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Promoter methylation analysis of seven clock genes in Parkinson's disease

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ABSTRACT

The expression of clock genes is altered in leukocytes from patients with Parkinson's disease (PD). However, the underlying mechanisms are unknown. To determine whether abnormal CpG methylation contributes to the dysregulated expression of these genes, the methylation status of the promoters of seven major human clock genes, PER1, PER2, CRY1, CRY2, Clock, NPAS2, and BMAL1, was examined using methylation-specific PCR (MSP) and sequencing in 206 PD patients and 181 healthy controls. This analysis revealed that most clock gene promoters were devoid of methylation. Methylation was only detectable in the CRY1 and NPAS2 promoters. Interestingly, the methylation frequency of the NPAS2 promoter was significantly decreased in PD patients. These results suggest that altered promoter methylation may contribute to the abnormal expression of clock genes in PD.

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the elderly, affecting around 2% of the population over 60 in China [24]. A variety of disruptions in different circadian rhythms, at both the behavioral and physiological levels, have been reported in patients with PD [4,23]. Parkinsonian symptoms undergo a circadian fluctuation, with patients often experiencing a worsening of motor symptoms in the afternoon and evening [17]. In addition, circadian rhythms in body temperature, blood pressure, and cortisol synthesis are also affected in PD [4,23]. Furthermore, most patients suffer from depression and sleep disorder, both of which are modulated by circadian cycles [18].

Circadian rhythms are controlled by clock genes. Several clock genes have been identified, including *period* (*PER1*, *PER2*, and *PER3*), *cryptochrome* (*CRY1* and *CRY2*), *CLOCK*, *aryl hydrocarbon receptor nuclear translocator-like* (*ARNTL1*, also called *BMAL1*), and *neuronal PAS domain protein 2* (*NPAS2*) [8]. These clock genes interact with each other at the transcriptional, translational, and post-translational levels, and form complex feedback loops to generate and sustain molecular, biological, and physiological circadian rhythms. It is believed that disruptions of circadian rhythms in PD are related to the abnormal expression of clock genes. Indeed, it was recently reported that the expression of clock genes is altered in both PD patients and in animal models of the disease [6,9,12]. However, the mechanisms underlying these changes are unknown.

DNA methylation is a chemical modification of the DNA structure that does not affect the genomic sequence and is an important epigenetic regulator of gene expression [11]. It has recently been reported that the methylation of clock genes is more prevalent in Dementia with Lewy Bodies (DLB), a disorder closely related to PD [16]. In addition, altered CpG methylation has also been associated with another neurodegenerative disorder, Alzheimer's disease [22]. Therefore, we hypothesized that abnormal CpG methylation of clock gene promoters might be responsible for the dysregulated expression of these genes observed in PD. In the present study, we analyzed the methylation status of the promoter regions of seven major clock genes using methylation-specific PCR (MSP) and sequencing to investigate the association between promoter methylation and PD.

PD cases used in this study were identified from the PD cohort of the Chinese National Consortium on Neurodegenerative Diseases (CNCPD, www.chinapd.cn), established by the Chinese Parkinson Study Group (CPSG), a collaboration of 42 clinical centers managed by the coordination center at Xuanwu Hospital of Capital Medical University in Beijing. PD was diagnosed by movement disorder specialists using the United Kingdom PD Society Brain Bank Criteria

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Table 1
The first set of primers used for methylation analysis of the promoters of seven clock genes.

Gene Name	Primer	Sequence	Amplicon (bp)
Per1	Mf	ATTTAGGTTTACGTGCGTTC	298
	Mr	CGACTCAAAAACGAAAATCG	
	Uf	TAGTATTAGTATTTAGGTTTATGTGTGTTT	318
	Ur	AACAACAATCCAACTCAAAAACAAAAATCA	
Per2	Mf	GCGGTTTCGTTGCGGTTTAC	140
	Mr	GCCGACGCCGTTTCAAACCG	
	Uf	GTGGTGTGGTGTGGTTTTGTTGTGGTTTAT	160
	Ur	ACACCCCCACACCAACACCATTTCAAACCA	
Cry1	Mf	TCGTTTTGTTTTTAGGGGTC	166
	Mr	GCAACCGCCTAAAAACGACG	
	Uf	ATTTTGGGTGGGTTGTTTTGTTTTTAGGGGTT	187
	Ur	ACACCCACCACAACCACCTAAAAACAACA	
Cry2	Mf	GTTTATTTTCGGTATTTCGC	149
	Mr	TAACGATTAACCCAAAAACG	
	Uf	TTGAGATTTGGTTTATTTTTGGTATTTTGT	170
	Ur	TACCTTCCACCTAACAATTAACCCAAAAACA	
Clock	Mf	GTTTTTTATTCGATTAGGTTTC	168
	Mr	CGTTAAACAACACGAAACCG	
	Uf	GTTGGTTGGTTGTGTTTTTTATTTGATTAGGTTTT	188
	Ur	CAACTTACCCCATTAAACAACACAAAACCA	
NPAS2	Mf	GGTTTAGTTCGCGTTCGGTTTC	140
	Mr	CCACGCTAACGAACAAATAACCG	
	Uf	TGTGTTTTTTTGGTTTAGTTTGTGTTTGGTTTTG	158
	Ur	ACATCCTCCACACTAACAAACAAATAACCA	
Bmal1	Mf	GGAGGTGTTTGTTTATTCGC	138
	Mr	AAATACGCGAAATCGCGTCG	
	Uf	TAGGTTAGGGATGGAGGTGTTTGTTTATTTGT	161
	Ur	AACCCCCAACAAAATACACAAAATCACATCA	

Table 2
The second set of primers used for methylation analysis of the promoters of seven clock genes.

Gene name	Primer	Sequence	Amplicon (bp)
Per1	Mf	TTTTATAGTCGATTTTTAGCGGGTATGTTTC	190
	Mr	CAACGCTAAAAACGAAATATTCTAATCCG	
	Uf	GGTTTTATAGTTGATTTTTAGTGGGTATGTTTTG	197
	Ur	AAAACCAACACTAAAAACAAAATATTCTAATCCA	
Per2	Mf	GGGGCGCGTTTTTGGTC	113
	Mr	GAAAAAACTAAACGCGAAACTCCTACG	
	Uf	GTTGGGGGTGTGTTTTTGGTTG	124
	Ur	TACAAACAAAAAAACTAAACACAAAACTCCTACA	
Cry1	Mf	GCGTTTTTGTTCGTCGGTTTC	116
	Mr	CCGAATAAACACGAAAATACGAAAAACTTACG	
	Uf	AATTGTTGAGTGTGTTTTTGTTTGTTGGTTTTG	129
	Ur	ACCCAAATAAACACAAAAATACAAAAAACTTACA	
Cry2	Mf	GTTCGGTACGGATAGCGTTTTTTC	161
	Mr	GATTAATCCCGACTAAAAAAAAAAACCGCG	
	Uf	GGTGTTAGTGTTTGGTATGGATAGTGTTTTTTTG	175
	Ur	CCATCAATTAATCCCAACTAAAAAAAAAACCACA	
Clock	Mf	GTTTTCGTCGGTCGTTTGAGTTC	81
	Mr	CGCAAACCGAATCCGTAATTACG	
	Uf	GAGTGTAGAGTTTTTGTTGGTTGTTTGAGTTTG	100
	Ur	AACCAACAACCACAAACCAAATCCATAATTACA	
NPAS2	Mf	TTCGTTTTTTATTTGTTTTCGGGTCGTATTTTC	173
	Mr	CGCGCAAAAAATAAAAAAAAACGAACACG	
	Uf	TTTTGTTTTTTATTTGTTTTTGGGTTGTATTTTTG	179
	Ur	TATCCCACACAAAAAATAAAAAAAAACAAACACA	
Bmal1	Mf	TTGTCGGTTTTAAAGGGTTCGTGATC	111
	Mr	AACCCCCGCGAAAAAAATAAAAACG	
	Uf	TGTTTGTTGTTGGTTTTAAAGGGTTTGTGATTG	125
	Ur	ACAACAACAACCCCCACAAAAAAAATAAAAACA	

[14]. Individuals with a family history of PD in a first- or second-degree relative or individuals with onset before the age of 50 were not included. Control subjects were selected from the community cohorts. Individuals with dementia, or with a family history of PD in a first- or second-degree relative were excluded. For PD patients, disease severity was evaluated using the United Parkinson's Disease Rating Scale (UPDRS) [10], sleep quality was measured by the Pittsburgh Sleep Quality Index (PSQI) [5]. Informed consent was obtained from all patients and controls. This study was approved by the Xuanwu Hospital Ethics Committee, and was in compliance with national legislation and the Code of the World Medical Association.

Seven key clock genes, including *PER1*, *PER2*, *CRY1*, *CRY2*, *CLOCK*, *NPAS2*, and *BMAL1* were selected for methylation analysis. Promoters for these clock genes were analyzed with online software (http://www.mspprimer.org/cgi-mspprimer/design.cgi) [2]. All of these promoters contain CpG island(s). Summary of promoter regions subject to MSP examination were provided in supplementary Fig. S1. Because methylation-specific PCR detected only three to four possible CpG sites located at the 3′ end of the primers, potentially methylated CpG sites located outside the primer region and between the primer sequences could not be detected and may have led to false negative results. To address this issue, two different pairs of primers were used for each gene in order to amplify two distinct regions from the same promoter. The first set of primers for *PER1*, *PER2*, *CRY1*, *CRY2*, *CLOCK*, and *BMAL1* have been described previously [21]. The other primers used for MSP on each clock promoter were designed with the aforementioned software. The sequences of the first and second set of primers are shown in Tables 1 and 2, respectively.

Genomic DNA was prepared using the QIAamp DNA mini kit (Qiagen, CA, USA), and sodium bisulfite treatment was performed as described [7]. Bisulfite-treated DNA was amplified with HS Taq (Takara, Japan). PCR products were then loaded and electrophoresed on 2% agarose gels and stained with ethidium bromide. To ensure specificity of clock promoter methylation, genomic DNA treated with SssI, a CpG methylase (New England Biolabs, UK), was used as a positive control. In order to confirm the results of methylation-specific and regular PCR reactions, the products were purified and sequenced by Beijing Genomics Institute (Beijing, PR China).

Methylation frequencies of the various clock genes in PD patients and controls were compared and analyzed by Fisher's exact test. In considering comparison of seven clock genes in total, using two sets of primers for each, we used Bonferroni correction for multiple comparison to set the α value. Significance levels were established at a value of $P < 0.00357$. Overall, 206 PD patients and 181 controls were recruited for this study. The two groups were matched for age and gender. The clinical features of the patients and controls are summarized in Table 3.

As shown in Table 4, the *PER1*, *PER2*, *CRY2*, and *CLOCK* promoters were free of methylation in both PD patients and healthy controls. Methylation frequencies were also very low for *BMAL1*; only 2 of 206 PD patients and 1 of 181 healthy controls exhibited partial methylation. Detectable methylation was present in *CRY1* and *NPAS2* (Figs. 1 and 2). 14 of 206 PD patients and 17 of 181 healthy controls had partial methylation of the *CRY1* promoter. 9 of 206 PD patients and 31 of 181 healthy controls had partial methylation of the *NPAS2* promoter. Of note, the methylation frequency of the *NPAS2* promoter was significantly different between PD patients and healthy controls ($P < 4.04 \times 10^{-5}$). Age, age at onset, UPDRS and PSQI score in PD patients with and without NPAS2

Table 3
Clinical summary of patients with PD and controls.

Characteristics	PD patients	Controls
No. of subjects	206	181
Male/female	122/84	103/78
Mean age (±SD) at sampling (years)	66.33 ± 3.36	66.08 ± 3.42
Mean age (±SD) at onset (years)	62.36 ± 4.72	–