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Research

Investigation of the Relationship Between Methylation of Circadian Rhythm Genes and Menopause

Sirkadiyen Ritm Genlerinin Metilasyonu ile Menopoz Arasındaki İlişkinin İncelenmesi

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ABSTRACT

Objective: The circadian system acts at whole levels of a woman's life as a cornerstone: from follicle generation to the arrangement of hormonal balance; and the process from embryo implantation to birth.

Methods: We compared the promoter methylation status of the circadian genes, *BMAL1* and *CLOCK*, between pre-menopausal and post-menopausal women to find an epigenetic explanation in women with menopause. In this perspective, 56 postmenopausal women and 48 premenopausal women were enrolled in this study.

Results: Menopause and methylation status of the *BMAL1* or *CLOCK* genes did not show any statistically significant correlations (p>0.05). Moreover, the correlation of the methylation pattern of the *BMAL1* and *CLOCK* genes with age could not be detected (p>0.05).

Conclusion: The methylation status of the *BMAL1* and *CLOCK* genes in menopause was characterized for the first time in our study. Further studies should shed light on this subject.

Keywords: Menopause, BMAL1, CLOCK, MS-HRM, methylation

ÖZ

Amaç: Sirkadiyen sistem, bir kadının yaşamının tüm seviyelerinde bir mihenk taşı olarak hareket etmektedir: Folikül oluşumundan hormonal dengenin düzenlenmesine kadar; ve embriyo implantasyonundan doğuma kadar olan süreçlerde aktif olmaktadır.

Gereç ve Yöntem: Sirkadiyen ritm genleri olan *BMAL1* ve *CLOCK*'nin promotör metilasyon durumunu menopoz öncesi ve menopoz sonrası kadınlar arasında karşılaştırılmıştır. Bu amaç ile 56 postmenopozal kadın ve 48 premenopozal kadın bu çalışmaya dahil edilmiştir. **Bulgular:** *BMAL1* ve *CLOCK* genlerinin DNA metilasyonu değerlendirilmiştir. *BMAL1* veya *CLOCK* genlerinin menopoz ve metilasyon durumu arasında istatistiksel olarak anlamlı bir ilişki saptanmamıştır (p>0,05). Ayrıca *BMAL1* ve *CLOCK* genlerinin metilasyon paterni ile yaş arasında da istatistiksel olarak anlamlı bir ilişki saptanmamıştır (p>0,05).

Sonuç: Literatürde postmenopozal dönemde *BMAL1* ve *CLOCK* genlerinin metilasyon durumu ilk kez çalışmamızda araştırılmış olup çalışmamız gelecekteki çalışmalara ışık tutacaktır.

Anahtar Kelimeler: Menopoz, BMAL1, CLOCK, MS-HRM, metilasyon

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INTRODUCTION

The menstruation cycle is a monthly-based rhythm and menopausal transition is an important factor that alters this rhythm. Menopausal condition is described as a lasting cessation of menses as a consequence of deficiency of ovarian follicular activity (1). The consequences of menopause include physical and psychological disturbances, hot flashes, jitters, depression, sleeplessness, and tiredness (2). Although, with the other problems, sleep problems also start during the postmenopausal term (3). Generally, the time of menopause may be affected by irregularities in the gonadal function due to the desynchronization of the signal of environmental circadian signs (4). The circadian rhythm is defined as all of the biological processing which is monitored between 24 h, and the rhythms that occur within 24 h are established by the circadian clock (5). The circadian system acts in whole levels of a woman's life as a cornerstone: from follicle generation to the arrangement of hormonal balance; and the process from embryo implantation to birth (6). In the transcriptional translation feedback loop of circadian rhythms, *BMAL1* (basic helix-loop-helix ARNT like 1) and *CLOCK* (Circadian locomotor output cycles protein kaput) genes play an important roles (7). In this study, we analyzed the DNA methylation level of the *BMAL1* and *CLOCK* genes in postmenopausal subjects.

METHODS

Subjects Characteristics

A total of 56 postmenopausal and 48 premenopausal women were enrolled in this study. To participate in the study, women should have been in the post-menopause phase for at least a year are requested. However, women in the period of menopause unnaturally, women on antidepressants, antianxiety, or exogenic hormones, or women with mental retardation or serious illnesses were excluded from the study. The research Scientific Research Ethics Committee of the Near East University approved this study protocol and conducted it according with the Declaration of Helsinki (no: YDU/2021/93-1830, date: 29.07.2021).

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Epigenetic Analyses

Blood samples were collected from menopause and control cases. We extracted DNA from all subjects using the Qiagen AllPrep DNA/RNA/Protein isolation kit (Qiagen, Manchester, UK). We measured the amount of DNA using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Sodium bisulfite treatment was performed using an Epitect Bisulfite Modification kit (Qiagen, Manchester, UK). The sequences of primers for the promotor regions were designed according to the EpiTect® HRM™ PCR Handbook (Qiagen, Manchester, UK). The BMAL1 and CLOCK promotor methylation were analyzed according to EpiTect® HRM™ PCR Handbook protocol (Rotor-Gene Q, Qiagen).

Statistical Analysis

The chi-square test and two-tailed Fisher's exact test were used for statistical analysis of participant characteristics and their relationships with statistical tests. Calculations were conducted using SPSS 15.0 software (SPSS, Chicago, IL, USA) with a statistical significance of $p<0.05$.

RESULTS

The mean age of 48 participants who are in the stage of premenopause was 33.4±6.8, and the mean age of 56 participants who are in the stage of menopause was 56.6±4.8.

DNA Promoter Methylation Status of BMAL1, CLOCK in Menopause and Non-menopause Subjects

The *BMAL1* gene promoter was methylated in 34 of 56 subjects in post-menopause (60.07%) and 22 of the 44 non-menopause subjects (50.0%). There was no statistically significant difference between methylation status and menopausal condition identified ($p>0.05$) (Table 1).

The *CLOCK* gene promoter was methylated in 22 of the 54 menopause subjects (40.7%) and 20 out of the 48 non-menopause subjects (41.7%). There was no significant difference between methylation status and menopausal conditions ($p>0.05$) (Table 2).

DISCUSSION

CLOCK and BMAL1 form a heterodimer and lead to the activation of transcription of *PER* and *CRY* genes, which create a heterodimer structure in the cytoplasm and then turn back into the nucleus to being able to repress their transcription activity by suppressing the CLOCK-BMAL1 complex. This process occurs in nearly 24 h (7).

The *CLOCK* gene is located on chromosome 4q12. The single-nucleotide polymorphisms (SNPs) in the *CLOCK* gene are associated with sleep diminution, the concentration of adipocytokine, body mass index, and uptake of energy (8). The location of the *BMAL1* gene is on chromosome 11p15.3. The SNPs of the *BMAL1* gene are responsible for the occurrence of high blood pressure, diabetes mellitus,

Table 1. Methylation status of the *BMAL1* gene in pre-menopause and post-menopause subjects

BMAL1		Menopause status			
		Pre-menopause	Post-menopause	Total	p-value
Unmethylated	Observed	22	22	44	p>0.05
	% within column	50.0%	39.3%	44.0%	
Methylated	Observed	22	34	56	
	% within column	50.0%	60.7%	56.0%	
Total	Observed	44	56	100	
	% within column	100.0%	100.0%	100.0%	

Table 2. Methylation status of the *CLOCK* gene in pre-menopause and post-menopause subjects

CLOCK		Menopause status			
		Pre-menopause	Post-menopause	Total	p-value
Unmethylated	Observed	28	32	60	p>0.05
	% within column	58.3%	59.3%	58.8%	
Methylated	Observed	20	22	42	
	% within column	41.7%	40.7%	41.2%	
Total	Observed	48	54	102	
	% within column	100.0%	100.0%	100.0%	

and metabolic diseases, which lead to a raised risk of myocardial infarction (9).

The function of methylation of DNA in gene regulation has been supported by different researchers. DNA methylation is the most known epigenetic mechanism which is related to gene expression (10). The involvement of DNA methylation in imprinting disorders and cancer has been demonstrated by several studies (11). Furthermore, according to recent studies, DNA methylation take a part in autoimmune disorders, metabolic diseases, psychological diseases, obesity and aging (12).

Epigenetic biomarkers of aging based on methylation levels have been reported in various articles, illustrating the reflectance of chronological age on DNA methylation levels. Levine et al. (3) concluded that the age of menopause was substantially related to epigenetic age acceleration. In other words, earlier menopause correlates with raised epigenetic age due to reason of higher level of DNA methylation rate than the expected rate (3). The relationship between the age of menopause and night shift workers was investigated by Stock et al. (4) night worker women are at higher risk of menopause at an earlier age was concluded by Stock et al. (4). The risk of menopause at an earlier age is more clear for females younger than 45 years old since they face exposure to night shift work at the current time and during their lives. A higher risk of menopause among night workers cannot be supported, as the circadian rhythm deficiency has a suppressing effect on ovulation due to the disturbed circadian rhythm (4).

Circadian rhythms are controlled by the *BMAL1*, *CLOCK*, *PER*, and *CRY* genes (7). The circadian clock system is regulated by both epigenetic and genetic factors (13). Due to the circadian rhythm's role in metabolism and physiological mechanisms, diseases such as cancer and metabolic syndrome can be detected when the system is compromised (5). The polymorphisms of the *BMAL1* and *CLOCK* genes and their actions on menopause have been analyzed by various researchers. Semenova et al. (14) analyzed (CLOCK) 3111T/C gene polymorphism in the participants who were in the stage menopause. They did not find any differences in CLOCK 3111T/C genotypes or allele frequency between the control group and the main group (14).

With age, the circadian clock-controlled genes that act in the regulation of the circadian system lose their responsiveness. Consequently, females in the menopausal transition stage experience impairment in homeostasis and this is exacerbated by the hormonal imbalance. Hernandez-Morante et al. (15) investigated the expression of circadian genes in adipose tissue and their relationship with circadian gene expression in metabolic syndrome. They found that, in the subcutaneous adipose tissue, the PER3 expression level

of women who are in menopause is 42% higher than their counterparts who are in the premenopausal stage (15).

DNA methylation is the most commonly studied epigenetic process and represents a potential biomarker of future health outcomes. However, to date, there have no DNA methylation studies in this field. Our study is the first study in which the association of the methylation status of the *BMAL1* and *CLOCK* genes in menopause was investigated.

In our study, the methylation status of the *CLOCK* and *BMAL1* genes in both pre- and postmenopausal women was identified. We detected the BMAL1 promoter methylation in 50.0% of premenopausal women, and 60.7% of postmenopausal women. *BMAL1* gene was unmethylated in 50.0% of premenopausal subjects, and 39.3% of postmenopausal subjects. We determined the CLOCK promoter methylation in 41.7% of women in the period of premenopause, and 40.7% of women in the period of postmenopause. However, the unmethylation of the *CLOCK* gene was detected in 58.3% of control samples, and 59.3% of menopause samples. Both the genes were unmethylated in 63.6% of participants, and methylated in 45.8% of participants. The *BMAL1* gene was unmethylated, whereas the *CLOCK* gene was methylated in 36.4% of subjects. However, the *BMAL1* gene was methylated and the *CLOCK* gene was unmethylated in 54.2% of subjects. The correlation between the methylation pattern of the analyzed two genes and menopause, and an important relationship between the methylation pattern of the analyzed two genes could not be found statistically significant ($p>0.05$).

CONCLUSION

Studies have demonstrated SNPs of circadian rhythm genes and their importance during menopause. There is no DNA methylation studies have been performed in this field. From this perspective, our study will shed light and provide critical information to further epigenetic studies.

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ETHICS

Ethics Committee Approval: The research Scientific Research Ethics Committee of the Near East University approved this study protocol and conducted it according with the Declaration of Helsinki (no: YDU/2021/93-1830, date: 29.07.2021).

Informed Consent: A written informed consent form was obtained from each subject.

Authorship Contributions

Surgical and Medical Practices: R.K., G.K., Concept: R.K., Design: R.K., Data Collection or Processing: R.K., G.K., Analysis or Interpretation: G.K., Ö.T., Literature Search: R.K., G.K., Writing: R.K., G.K., Ö.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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