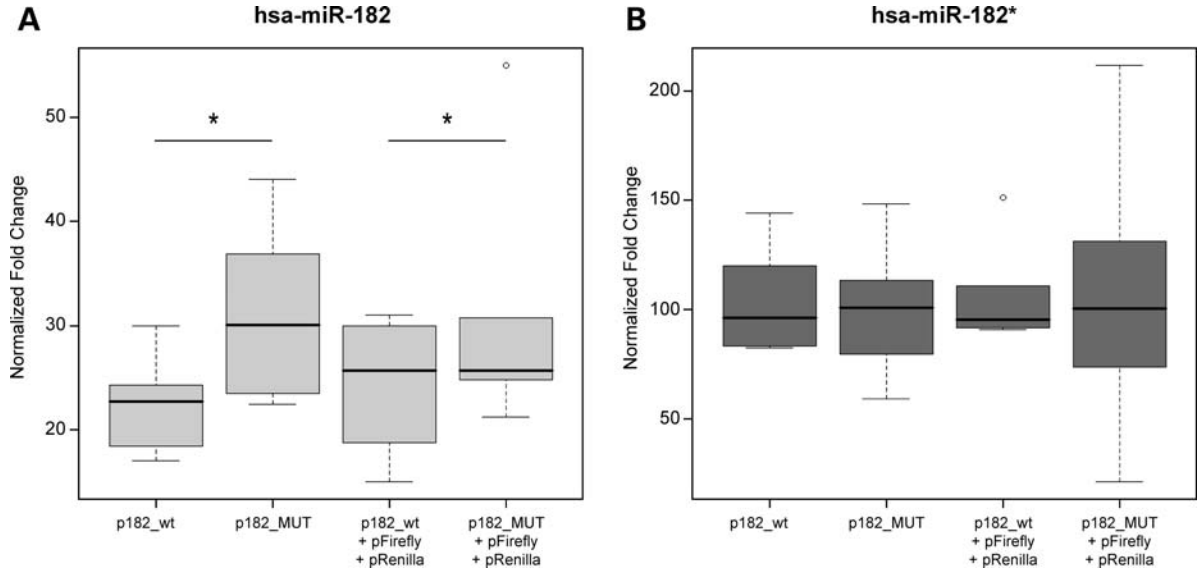

Figure 1. *In vitro* functional effects of variant rs76481776 in the pre-miR-182 on expression levels of miR-182 and mir-182* measured by RT quantitative PCR. Box plots of FCs distribution in of miR-182 (**A**) and miR-182* (**B**) are represented (bold line: median; bottom and top line of the box: first and third quartiles, respectively). Comparisons of miR-182 and miR-182* expression levels between HeLa cells transfected with p182_wt (encoding for normal precursor form of miR-182) or p182_MUT (encoding for pre-miR-182 carrying the variant rs76481776) were performed for different experimental situations (transfecting only p182_wt or p182_MUT or cotransfecting them with pFirefly and pRenilla vectors to check for possible cotransfection effects). The expression values of miR-182 and miR-182* were normalized to that of RNU58B (used as an endogenous control) and the results in different cases were then referred to the expression levels of non-transfected HeLa cells treated with Lipofectamine 2000. Cotransfection of p182_wt or p182_MUT with pFirefly and pRenilla produced no effect on the overexpressed levels of mature form miR-182 in cells transfected with p182_MUT. *Statistically significant *P*-values for differences in FCs ($P < 0.05$).

checked the expression levels of miR-182 and miR-182* (the less predominantly expressed mature form originating from the passenger strand of the precursor) in cells transfected with a plasmid encoding for the wild-type pre-miR-182 (p182_wt) or one carrying the change that we identified (p182_MUT). We found that, in comparison with cells transfected with p182_wt, cells transfected with p182_MUT overexpressed the mature form of miR-182 [fold change (FC) = 1.29; $P = 0.04$; Fig. 1A], but not the mature form of miR-182* (Fig. 1B). Fold changes and *P*-values for all comparisons performed are shown in Supplementary Material, Table S3.

Target prediction of hsa-miR-182

In order to test whether the newly discovered variant has a role in the regulation of circadian rhythmicity, we first performed an *in silico* analysis looking for potential targets of miR-182 that take part in the regulation of the clock machinery. We found 16 predicted target genes involved in molecular clock regulation, the most interesting ones for the present study being *ADCY6*, *CLOCK* and *DSIP* (Supplementary Material, Table S4). We selected *ADCY6* gene as a positive control, since it is a validated target of miR-182 in mice and is known to be involved in circadian rhythm regulation (6). We also selected *CLOCK* gene because it is an important molecular clock regulator which has been previously associated with insomnia in unipolar and bipolar MD patients (15). Finally, we also selected the delta sleep-inducing peptide (DSIP) because it is known to affect the sleep-wakefulness cycle (19).

Functional analysis of miR-182 targeting by luciferase assays

To test whether the presence of the variant rs76481776 in the sequence of the pre-miR-182 could affect the regulation of the above target genes, we compared the effects of the regulation produced by p182_wt and by p182_MUT by means of luciferase assays. Regarding *ADCY6*, as expected, we observed a trend towards a decreased luciferase activity in cells transfected with p182_wt [difference (diff) = 0.80; $P = 0.056$; Fig. 2A], and also a significant and more intense reduction when cells were transfected with p182_MUT (diff = 0.66; $P = 0.0005$; Fig. 2A). The same pattern was observed when studying 3′ UTR of the *CLOCK* gene: we detected a reduction of the luciferase activity when transfecting cells with p182_wt (diff = 0.79; $P = 0.048$; Fig. 2B) and a more pronounced reduction in cells transfected with p182_MUT (diff = 0.64; $P = 0.0004$; Fig. 2B). Finally, for *DSIP* gene, a significant decrease in the luciferase activity was seen only in cells transfected with the p182_MUT compared with p182_NULL (diff = 0.72; $P = 0.0056$; Fig. 2C). Differences and *P*-values for all comparisons performed are shown in Supplementary Material, Table S5.

DISCUSSION

The aim of the present study was to explore whether genetic variants in five miRNAs and in some of their target genes, all related to the regulation of circadian rhythmicity (5,6), are involved in the pathophysiology of MD and in the circadian phenotypes typically observed in these patients. To our

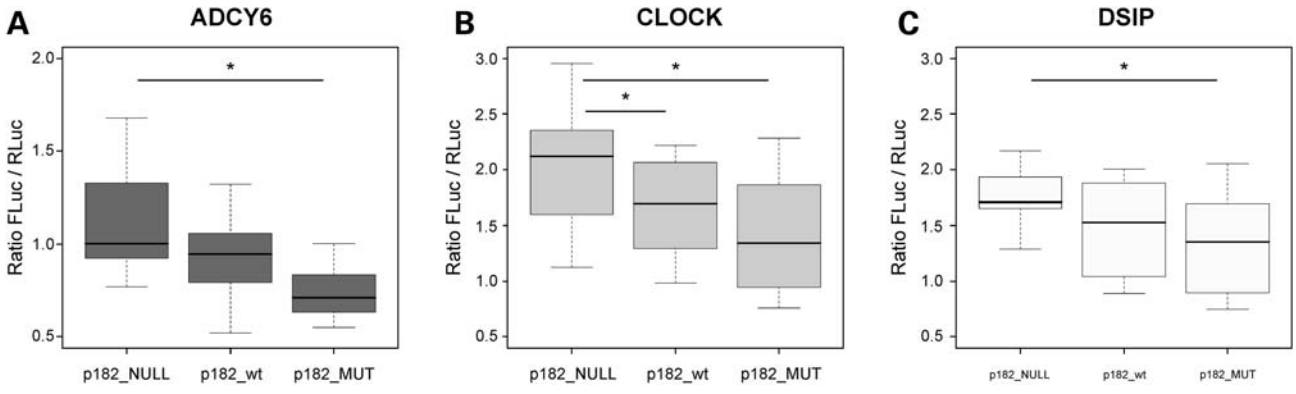

Figure 2. *In vitro* functional effects of variant rs76481776 in the pre-miR-182 on reporter gene expression measured by luciferase-reporter assays. pFirefly constructs containing 3′ UTR of *ADCY6* (pADCY6) (**A**), *CLOCK* (pCLOCK) (**B**) or *DSIP* (pDSIP) (**C**) genes were cotransfected to HeLa cells with pRenilla (as a control of transfection efficiency) and with p182_wt (encoding for normal precursor form of miR-182), p182_MUT (encoding for pre-miR-182 carrying rs76481776) or p182_NULL (expressing no mature form of miR-182 and used as an unspecific effect control). Luciferase activities were measured 24 h after transfection and firefly luciferase activity was normalized to Renilla luciferase activity. Box plots represent the distribution of ratios of firefly/Renilla luciferase activities in each experiment condition (bold line: median; bottom and top line of the box: first and third quartile, respectively). FLuc/RLuc (ratio of firefly luciferase activity/Renilla luciferase activity). *Statistically significant *P*-values for differences in FLuc/RLuc ratio ($P < 0.05$).

knowledge, this is the first attempt to evaluate whether variations in miRNAs are potential regulators of the molecular clockwork in MD patients.

In our study we found that the common variant rs76481776, located in the precursor form of miR-182, is significantly associated with late insomnia in MD patients. In addition, we provide functional evidence for a role of this variant in mature form processing. Pre-miR-182 carrying rs76481776 overexpresses the mature form of miR-182 compared with the wild-type precursor form and, in concordance with these results, it causes a significant reduction in luciferase activity when testing the 3′ UTR of *ADCY6*, *CLOCK* and *DSIP* genes, all of them encoding proteins that are possibly involved in sleep/wake cycle regulation. This is in agreement with previous studies which showed that SNPs or mutations affecting not only the miRNAs but also their precursor forms, as well as the miRNA-binding sites, could potentially be associated with different human diseases such as cancers, hypertension, asthma or other neuropsychiatric disorders (reviewed in 20,21).

The most widely accepted model of sleep regulation involves the interaction of a homeostatic process and a circadian timing process which, although generated independently, mediates the propensity, length, incidence of episodes and intensity of sleep (22). It is known that clock genes have a crucial role in sleep homeostasis processes (reviewed in 23), and it has been widely demonstrated that sleep, sleep deprivation and wakefulness not only alter brain gene expression (24–27), but also change the expression of ∼50 miRNAs, which can be either up or downregulated depending on the brain area examined (28). Thus, it would be no surprise if molecular changes not only in particular miRNAs but also in their mRNA clock targets could regulate sleep homeostasis. This hypothesis is best exemplified by our results on the *CLOCK* gene. First, we found a significant association of miR-182 and late insomnia in MD patients. Second, this miRNA has *CLOCK* as a target effector. Finally, there is evidence in animals and humans that molecular changes in *CLOCK* influence sleep-related traits (reviewed in 29). *Clock* mutation in mice, for example, acts not only on sleep architecture but also on the amount of sleep without affecting sleep intensity, the most striking effect being that mutant mice spend less time asleep (30).

In healthy human adults, a polymorphism in the *CLOCK* gene (3111T/C; rs1801260) has been associated with evening preference and delayed timing of the sleep-wake cycle (31,32). In relation to circadian rhythm sleep disorders, in a patient with non-24 h sleep–wake disorder, the expression profile of clock genes, *CLOCK* among them, was normalized after successful treatment resulting in synchronization of his sleep–wake cycle to a 24 h day (33). Moreover, the 3111T/C SNP mentioned above has been associated with early, middle and late insomnia, diurnal activity pattern and insomnia evolution during antidepressant treatment in bipolar disorder patients (15–17). This particular variant was not associated with any of the phenotypes studied in our sample (data not shown), including the three types of insomnia considered. The methodological strategies used in the original report (15) and in our study are the same (HAM-D-21), and the genotype frequencies in both populations for this single nucleotide polymorphism (SNP) are very similar (data not shown). Thus, the most probable explanation for the inconsistency of the results between the two studies is a type II error due to a smaller sample size in our case, which could have led to a false-negative result. Alternatively, another untyped potential functional variant in linkage disequilibrium with the rs1801260 SNP could be the true causal variant of the phenotype effects. In this situation, population differences in the linkage disequilibrium of the region could account for the lack of association of the rs1801260 SNP and insomnia in our sample. Apart from this SNP, two other rare changes in the 3′ UTR of *CLOCK* gene have been found in two MD patients with sleep disturbances but not in healthy subjects (34). One of these changes (rs70965446) was indeed found in two of our MD patients, both of whom presented severe late insomnia. None of the above SNPs in the 3′ UTR of *CLOCK* is located in the predicted target site of miR-182, and none of them has been shown to alter the *CLOCK*

transcriptional activity but, in view of our results, which implicate miR-182 in its regulation, it is tempting to speculate that they could influence the accessibility of the miRNA–RISC complex or the coordination of miRNA with other regulatory elements, as has been previously suggested for SNPs near an miRNA target site (21).

Besides posttranscriptional regulation of circadian rhythms by miRNAs, other mechanisms are possibly contributing to control expression of core components circadian clock genes. Disruption of circadian rhythms is believed to play a critical role in cancer development and, indeed, there are different examples of alternative methylation patterns in clock genes in these pathologies, such as hypermethylation of *PER3* in patients with chronic myeloid leukemia (35), transcriptionally silencing of *BMAL1* gene by means of hypermethylation in hematologic malignancies (36), and hypermethylation in the *CLOCK* promoter reducing the risk of breast cancer (37). Moreover, there is recent evidence that epigenetic mechanisms such as DNA methylation might also contribute to the developmental expression of clock genes (38). Other regulation steps in the control of circadian clock account for posttranslational modifications of clock proteins by phosphorylation, ubiquitination and acetylation (39). Thus, for a complete knowledge and understanding of clock genes regulation, it is important to focus not only in the study of transcription factors and modulators of the mammalian circadian clock, but also to take into account other mechanisms, such as posttranscriptional regulation by miRNAs (widely discussed in this work), posttranslational mechanisms and epigenetic factors.

There are some limitations in the present study that must be acknowledged. First, the sample size limits the statistical power of the study since most changes found are rare alleles with very low frequencies (MAF ranging from 0.1 to 1, Table 2), which made it impossible to perform association analyses except for the common variant rs76481776. In this case, the statistical power to detect a reliable association with the rs76481776 polymorphism (presenting an MAF of 7.1) is 80%. Nonetheless, it is noticeable that three out of these five rare changes found in the pre-miR-96 and pre-miR-182 were present only in MD patients and in none of the control individuals screened, which supports the emerging hypothesis of rare alleles underlying complex disorders (40). Second, it is not possible to rule out an association between the rs76481776 SNP and late insomnia regardless of the presence of an MD. In our study, information about insomnia was not available for controls, since insomnia variables were obtained through the items 4, 5 and 6 of the HAM-D-21 which evaluate the presence and severity of the insomnia subtypes as symptoms of depression in individuals undergoing a major depressive episode. Consequently, further studies between this variant and insomnia in general population should be performed. Third, all the functional experiments performed were *in vitro* and, consequently, we suggest that further evidence is required from experiments carried out on other *in vivo* systems in order to better understand the potential role of the variant rs76481776 and miR-182 itself in sleep regulation. Finally, these potentially exciting results must be considered as preliminary until further confirmation is obtained in independent samples.

In summary, in view of the functional consequences of the rs76481776 described here, and considering the association of this SNP with late insomnia in MD patients demonstrated in this study, we suggest a precise posttranscriptional regulation of circadian rhythms by miRNAs, and specifically by miR-182, in which the rs76481776 SNP in its precursor form could play an important role in fine-tuning its target sites involved in the control of sleep and wakefulness.

MATERIALS AND METHODS

Subjects and clinical assessment

We studied a clinical sample of 359 unrelated patients and 341 control individuals. Patients with a major depressive episode of at least moderate severity were consecutively recruited from the Psychiatry Department at the 'Hospital Universitari de Bellvitge' between 2004 and 2006 and diagnosed by experienced psychiatrists through a structured interview (41) according to DSM-IV criteria (42) (217 unipolar major depressive disorder, 142 bipolar depression). Depression severity was assessed using the 21-item HAM-D scale (43) administered by a psychiatrist during the depressive index episode. All patients completed the Spanish versions of the Seasonal Pattern Assessment Questionnaire [SPAQ (44)] and the Horne-Östberg Morningness-Eveningness Questionnaire [MEQ (45)]. Other sociodemographic and clinical variables were obtained by means of an interview conducted in person and supported by the medical information available. The exclusion criteria were age <18, additional past or present psychiatric diagnosis other than MMD, past or present history of psychoactive substance abuse and severe medical disease.

The control group consisted of 341 unrelated blood donors recruited from the Blood and Tissue Bank from the Catalan Health Service, who were matched to cases for ethnicity.

All subjects provided written informed consent through the local Institutional Review Board-approved procedures prior to participation. The study complied with the guidelines of the Medical Ethical Committee from each of the participating institutions.

Identification of miRNAs and their targets in humans

MiRNAs of interest (miR-132, miR-219-1 and miR-183/96/182 cluster) were first explored for their conservation between species through the comparison of their respective sequences available from the public miRBase database (46) from The Wellcome Trust Sanger Institute (http://microrna.sanger.ac.uk/sequences). We then checked the conservation in humans of the *RFX4*, *PHLPP*, *CLOCK* and *ADCY6* gene target sites in mice, using the web-based versions of TargetScan (http://genes.mit.edu/tscan/targetscanS2005.html), PicTar (http://pictar.bio.nyu.edu) and MiRanda (http://microrna.sanger.ac.uk/targets; http://www.microrna.org). We also used these programs to screen for predicted target genes for hsa-miR-182.

Mutational screening

The reference sequences of the miRNAs and their target sites were obtained from human genome assembly hg18

available at the public repository of the University of Santa Cruz, California (UCSC, http://genome.ucsc.edu/), March 2006 freeze (NCBI Build 36.1). PCR primers for each genomic DNA fragment were designed by Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were also used as primers for forward and reverse direct sequencing (Supplementary Material, Table S6). PCR products were confirmed by 1.5% agarose gel electrophoresis and were then enzymatically purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA). The purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and sequence purification was performed with Millipore Montage seq96 (Millipore Corporation, Billerica, MA, USA). Sequencing was performed using an ABI Prism® 3730*xl* DNA Analyzer (Applied Biosystems). We used the software SeqScape (version 2.5, Applied Biosystems) to assist in mutation detection, and we visually verified each mutation. We first tested all the MD samples for the different regions and, when sequence variants were found, we analyzed the control samples for that specific region.

Statistical analysis of polymorphisms

Power calculations used Quanto software v.1.2.3 (47,48). Polymorphisms with an MAF of >0.5 were tested in controls to ensure the fitting with Hardy–Weinberg equilibrium. Case–control association analyses to test for diagnosis phenotype (total MD sample and stratified analysis considering unipolar and bipolar depressives) and association analyses of the insomnia (early, middle or late), defined by a score of 1 or 2 on items 4, 5 and 6, respectively, in the HAM-D-21 in MD patients were performed by logistic regression, whereas linear regression was used for quantitative traits (scores in SPAQ and MEQ). All analyses testing diagnosis phenotype were adjusted for the covariate sex, and when interrogating circadian rhythm-related phenotypes (namely insomnia subtypes, seasonality and diurnal preference scores) all the association analyses were adjusted for the following covariates: sex, age at collection and polarity. Four different genetic models were considered in these analyses: codominant, dominant, recessive and additive. Finally, *P*-values were computed using the likelihood ratio test and the best model was selected based on the Akaike information criteria (49). All analyses were performed using the SNPassoc R package (50). As we performed eight different variable association analyses, a *P*-value of <0.00625 was considered statistically significant after Bonferroni correction for multiple testing.

Plasmids construction

We used an expression vector that encodes the precursor form of miR-182 under an EF-1α promoter, which ensures a high level of expression in mammalian cells (miRNASelect™ pEP-miR-182 Expression Vector; Cell Biolabs, Inc.). From pEP-miR-182 (p182_wt), we generated two constructs: one lacking a region of the insert and not expressing pre-miR-182 (p182_NULL), and another one, which was generated through site-directed mutagenesis, carrying the C/T change (rs76481776) in the pre-miR-182 sequence (p182_MUT). First, to generate p182_NULL, we performed a sequential double-digestion of the p182_wt vector with *Bam*HI and *Nhe*I restriction enzymes (New England Biolabs, Inc.) and a posterior filling-in of 5′ overhangs to form blunt ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs, Inc.). Second, to obtain p182_MUT construct, single nucleotide substitution (C>T) corresponding to rs76481776 was introduced into the DNA sequence of p182_wt with the use of a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions and using the following primers: 5′-CGAGGACTCAG CCAGCACCCTGTGCAC-3′ (forward) and 5′-GTGCACAG GGTGCTGGCTGAGTCCTCG-3′ (reverse).

For the luciferase assays, we generated three different firefly luciferase pGL4.13 constructs (pFirefly). DNA fragments corresponding to 929, 704 and 936 bp of the 3′ UTR regions with predicted target sites for miR-182 of *ADCY6*, *CLOCK* and *DSIP* genes, respectively, were amplified by PCR with *PfuTurbo*® DNA polymerase (Stratagene) from control individuals' DNA samples containing no variation in the sequences. The primers used for each of the genes were the following, all of them containing an *Xba*I restriction site (New England Biolabs, Inc.) at the 5′ end: *ADCY6* (forward: 5′-AC ACACTCTAGAATGCTTGCCTTTGCAATACC-3′; reverse: 5′-ACACACTCTAGAACCCCAGACAGTTCAAGTGC-3′), *CLOCK* (forward: 5′-ACACACTCTAGACTAGGTTGCTCC ATGGGAAT-3′; reverse: 5′-ACACACTCTAGATGAACCC ACACAACATTCTGA-3′) and *DSIP* (forward: 5′-ACACAC TCTAGACAAGCATCATCTCACGAGGA-3′; reverse: 5′-AC ACACTCTAGACACTGTGAAGCCCAAACTGA-3′). The PCR fragments obtained were purified, *Xba*I digested and cloned into an *Xba*I site located downstream of the firefly luciferase-reporter gene in the pGL4.13 vector (Promega Corporation, Madison, WI, USA). Finally, the pFirefly constructs obtained (pADCY6, pCLOCK and pDSIP) were propagated in *E. coli* One Shot® TOP 10 cells (Invitrogen).

For all constructs, the authenticity, the orientation of the inserts and the presence of the mutations were confirmed by direct sequencing.

Real-time quantitative reverse transcription PCR

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml Streptomycin (GIBCO™, Invitrogen). HeLa cells were seeded at 6.75×10^4 cells/well in 24-well plate and transfected 24 h later using Lipofectamine 2000 (Invitrogen) with the pEP-miR-182 constructs described above (1 μg of p182_wt, p182_MUT or p182_NULL) and with the pFirefly (50 ng) and the pRenilla (15 ng of pGL4.75) if required. Total RNA, including miRNA, was isolated using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions 24 h after transfection (when optimal miR-182 and miR-182* expression was found). Ten nanograms of total RNA were used for reverse transcription as measured by a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). cDNA was made using the TaqMan® MicroRNA Reverse Trancription Kit (Applied Biosystems) and the specific reverse transcription primers for hsa-mir-182, hsa-mir-182* and the housekeeping gene RNU58B (Applied

Biosystems). The corresponding PCR primers were used with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) using ABI PRISM 7900HT Fast PCR systems (Applied Biosystems). Control reactions containing no reverse transcriptase and no cDNA template were also performed. Crossing-point values, the point at which the fluorescence of a sample rises above the background fluorescence, were obtained for each reaction in the different experimental conditions performed and were used for relative quantification analyses. Three different transfections with their subsequent RNA extraction were performed for each experimental condition. Two different reverse transcription assays with follow-up qRT-PCR were made from each isolated RNA, and each experimental condition was measured in triplicate. Finally, some verifications were done before performing the final experiments in order to corroborate the overexpression of miR-182 and miR-182* in cells transfected with p182_wt compared with non-transfected cells, and also that p182_NULL plasmid used in further experiments expressed none of the mature forms of the miRNA.

Luciferase activity assays

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml Streptomycin (GIBCO™, Invitrogen). HeLa cells were seeded at 1.35×10^4 cells/well in 96-well plates and cotransfected 24 h later using Lipofectamine 2000 (Invitrogen) with the appropriate pEP-miR-182 constructs (200 ng of p182_wt, p182_NULL or p182_MUT), the pRenilla (3 ng) and the Firefly reporter constructs described above (24 ng of pADCY6, pCLOCK or pDSIP) or the empty pFirefly vector (10 ng). Activities of Firefly and Renilla luciferases were determined 24 h after transfection using the Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's instructions. Relative reporter activity was obtained by normalization to Renilla luciferase activity (ratio Firefly luciferase/Renilla luciferase). Each experimental condition was measured in triplicate for each luciferase assay, and each luciferase assay was performed four times.

Data and statistical analyses of functional assays

For the qRT-PCR assays, FCs were calculated to compare expression values of both miR-182 and miR-182* normalized to that of RNU58B among different experimental conditions. These FCs were obtained from a linear mixed effects model (51) that accounted for different sources of variations; three different transfections and two reverse transcriptions per transfection (see Supplementary Material, Methods for details). For the luciferase assays, FCs were also calculated to compare the relative reporter activity among the different experimental conditions via a linear mixed effects model, which also accounted for experimental day variability (see Supplementary Material, Methods for details).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

The authors thank all the study participants, clinicians and nurses from the Psychiatry Department of 'Hospital Universitari de Bellvitge' who have collaborated to obtain the sample of this study. We also thank Josiane Wyniger and Daniel Trujillano for their experimental support, and Sílvia Porta, Elena Miñones and Eulàlia Martí for scientific support and suggestions and Eulàlia Martí for critically reading the manuscript. We thank the support from Genome Spain to the National Genotyping Center (CeGen). This publication includes part of the work of E.S.'s doctoral thesis attached to University Pompeu Fabra (UPF), Barcelona.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Ministry of Science and Innovation, MICINN (SAF2005-01005, SAF2008-00357); the Spanish Ministry of Health, 'Instituto de Salud Carlos III' (Centro de Investigación en Red de Salud Mental, CIBERSAM; Centro de Investigación en Red en Epidemiología y Salud Pública, CIBERESP; PI050960; PI000954; PI040632; PI040619), the Department of Health and the Department of Universities Research and Information Society (2005SGR00008; 2005SGR00322; 2009SGR1554) (Generalitat de Catalunya). E.S. was supported by the CRG under project (SAF2005-01005) with fellowship BES-2006-13917 (Spanish Ministry of Science and Innovation, MICINN).

REFERENCES

1. Reppert, S.M. and Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.*, **63**, 647–676.
2. Merrow, M., Spoelstra, K. and Roenneberg, T. (2005) The circadian cycle: daily rhythms from behaviour to genes. *EMBO Rep.*, **6**, 930–935.
3. Bushati, N. and Cohen, S.M. (2007) microRNA functions. *Annu. Rev. Cell. Dev. Biol.*, **23**, 175–205.
4. Pegoraro, M. and Tauber, E. (2008) The role of microRNAs (miRNA) in circadian rhythmicity. *J. Genet.*, **87**, 505–511.
5. Cheng, H.Y., Papp, J.W., Varlamova, O., Dziema, H., Russell, B., Curfman, J.P., Nakazawa, T., Shimizu, K., Okamura, H., Impey, S. *et al.* (2007) microRNA modulation of circadian-clock period and entrainment. *Neuron*, **54**, 813–829.
6. Xu, S., Witmer, P.D., Lumayag, S., Kovacs, B. and Valle, D. (2007) MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J. Biol. Chem.*, **282**, 25053–25066.
7. Mendlewicz, J. (2009) Disruption of the circadian timing systems: molecular mechanisms in mood disorders. *CNS Drugs*, **23**(Suppl. 2), 15–26.
8. McClung, C.A. (2007) Circadian genes, rhythms and the biology of mood disorders. *Pharmacol. Ther.*, **114**, 222–232.
9. Johansson, C., Willeit, M., Smedh, C., Ekholm, J., Paunio, T., Kieseppa, T., Lichtermann, D., Praschak-Rieder, N., Neumeister, A., Nilsson, L.G. *et al.* (2003) Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference. *Neuropsychopharmacology*, **28**, 734–739.
10. Partonen, T., Treutlein, J., Alpman, A., Frank, J., Johansson, C., Depner, M., Aron, L., Rietschel, M., Wellek, S., Soronen, P. *et al.* (2007) Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. *Ann. Med.*, **39**, 229–238.
11. Soria, V., Martinez-Amoros, E., Escaramis, G., Valero, J., Perez-Egea, R., Garcia, C., Gutierrez-Zotes, A., Puigdemont, D., Bayes, M., Crespo, J.M. *et al.* (2010) Differential association of circadian genes with mood disorders:

CRY1 and NPAS2 are associated with unipolar major depression and CLOCK and VIP with bipolar disorder. *Neuropsychopharmacology*, **35**, 1279–1289.
12. Mansour, H.A., Wood, J., Logue, T., Chowdari, K.V., Dayal, M., Kupfer, D.J., Monk, T.H., Devlin, B. and Nimgaonkar, V.L. (2006) Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia. *Genes Brain Behav.*, **5**, 150–157.
13. Shi, J., Wittke-Thompson, J.K., Badner, J.A., Hattori, E., Potash, J.B., Willour, V.L., McMahon, F.J., Gershon, E.S. and Liu, C. (2008) Clock genes may influence bipolar disorder susceptibility and dysfunctional circadian rhythm. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **147B**, 1047–1055.
14. Artioli, P., Lorenzi, C., Pirovano, A., Serretti, A., Benedetti, F., Catalano, M. and Smeraldi, E. (2007) How do genes exert their role? Period 3 gene variants and possible influences on mood disorder phenotypes. *Eur. Neuropsychopharmacol.*, **17**, 587–594.
15. Serretti, A., Benedetti, F., Mandelli, L., Lorenzi, C., Pirovano, A., Colombo, C. and Smeraldi, E. (2003) Genetic dissection of psychopathological symptoms: insomnia in mood disorders and CLOCK gene polymorphism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **121B**, 35–38.
16. Benedetti, F., Dallaspezia, S., Fulgosi, M.C., Lorenzi, C., Serretti, A., Barbini, B., Colombo, C. and Smeraldi, E. (2007) Actimetric evidence that CLOCK 3111 T/C SNP influences sleep and activity patterns in patients affected by bipolar depression. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **144B**, 631–635.
17. Serretti, A., Cusin, C., Benedetti, F., Mandelli, L., Pirovano, A., Zanardi, R., Colombo, C. and Smeraldi, E. (2005) Insomnia improvement during antidepressant treatment and CLOCK gene polymorphism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **137B**, 36–39.
18. Ebisawa, T., Uchiyama, M., Kajimura, N., Mishima, K., Kamei, Y., Katoh, M., Watanabe, T., Sekimoto, M., Shibui, K., Kim, K. *et al.* (2001) Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. *EMBO Rep.*, **2**, 342–346.
19. Pollard, B.J. and Pomfrett, C.J. (2001) Delta sleep-inducing peptide. *Eur. J. Anaesthesiol.*, **18**, 419–422.
20. Sethupathy, P. and Collins, F.S. (2008) MicroRNA target site polymorphisms and human disease. *Trends Genet.*, **24**, 489–497.
21. Mishra, P.J. and Bertino, J.R. (2009) MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. *Pharmacogenomics*, **10**, 399–416.
22. Borbely, A.A. (1982) A two process model of sleep regulation. *Hum. Neurobiol.*, **1**, 195–204.
23. Franken, P. and Dijk, D.J. (2009) Circadian clock genes and sleep homeostasis. *Eur. J. Neurosci.*, **29**, 1820–1829.
24. Cirelli, C., Gutierrez, C.M. and Tononi, G. (2004) Extensive and divergent effects of sleep and wakefulness on brain gene expression. *Neuron*, **41**, 35–43.
25. Guzman-Marin, R., Ying, Z., Suntsova, N., Methippara, M., Bashir, T., Szymusiak, R., Gomez-Pinilla, F. and McGinty, D. (2006) Suppression of hippocampal plasticity-related gene expression by sleep deprivation in rats. *J. Physiol.*, **575**, 807–819.
26. Taishi, P., Sanchez, C., Wang, Y., Fang, J., Harding, J.W. and Krueger, J.M. (2001) Conditions that affect sleep alter the expression of molecules associated with synaptic plasticity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **281**, R839–R845.
27. Terao, A., Wisor, J.P., Peyron, C., Apte-Deshpande, A., Wurts, S.W., Edgar, D.M. and Kilduff, T.S. (2006) Gene expression in the rat brain during sleep deprivation and recovery sleep: an Affymetrix GeneChip study. *Neuroscience*, **137**, 593–605.
28. Davis, C.J., Bohnet, S.G., Meyerson, J.M. and Krueger, J.M. (2007) Sleep loss changes microRNA levels in the brain: a possible mechanism for state-dependent translational regulation. *Neurosci. Lett.*, **422**, 68–73.
29. Lamont, E.W., James, F.O., Boivin, D.B. and Cermakian, N. (2007) From circadian clock gene expression to pathologies. *Sleep Med.*, **8**, 547–556.
30. Naylor, E., Bergmann, B.M., Krauski, K., Zee, P.C., Takahashi, J.S., Vitaterna, M.H. and Turek, F.W. (2000) The circadian clock mutation alters sleep homeostasis in the mouse. *J. Neurosci.*, **20**, 8138–8143.
31. Katzenberg, D., Young, T., Finn, L., Lin, L., King, D.P., Takahashi, J.S. and Mignot, E. (1998) A CLOCK polymorphism associated with human diurnal preference. *Sleep*, **21**, 569–576.
32. Mishima, K., Tozawa, T., Satoh, K., Saitoh, H. and Mishima, Y. (2005) The 3111T/C polymorphism of hClock is associated with evening preference and delayed sleep timing in a Japanese population sample. *Am. J. Med. Genet. B Neuropsychiatr. Genet.*, **133B**, 101–104.
33. Takimoto, M., Hamada, A., Tomoda, A., Ohdo, S., Ohmura, T., Sakato, H., Kawatani, J., Jodoi, T., Nakagawa, H., Terazono, H. *et al.* (2005) Daily expression of clock genes in whole blood cells in healthy subjects and a patient with circadian rhythm sleep disorder. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **289**, R1273–R1279.
34. Pirovano, A., Lorenzi, C., Serretti, A., Ploia, C., Landoni, S., Catalano, M. and Smeraldi, E. (2005) Two new rare variants in the circadian 'clock' gene may influence sleep pattern. *Genet. Med.*, **7**, 455–457.
35. Yang, M.Y., Chang, J.G., Lin, P.M., Tang, K.P., Chen, Y.H., Lin, H.Y., Liu, T.C., Hsiao, H.H., Liu, Y.C. and Lin, S.F. (2006) Downregulation of circadian clock genes in chronic myeloid leukemia: alternative methylation pattern of hPER3. *Cancer Sci.*, **97**, 1298–1307.
36. Taniguchi, H., Fernandez, A.F., Setien, F., Ropero, S., Ballestar, E., Villanueva, A., Yamamoto, H., Imai, K., Shinomura, Y. and Esteller, M. (2009) Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies. *Cancer Res.*, **69**, 8447–8454.
37. Hoffman, A.E., Yi, C.H., Zheng, T., Stevens, R.G., Leaderer, D., Zhang, Y., Holford, T.R., Hansen, J., Paulson, J. and Zhu, Y. (2010) CLOCK in breast tumorigenesis: genetic, epigenetic, and transcriptional profiling analyses. *Cancer Res.*, **70**, 1459–1468.
38. Ji, Y., Qin, Y., Shu, H. and Li, X. (2010) Methylation analyses on promoters of mPer1, mPer2 and mCry1 during perinatal development. *Biochem. Biophys. Res. Commun.*, **391**, 1742–1747.
39. Hirota, T. and Kay, S.A. (2009) High-throughput screening and chemical biology: new approaches for understanding circadian clock mechanisms. *Chem. Biol.*, **16**, 921–927.
40. Schork, N.J., Murray, S.S., Frazer, K.A. and Topol, E.J. (2009) Common vs. rare allele hypotheses for complex diseases. *Curr. Opin. Genet. Dev.*, **19**, 212–219.
41. First, M.B., Spitzer, R.L., Gibbon, M. and Williams, J.B.W. (1997) *Structured Clinical Interview for DSM-IV Axis I Disorders (SCID)*, American Psychiatric Press, Washington, DC.
42. American Psychiatric Association (2000) *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), Text Revision.*, American Psychiatric Press, Washington, DC.
43. Hamilton, M. (1960) A rating scale for depression. *J. Neurol. Neurosurg. Psychiatry*, **23**, 56–62.
44. Goikolea, J.M., Miralles, G., Bulbena Cabre, A., Vieta, E. and Bulbena, A. (2003) [Spanish adaptation of the Seasonal Pattern Assessment Questionnaire (SPAQ) in the adult and children–adolescent versions]. *Acta. Esp. Psiquiatr.*, **31**, 192–198.
45. Adan, A. and Almirall, H. (1990) Adaptation and standardization of a Spanish version of the morningness–eveningness questionnaire: individual differences. *Pers. Individ. Dif.*, **11**, 1123–1130.
46. Griffiths-Jones, S., Saini, H.K., van Dongen, S. and Enright, A.J. (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res.*, **36**, D154–D158.
47. Gauderman, W.J. (2002) Sample size requirements for matched case–control studies of gene–environment interaction. *Stat. Med.*, **21**, 35–50.
48. Gauderman, W.J. and Morrison, J.M. (2006) QUANTO 1.1: a computer program for power and sample size calculations for genetic-epidemiology studies. http://hydra.usc.edu/gxe [program].
49. Akaike, H. (1973) Information theory as an extension of the maximum likelihood principle. In: Petrov, B.N. and Csaki, F. (eds), *Proc. 2nd Int. Symp. Info. Theory*. Akademiai Kiado, Budapest, Hungary, pp. 267–281.
50. Gonzalez, J.R., Armengol, L., Sole, X., Guino, E., Mercader, J.M., Estivill, X. and Moreno, V. (2007) SNPassoc: an R package to perform whole genome association studies. *Bioinformatics*, **23**, 644–645.
51. Steibel, J.P., Poletto, R., Coussens, P.M. and Rosa, G.J. (2009) A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics*, **94**, 146–152.