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Genetic polymorphisms in the aryl hydrocarbon receptor–signaling pathway and sleep disturbances in middle-aged women

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Abstract

Objective—We aimed to determine if selected genetic polymorphisms in the aryl hydrocarbon receptor (AHR)–signaling pathway and circadian locomotor output cycles kaput (*CLOCK*) are associated with insomnia and early awakening in middle-aged women.

Methods—Women aged 45 to 54 years (n=639) were recruited into a middle-aged health study and agreed to complete questionnaires and donate blood samples. Questionnaires were used to assess sleep outcomes. Blood samples were processed for genotyping the selected polymorphisms: *AHR* (rs2066853), *AHR* repressor (*AHRR*) (rs2292596), aryl hydrocarbon nuclear translocator (*ARNT*) (rs2228099), and circadian locomotor output cycles kaput (*CLOCK*) (rs1801260). Data were analyzed using multivariable logistic regression.

Results—Women heterozygous for the *AHRR* alleles (GC) had decreased odds of insomnia compared to women homozygous for the *AHRR*_C allele (adjusted odds ratio [aOR], 0.69; 95% confidence interval [CI], 0.49–0.96). Women with at least one of the *AHRR*_G or *CLOCK*_C alleles had significantly decreased odds of insomnia compared to women homozygous for the *AHRR*_C and *CLOCK*_T alleles (aOR, 0.64; 95% CI, 0.43–0.96). Additionally, women homozygous for the *AHRR*_G and *CLOCK*_C alleles had significantly decreased odds of insomnia compared to women homozygous for the *AHRR*_C and *CLOCK*_T alleles (aOR, 0.56; 95% CI, 0.35–0.89). None of the selected single nucleotide polymorphisms (SNPs) or combinations of SNPs were significantly associated with early awakening.

Conclusions—Selected genetic polymorphisms in the AHR-signaling pathway (i.e., *AHRR*) and *CLOCK* may play a role in decreasing the risk for experiencing insomnia during the menopausal transition.

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Keywords

polymorphism; *CLOCK*; *AHR*; insomnia; early awakening; middle-aged women

1. Introduction

As women age and go through the menopausal transition, the likelihood of experiencing sleep disturbances increases [1–3]. Previous studies have examined potential factors associated with sleep disturbances during this time period; however, these studies mainly have focused on hormonal changes, vasomotor symptoms, or stress [4–8]. Although some of these factors have been found to be significantly associated with sleep disturbances [7,8], the data are equivocal [9]. For example, sleep disturbances can be experienced by healthy women without vasomotor symptoms [2,9]. Additionally, even after vasomotor symptoms have diminished, some women still experience sleep disturbances [9]. Similarly, women experiencing stress or altered hormonal levels may not have sleep disturbances. Therefore, our study focused on whether or not other factors such as selected genetic polymorphisms are associated with sleep disturbances during the menopausal transition.

The main master regulators of circadian rhythms related to sleep-wake cycles are circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like or BMAL1. These regulators are members of the basic helix-loop-helix (bHLH)/period-ARNT-single minded (PAS) domain family. These domains are needed for binding and transcription regulation [10]. Overall, the clock machinery acts in a feedback activation/inhibition manner [11]. Basically, the CLOCK/BMAL1 heterodimer complex binds to the enhancer boxes of their target genes and further initiates the transcription/translation of inhibiting factors: periods (PER 1,2, and 3) and cryptochromes (CRY1 and 2). This binding leads to increased levels of the inhibiting complexes and results in reduced transcription/translation. This then allows consecutive action of CLOCK and BMAL1 [11].

Other members of the bHLH/PAS domain protein family belong to the aryl hydrocarbon receptor (AHR)–signaling pathway. This highly conserved signaling pathway is present in most cell types. In addition, members of this pathway have a daily oscillation rhythm of their own that operates in a tissue specific manner in rodents [12,13]. The main members of this signaling pathway are the AHR, its repressor (AHRR), and ARNT. The bHLH/PAS domains of BMAL1 and ARNT are extremely similar and are thought to originate from the same ancestral gene [14]. Furthermore, researchers have proposed a potential functional link between the AHR-signaling pathway and the circadian clock [15,16].

Alterations in circadian rhythms such as those resulting from genetic variations have been examined by other researchers [17–19]. In a few studies, a single nucleotide polymorphism (SNP) in *CLOCK* (rs1801260; T/C) has been associated with sleep differences such as evening preference and delayed timing of the sleep-wake cycle [18,19]. Subjects carrying one or two copies of the *CLOCK*_C allele showed increased eveningness and reduced morningness, while subjects homozygous for *CLOCK*_T showed higher morningness scores [18,19] and shorter total sleep duration [19]. The *CLOCK*_CC genotype was also associated with delayed sleep timing and greater daytime sleepiness in Japanese [19] but not in white women [18]. However, no studies have explored the associations between SNPs in *CLOCK*, the AHR pathway related genes, and sleep disturbances in middle-aged women. Hence, in our study we tested the hypothesis that selected SNPs (individual and combined SNPs) in the *CLOCK* gene (rs1801260), and the AHR-signaling pathway (*AHR* rs2066853, *AHRR*

rs2292596, *ARNT* rs2228099) are associated with the risk for insomnia and early awakening among middle-aged women.

2. Materials and methods

2.1. Study population and design

Study methods have been described in detail elsewhere [20,21]. Between 2000 and 2004, a population-based cross-sectional study of middle-aged women's health was conducted in the Baltimore metropolitan area and included 639 generally healthy women between the aged of 45 to 54 years. All women from the target population were invited by mail to participate in the study. A woman was eligible for study participation based on age (45–54 y) and on having intact ovaries and uteri. Reproductive stage was based on self-reported menstrual period history and categorized as pre- and perimenopausal as follows: premenopausal, last menstrual period within the past 3 months and no changes in bleeding or regularity in the past year; and perimenopausal, last menstrual period within the past year but not within the past 3 months or last menstrual period within the past 3 months and changes in either bleeding or regularity in the past year. Postmenopausal women were excluded from the study.

Women also were excluded if they were pregnant, were taking any exogenous hormones, or had a history of cancer. Eligible women were scheduled for a morning visit in the clinic after fasting overnight. At the clinic visit, participants were weighed, measured, and had their blood drawn for genotyping for selected SNPs. Additionally, study participants completed a detailed study questionnaire that included questions regarding their sleep quality, medical and family history, lifestyle habits, and reproductive history. All participants gave written informed consent according to procedures approved by the University of Illinois at Urbana-Champaign and Johns Hopkins University institutional review boards.

Participants were asked if “they experienced early awakening or insomnia (difficulty sleeping) on a regular basis (once a week or more) anytime during a month”. Dichotomous sleep outcomes examined in the analyses were experienced insomnia in the past year (yes or no) and experienced early awakening in the past year (yes or no). Age and ethnicity were self-reported. Smoking status (current, former, never) was based on participants' answers to the questions, “Have you ever smoked cigarettes?” and “Do you still smoke cigarettes?” Lastly, body mass index (BMI) was calculated based on height and weight measurements of the participant at the clinic visit and were categorized either as normal BMI (≤24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), or obese (≥30.0 kg/m^2).

2.2. Genotyping

Genomic DNA was isolated from whole blood using GenElute Blood Genomic DNA kits (Sigma, St. Louis, MO). DNA samples were genotyped for polymorphisms, including *CLOCK* (rs1801260; T/C), *AHR* (rs2066853; G/A), *ARNT* (rs2228099; G/C), and *AHRR* (rs2292596; C/G). DNA extracts were amplified by polymerase chain reactions as previously published [22,23]. Genotypes were determined based on amplicon size using agarose gel electrophoresis.

2.3. Statistical analyses

The associations between the selected genetic polymorphisms, categorical covariates, and sleep disturbance outcome variables were analyzed using χ^2 tests. Unadjusted odds ratios (ORs), covariate-adjusted ORs, and 95% confidence intervals (CI) for associations between genetic polymorphisms, or combination of polymorphisms, and sleep disturbance outcome variables were generated using logistic regression models. Age, race, BMI, and smoking

status were selected a priori as variables to adjust for, and thus were included in all logistic regression models. Specifically, age was included because studies indicate that the prevalence of sleep disturbances in women increases with age [2,4]. Race was included because it was previously reported to be associated with early awakening in middle-aged women [2]. Specifically, white women have been shown to have higher rates of early awakening compared to Hispanic women [2]. BMI was included because obesity was previously shown to be a risk factor for insomnia [24], and it was significantly associated with early awakening in our sample. Lastly, smoking status was added to the statistical model because our study includes selected SNPs in the AHR-signaling pathway, and it is well-known that compounds present in cigarette smoke can activate the AHR-signaling pathway [25]. Additionally, smoking status was significantly associated with insomnia and early awakening in our sample.

Prior to including age, race, BMI, and smoking status in the covariate-adjusted models, these variables were each examined as potential effect modifiers using stratified analyses. In all of the stratified analyses, the resulting ORs did not differ in strata of the examined variable (strata were age, 45–49 y and 50–54 y; race, white and black; BMI, <25kg/m^2, 25–29.9 kg/m^2, and >30 kg/m^2; smoking, ever and never). Therefore, none of the stratified analyses are presented. All statistical analyses were performed using SAS version 9.1 (Cary, NC). A *P* value of less than .05 was considered to be statistically significant.

3. Results

Characteristics of our study sample by sleep disturbance outcomes and genotype distributions are presented in Table 1. Women who experienced insomnia were more likely to be current smokers compared to women who did not experience insomnia. Similarly women who experienced early awakening were more likely to be former or current smokers and were less likely to be obese compared to women who did not experience early awakening. Age, race, and menopausal status distributions of women who experienced insomnia or early awakening were not significantly different than women who did not experience insomnia or early awakening.

We used the Hardy-Weinberg equation to estimate if the observed genotype frequencies in our study sample differed from the predicted genetic variation at equilibrium of the general population. Only the *ARNT* genotype distribution met the assumptions of the Hardy-Weinberg Equilibrium Theory (*P*=.54), while *AHR*, *AHRR*, and *CLOCK* did not meet these assumptions (*P*=.012, *P*=.0001, and *P*=.04, respectively). This finding indicates a stable frequency distribution of *ARNT* in our study compared to the predicted variation of the general population.

Women who experienced insomnia were less likely to carry the *AHRR*_G compared to women who did not experience insomnia. However, the genotype distribution of *AHR*, *ARNT*, and *CLOCK* did not differ in women with or without insomnia. Additionally, the percentages of women with genetic polymorphisms in *AHRR*, *AHR*, *ARNT*, and *CLOCK* were not significantly different between women who experienced early awakening and women who did not experience early awakening.

The unadjusted and covariate-adjusted associations between the selected polymorphisms and insomnia and early awakening are presented in Table 2. The selected SNP in *AHRR* was associated with insomnia after adjusting for age, BMI, race, and smoking status. Specifically women heterozygous for the *AHRR* alleles (C*G*) had lower odds of insomnia compared to women homozygous for the *AHRR*_C allele (adjusted odds ratio [aOR], 0.69; 95% CI,

0.49–0.96). SNPs in *AHR*, *ARNT*, and *CLOCK* were not associated with insomnia. None of the SNPs (i.e., *AHRR*, *AHR, ARNT*, or *CLOCK)* were associated with early awakening.

The associations between combinations of SNPs and insomnia or early awakening are presented in Table 3. The combined SNPs of *CLOCK* and *AHRR* were significantly associated with insomnia. Specifically, women heterozygous for one of the following alleles *CLOCK*_C or *AHRR*_G had lower odds of experiencing insomnia (aOR, 0.64; 95% CI, 0.43–0.96) compared to women homozygous for *CLOCK*_T and *AHRR*_C. Similarly women who carried both of the alleles of *CLOCK*_C and *AHRR*_G had decreased odds of experiencing insomnia (aOR, 0.56; 95% CI, 0.35–0.89) compared to women homozygous for *CLOCK*_T and *AHRR*_C. Additionally, the combined SNPs of *CLOCK* and *ARNT* were significantly associated with insomnia only in the crude analysis and were no longer significant after adjusting for the covariates. Women heterozygous for one of the *CLOCK*_C or *ARNT*_C alleles had decreased odds of insomnia compared to women homozygous for the *CLOCK*_T and *ARNT*_G alleles (OR, 0.62; 95% CI, 0.41–0.95). In contrast the combined SNPs of *CLOCK* and *AHR* had similar odds of insomnia for all genotypes. Further, none of the combined SNPs of *CLOCK* and *AHRR*, *ARNT*, or *AHR* were significantly associated with early awakening.

4. Discussion

Our study examined the relationship between genetic polymorphisms in the AHR-signaling pathway, the *CLOCK* gene, and 2 the common sleep disturbance outcomes, insomnia and early awakening. Findings from our study suggest a potential role of the AHR-signaling pathway in experiencing insomnia and early awakening during midlife. The AHR-signaling pathway may act alone or in combination with *CLOCK*.

In our study, the *AHRR* was associated with insomnia. At a cellular level, there is a relationship of regulator and activator between the AHRR and the AHR. Interestingly in our study, the *AHRR* was significantly associated with a decreased risk for insomnia. Because the AHRR plays a role as a repressor of the AHR by competitive binding with ARNT, it is possible that the AHRR acts on components in circadian rhythm such as BMAL1 that serve as a key regulator (along with PER1) in circadian rhythms. The BMAL1/CLOCK complex forms a positive element of the transcriptional-translational feedback loop that activates the expression of *PER* and *CRY* genes. The PER/CRY complex leads to the inhibition of BMAL1/CLOCK activity. We can only speculate that women heterozygous for the selected SNP in the *AHRR* have an altered feedback transmission to the PER/CRY leading to delayed inhibition of BMAL1/CLOCK activity.

Although the *CLOCK* gene plays a central role in circadian rhythm regulation, the selected SNP of *CLOCK* (rs1801260) was not associated with insomnia or early awakening. While previous studies have examined the association between *CLOCK* and sleep outcomes, they have not focused on insomnia or early awakening. Mishima et al [19] reported a significant association between the homozygosity of the *CLOCK*_C allele and shorter sleep time. In contrast, Robilliard et al [17] found no significant association between the selected *CLOCK* SNP and morning or evening preference. Currently, the biologic effects of this SNP on the functionality or stability of the genomic sequences are unknown. Thus, it is unclear why the selected *CLOCK* polymorphism is associated with some sleep outcomes (e.g., shorter sleep time) but not all (e.g., morning/evening preference, insomnia, early awakening). This association also may be that it interacts with factors that regulate sleep or sleep-related processes that were not fully explored in our study.

Further, it also may be that CLOCK interacts with other factors in the AHR-signaling pathway, as we observed in our study. Specifically our results suggest that the *CLOCK* polymorphism may affect sleep outcomes when combined with other SNPs, such as *AHRR* or *ARNT*. Importantly the combined SNPs of *CLOCK*_C and *AHRR*_G maintain the same statistical effect as the *AHRR* alone (i.e., decreased odds of insomnia). Hence, it is possible that the AHRR acts directly on CLOCK and not only on BMAL1. Future studies on other populations along with mechanistic studies should explore whether or not CLOCK interacts with or moderates the association between the AHR-signaling pathway and sleep disturbances despite having no independent main effect.

Overall, findings from our current study along with previous publications [10, 27, 28], are highly suggestive for an involvement of the AHR-signaling pathway in the regulation of circadian rhythmicity. Our study has several strengths; it is innovative in its research approach and the examined study questions. In addition we studied a sample of generally healthy women during their menopausal transition, a time in which many women start suffering from sleep disturbances. Despite these strengths, we were only able to examine a limited number of SNPs and 2 sleep outcomes. Hence, it is possible that the selected SNPs are significantly associated with other sleep disturbance outcomes (e.g., hypersomnia, sleep quality, daytime sleepiness). It also is possible that other SNPs in the genes that we investigated also may be associated with insomnia or early awakening. Lastly the sleep outcomes were subjective and did not include levels or degrees of sleep disturbance.

In conclusion our results suggest that the AHR-signaling pathway may act with known regulators in sleep-wake rhythms to alter the risk for insomnia. Further studies should investigate the potential roles of individual SNPs and the combinations of the selected SNPs to obtain a better understanding of their potential role as risk factors for sleep disturbances during the menopausal transition.

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Table 1

Sample characteristics.

	Insomnia		*P* value	Early awakening		*P* value
(n)	**No (344)**	**Yes (288)**		**No (330)**	**Yes (307)**	
Age n (%)			.44			.81
45–9	218 (53.3)	191 (46.7)		212 (51.5)	200 (48.5)	
50–54	126 (56.5)	97 (43.5)		118 (52.4)	107 (47.6)	
Race n (%)			.38			.56
White	282 (53.7)	243 (46.3)		280 (52.6)	252 (47.4)	
Black	54 (57.4)	40 (42.6)		44 (47.3)	49 (52.7)	
Other	8 (72.7)	3 (27.3)		6 (60.0)	4 (40.0)	
BMI n (%)			.06			.04
<25.0 kg/m^2	158 (58.1)	114 (41.9)		85 (45.0)	104 (55.0)	
25.0–29.9 kg/m^2	97 (56.7)	74 (43.3)		90 (51.7)	84 (48.3)	
≥30 kg/m^2	89 (47.3)	99 (52.7)		155 (56.8)	118 (43.2)	
Smoking status n (%)			.04			.04
Never	189 (57.6)	139 (42.4)		186 (56.0)	146 (44.0)	
Former	132 (53.9)	113 (46.1)		120 (48.8)	126 (51.2)	
Current	23 (39.7)	35 (60.3)		23 (39.7)	35 (60.3)	
Menopausal status n (%)			.7			.2
Premenopause	133 (55.9)	105 (44.1)		133 (55.6)	106 (44.4)	
Perimenopause	207 (54.2)	175 (45.8)		194 (50.3)	192 (49.7)	
AHRR n (%)			.03			.37
CC	137 (51.3)	130 (48.7)		140 (52.6)	126 (47.4)	
CG	197 (58.5)	140 (41.5)		180 (52.5)	163 (47.5)	
GG	9 (34.6)	17 (65.4)		10 (38.5)	16 (61.5)	
AHR n (%)			.20			.12
GG	238 (55.2)	193 (44.8)		237 (54.7)	196 (45.3)	
GA	93 (55.4)	75 (44.6)		79 (45.9)	93 (54.1)	
AA	12 (38.7)	19 (61.3)		14 (46.7)	16 (53.3)	
ARNT n (%)			.73			.96
GG	108 (52.7)	97 (47.3)		108 (51.4)	102 (48.6)	
CG	173 (54.6)	144 (45.4)		167 (52.5)	151 (47.5)	
CC	62 (57.4)	46 (42.6)		55 (51.4)	52 (48.6)	
CLOCK n (%)			.32			.17
TT	191 (51.9)	177 (48.1)		192 (52.0)	177 (48.0)	
TC	139 (58.2)	100 (41.8)		130 (53.5)	113 (46.5)	
CC	13 (54.2)	11 (45.8)		8 (33.3)	16 (66.7)	

Abbreviations: BMI, body mass index; *AHHR*, aryl hydrocarbon receptor repressor; *ARNT*, aryl hydrocarbon receptor nuclear translocator; *CLOCK*, circadian locomotor output cycles kaput.

Table 2

Associations between selected single nucleotide polymorphisms and sleep outcomes.

	Insomnia				Early awakening			
	Unadjusted model		Adjusted model		Unadjusted model		Adjusted model	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
AHRR								
CC	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
CG	0.75	0.54–1.04	0.69	0.49–0.96	1.01	0.73–1.39	1.02	0.73–1.43
GG	1.99	0.86–4.62	1.63	0.68–3.88	1.78	0.78–4.06	1.70	0.73–3.96
AHR								
GG	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
GA	0.99	0.70–1.42	1.06	0.72–1.57	1.42	1.00–2.03	1.36	0.93–2.01
AA	1.95	0.93–4.12	2.19	0.99–4.85	1.38	0.66–2.90	1.32	0.61–2.85
ARNT								
GG	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
CG	0.93	0.65–1.32	0.96	0.67–1.37	0.96	0.68–1.36	0.97	0.68–1.38
CC	0.83	0.52–1.32	0.85	0.53–1.38	1.00	0.63–1.60	0.97	0.61–1.56
CLOCK								
TT	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
TC	0.78	0.56–1.08	0.75	0.54–1.06	0.94	0.68–1.30	0.96	0.69–1.34
CC	0.91	0.40–2.09	0.83	0.35–1.94	2.17	0.91–5.19	2.20	0.91–5.33

Abbreviations: OR, odds ratio; CI, confidence interval; *AHHR*, aryl hydrocarbon receptor repressor; *AHR*, aryl hydrocarbon receptor; *ARNT*, aryl hydrocarbon receptor nuclear translocator; *CLOCK*, circadian locomotor output cycles kaput.

Adjusted OR is adjusted for age (<50/50+ y), race, body mass index (≥30, 25–29, <25), and smoking status (never, former, current).

Table 3

Associations between combinations of the selected single nucleotide polymorphisms and sleep outcomes.

	Insomnia				Early awakening			
	Unadjusted model		Adjusted model		Unadjusted model		Adjusted model	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
CLOCK and *AHRR*								
CLOCK _TT+ *AHRR*_CC	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
Carriers of *CLOCK_T* or *AHRR_G*	0.71	0.49–1.04	0.64	0.43–0.96	0.97	0.66–1.42	0.99	0.67–1.47
Carriers of *CLOCK_T* and *AHRR_G*	0.64	0.41–1.01	0.56	0.35–0.89	1.07	0.69–1.66	1.10	0.69–1.74
CLOCK and *ARNT*								
CLOCK _TT+ *ARNT*_GG	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
Carriers of *CLOCK_T* or *ARNT_C*	0.62	0.41–0.95	0.66	0.43–1.01	0.88	0.58–1.33	0.92	0.60–1.41
Carriers of CLOCK_T and ARNT_C	0.67	0.42–1.07	0.67	0.42–1.09	0.97	0.61–1.53	0.99	0.62–1.59
CLOCK and *AHR*								
CLOCK _TT+ *AHR*_GG	1.00	Referent	1.00	Referent	1.00	Referent	1.00	Referent
Carriers of *CLOCK_T* or *AHR_G*	0.90	0.64–1.26	0.95	0.67–1.34	1.36	0.97–1.91	1.38	0.98–1.96
Carriers of *CLOCK_T* and *AHR_A*	0.87	0.51–1.47	0.82	0.47–1.43	1.31	0.78–2.19	1.20	0.70–2.05

Abbreviations: OR, odds ratio; CI, confidence interval; *CLOCK*, circadian locomotor output cycles kaput; *AHHR*, aryl hydrocarbon receptor repressor; *ARNT*, aryl hydrocarbon receptor nuclear translocator; *AHR*, aryl hydrocarbon receptor.

Adjusted OR is adjusted for age (<50/50+ y), race, body mass index (≥30, 25–29, <25), and smoking status (never, former, current).