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A pilot study for circadian gene disturbance in dementia patients

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Abstract

Disturbance of circadian gene regulation might contribute to behavioral and psychological symptoms in dementia patients. This study was to evaluate the CpG island methylation status on the circadian gene promoters in dementia patients. We conducted a set of methylation specific polymerase chain reaction (mPCR) followed by nucleotide sequencing to analyze the methylation status within the promoters of nine circadian-related genes, including *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *TIM* and *CK1ε*, in the genomic DNA from the peripheral blood leukocytes of 80 dementia patients and 80 age- and gender-matched controls. A total of seven dementia patients (7/80) had CpG island methylation in the circadian genes and none of the controls had methylation. There were three and four patients had CpG island methylation on the promoters of *PER1* and *CRY1*, respectively. Dementia with Lewy body (DLB) patients had the significantly highest frequency of circadian gene CpG island methylation (35.7%). It suggested that epigenetic methylation of circadian gene was more prevalent in dementia patients, especially for the DLB patients. The significance of circadian gene methylation in clinical behavior/sleep disturbance in dementia patients needs further study.

Keywords: Circadian rhythm; Methylation; Epigenetic; Dementia with Lewy body; Alzheimer's disease

Dementia is a group of heterogeneous neurodegenerative diseases. It mainly consists of Alzheimer's disease (AD), dementia with Lewy body (DLB), frontotemporal lobe dementia (FTD) and others, such as vascular dementia [33]. In addition to cognitive deficits, there are many behavioral and psychological symptoms in dementia (BPSD), including sleep disturbance, delusions, hallucinations and depression, etc. [15]. Sleep problem, a common BPSD symptom, usually causes a heavy burden for care-givers of dementia patients [6,7]. Additionally, the activities of dementia patients could be markedly disarranged in day–night cycle [20]. Sun-downing phenomenon, which presents as confusion and agitation in the early night frequently occurs in the dementia patients [18,40].

Many physiological functions and behaviors are expressed rhythmically according to the circadian rhythm, which is mainly entrained by light [5,34]. The circadian rhythm regulates many physiological presentations in human, including sleep and wakefulness, body temperature, blood pressure, hormone production, digestive secretion and immune responses [31]. Sleep/wake cycle is the mostly obvious outward presentation of circadian rhythm behavior. The circadian clock is executed by a group of self-sustained biological oscillators, which is located in suprachiasmatic nucleus (SCN) of the anterior hypothalamus [42]. Circadian rhythms similar to those operating in the SCN have been found in peripheral tissues, and the peripheral circadian gene expression is slaved by the central clock in the SCN [4]. Molecular components of the circadian oscillator have been characterized to be a transcriptional–translational feedback loop consisting of at least nine genes, including *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *TIM* and *CK1ε* [1]. Of these, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2* and *TIM* act as negative regulators, whereas *BMAL1* and *CLOCK* are positive regulators. *CK1ε* binds and phosphorylates *PER* proteins, and regulates their stability post-transcriptionally. Disturbance of these genes would result in profound influence on circadian rhythms [9,19]. Studies using genetically mutant animals have revealed that dis-

Abbreviations: DLB, dementia with Lewy body; AD, Alzheimer's disease; Others, dementia of other causes.

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turbance of the circadian gene expression resulting in both sleep problems and behavioral pattern changes [12,13].

DNA methylation is a chemical modification on DNA structure without changes of the genomic sequence and is an important epigenetic regulation mechanism in gene expression. DNA is methylated mostly on the CpG dinucleotide, in terms of CpG island [21]. CpG methylation on the promoter of specific genes can interfere with the interaction between the transcription factors and DNA, then lead to inhibition of gene expression [3]. CpG island methylation plays important roles in many physiological changes, such as X-chromosome inactivation and aging [32,38]. Although the impacts of CpG island methylation in dementia remains elusive, CpG methylation status in the relevant genes might be altered and these alterations might be associated with some clinical manifestations. It has been shown that amyloid precursor protein gene is hypomethylated in AD patients [41].

Therefore, we hypothesized that the abnormal behaviors observed in dementia patients were in part derived from dysregulation of circadian gene expression and abnormal CpG methylation on the promoter of the circadian genes might be the cause of this gene expression dysregulation. In this study, we used methylation specific PCR (mPCR) and sequencing methods to analyze the methylation status on the promoter of nine circadian-related genes to explore the association between the CpG methylation of circadian gene promoters and dementia.

We selected 80 dementia patients randomly from the Geriatric Neuropsychiatry DNA Bank of Taipei City Psychiatric Center (TCPC), Taipei City Hospital. The genomic DNA was extracted from the peripheral leukocytes of venous blood as standard procedure. The dementia patients were all sampled when they were admitted to the geriatric neuropsychiataric ward of TCPC for treating their BPSD problems. The clinical variable of total sleeping time was recorded daily by ward nurse. The BPSD was assessed by Monitor Sheet for Psychiatric Inpatient (MSPI) [23]. The MSPI was a 20 items clinical rating scales routinely used by nursing staff in TCPC for evaluation the psychiatric patients. The 20 items include irritability, agitation, aggression, hyperactivity, unusual aberrant behaviors, self-harm, withdrawal, taciturn, hypertalktive, uncooperation, hallucination, delusion, unusual thought content, incoherence, orientation, sleep disturbance, oral intake, hypoactivity, hypersexuality and motor disability. All the dementia patients had undergone laboratorial tests to search for other dementia causes, including complete blood count, folic acid, vitamin B12, VDRL, thyroid function test and CT of brain. All the dementia patients were mild to moderate cases in terms of scoring 10–26 in Mini-Mental State Examination [16] or 1–2 in Clinical Dementia Rating [22,29]. The diagnosis of dementia subtype was done at the time of discharge. The AD diagnosis was according to the criteria of the National Institute of Neurological and Communicative Disorders and Strokes (NINCDS), and the Alzheimer's Disease and Related Disorders Association (ADRDA), for probable and possible AD [28]. The DLB diagnosis was according to the Consensus Criteria for the Clinical Diagnosis of possible or probable DLB [26,27]. Familial dementia was excluded through information on family history. Another 80 healthy controls were age- and gender-matched with dementia patients. They were subjects who received routine health examinations at Taipei City Hospital. Each control subject was given clinical, mental and neurological examinations, and none of them showed any cognitive defects or sleep disorders. This study has been approved by the institutional review board of Taipei City Hospital. The informed consents were obtained from the controls themselves. For the dementia patients, written informed consents were obtained by patients themselves in the presence of their family or from the patients' family if the patients cannot write efficiently.

Genomic DNA was modified with sodium bisulfite and mPCRs were performed as previously described [11]. The primers used for mPCRs of the promoters the nine circadian genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *CKIε* and *TIM*) were as described [36]. CpG methylase (SssI) (NEB, Ipswich, MA)-treated genomic DNA was used as the template of a positive control for the mPCR and unmodified genomic DNA was as a negative control. In order to confirm the results of the methylation and unmethylation specific PCRs, the PCR products were subject to direct sequencing by ABI Prism 310 Genetic Analyzer and the Big Dye Terminator Cycle sequencing kit (Applied Biosystem). The procedures were according to the manufacturer's protocol.

The frequencies of circadian gene promoter methylation in DLB, AD, dementia due to other causes were compared and analyzed by Fisher's exact test. In considering comparison of nine circadian-related genes in total, we used Bonferroni correction for multiple comparison to set the α value to be 0.00556. Significance levels were established at a value of $P < 0.0056$.

Fifty nine AD, 14 DLB and 7 dementia of other causes were randomly selected for this study. The patients of dementia of other causes included two vascular dementia, one Parkinson's disease with dementia and four dementia of multiple etiologies.

Among the nine circadian genes studied, the methylation was detected in the promoter of *PER1* and *CRY1* genes. The mPCR results are shown in Fig. 1. Methylation could be easily identified by using pairs of methylation and unmethylation specific primers in concomitant with methylase treated positive controls. Fig. 1 only shows the representative results of *PER1* and *CRY1* genes, methylation analysis for seven other genes were carried out in the same way. In order to confirm the results of mPCRs, we sequenced some of the PCR products from the mPCRs and found that the sequencing results were in accordance with mPCRs (data not shown). Since the mPCR in this study only detected two or three possible methylation sites in each gene promoter, methylated CpG sites which do not locate within the mPRC primer amplification region may result in falsely negative results. Therefore, we further used another pair of primers for each gene promoter to amplify each interested promoter region possibly containing other methylated CpG sites other than regions detected by mPCR. We sequenced these PCR products and found no false negative results in all nine circadian genes (data not shown).

Seven dementia patients were found to have methylation in either *PER1* ($N = 3$) or *CRY1* ($N = 4$) promoter. None of the con-

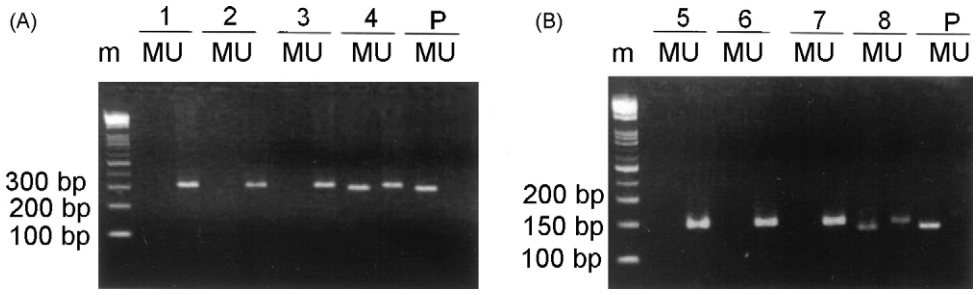

Fig. 1. Methylation analysis of circadian genes promoter. The figure shows the representative mPCR analysis of *PER1* (A) and *CRY1* (B) genes. (A) In *PER1* promoter, cases 1–3 were cases of no methylation. They only had a 318 bp PCR product in using unmethylation specific primers and they had no PCR product in methylation specific primers. The case 4 was a case with *PER1* methylation, this patient had a 298 bp PCR product in using methylation specific primers. (B) In *CRY1* promoter, cases 5–7 were cases of no methylation. They only a 187 bp PCR product in using unmethylation specific primers and they had no PCR product in methylation specific primers. The case 8 was a case with *CRY1* methylation, this patient had a 166 bp PCR product in using the methylation specific primers. *Abbreviations*: m: 100 bp ladder markers; P: methylase treated DNA positive control; M: PCR using methylation specific PCR primers; U: PCR using unmethylation specific PCR primers.

trols had circadian gene methylation. The association of *PER1* and *CRY1* promoter methylation and the clinical categories is shown in Table 1. The frequency of CpG methylation in the dementia group was higher than that of controls with borderline significance (Fisher's exact test with Bonferroni correction, $P = 0.0136$). Among the seven methylated dementia patients, five were DLB patients, one was AD patient and one was vascular dementia (in the group of dementia of other causes). DLB patients had the statistical significantly higher frequency (35.7%) of CpG methylation than controls (Fisher's exact test with Bonferroni correction, $P = 0.0000365$). In comparison to AD patients, DLB patients also had significantly higher CpG methylation (Fisher's exact test with Bonferroni correction, $P = 0.0007115$).

There are several CpG islands on the promoter of each circadian gene. Based on the understanding of maps of CpG islands fore each gene, the regions surveyed in this study by mPCR contain the mostly dense concentration of CpG for each gene. Although, we did not survey all the CpG, the selected areas can represent the major CpG islands on the promoter. The major problem is that we just examined circadian gene from the peripheral leukocytes rather than the brain circadian center. However, compelling data support that there are synchronized expression of circadian genes between central circadian clock and peripheral tissues, such as monocytes [8]. Thus, we speculated that the circadian gene regulation status in the peripheral tissues might represent the central status to some degree. Of course, it is necessary for further study to examine if the status of CpG methylation on circadian genes in peripheral leukocytes reflects the methylation status of the central circadian clock. Both *PER1* and *CRY1* are important genes participating in the circadian clock [31,34]. Activation of these genes is regulated by *BMAL1* and *CLOCK* through the E-box, a transcriptional consensus of DNA sequence on *PER*, which induce the expression of PER and CRY proteins. The homozygous *PER1* and *CRY1* mutant animals displayed a shorter circadian period with reduced precision and stability [10,39]. As the methylation epigenetic regulation, *PER1* and *CRY1* promoter methylation are supposed to decrease their protein levels. It is reasonable to assume that this dysregulation might contribute to the abnormal behavioral phenotype in part as the mutant animals.

Table 1
Circadian gene methylation in the dementia patients and controls

Dementia category	Methylation gene		Total	Age (sex: M/F)
	PER1	*CRY1*		
DLB ($n = 14$)	3	2	5	72.5 ± 9.3 (6/8)
AD ($n = 59$)	0	1	1[a]	72.0 ± 9.7 (27/32)
Others ($n = 7$)	0	1	1	60.8 ± 7.7 (4/3)
Controls ($n = 80$)	0	0	0[b]	71.1 ± 9.4 (37/43)

DLB patients was found to have significant increase of methylation in comparison to controls. DLB patients also had significantly higher CpG methylation in comparison to AD patients.

[a] Fisher's exact test with Bonferroni correction, $P = 0.0007115$.

[b] Fisher's exact test with Bonferroni correction, $P = 0.0000365$.

Therefore, we compared the behavioral disturbances in dementia patients between patients with and without circadian gene methylation. The total sleeping time and MSPI severity were analyzed, however, there were no significant differences in the severity of behavioral disturbances in terms of MSPI rating score and sleeping time between patients with and without methylation (data not shown). Though MSPI is not a rating scale designated for dementia patients, the items are similar to the common BPSD rating scales, such as BEHAVE-AD [30]. The failure to demonstrate the association of circadian gene methylation and behavioral disturbance in this study suggested that the behavioral disturbances in dementia patients might derive from multiple etiologies. The role of circadian gene methylation in behavior and sleep disturbances of dementia patients needs larger sample size and prospective study for clarification and using dementia specific rating scale. Additionally, the enrolled dementia patients in this study were only mild to moderate degree. Further study to enroll more severe dementia patients is necessary to explore if the methylation would increase in accordance with disease progression.

The most striking finding of this study was that DLB patients had the highest frequency (35.7%) of CpG methylation in comparison to controls ($P = 0.0000365$) and AD patients ($P = 0.0007115$). Fluctuation of cognition is one of the core features in diagnosis of DLB and rapid eye movement behavior disorder (RBD) is one of the suggestive features [14,24,25]. In review of the clinical features of our DLB patients, all of them had the presentation of fluctuation of cognition and it was hard to differentiate the fluctuation severity between patients with and without methylation from the chart review. RBD was reported in two DLB patients with methylation and no report in non-methylation patients. We suggested that these rhythmic activity disarrangement symptoms may derive from circadian dysregulation. DLB patients have been shown to manifest greater circadian rhythm disturbance behaviors than the AD patients and the severity of circadian rhythm disarrangement is paralleled with the amount of Lewy bodies in the brain [17]. Our study conformed to this finding with significantly higher frequency of methylation in DLB than in AD patients. DLB and Parkinson disease are members of a family of disorders characterized by the presence of alpha-synuclein Lewy body. There must be some pathogenesis differences in epigenetic methylation between amyloidopathy and synucleinopathy and further basic studies are necessary for clarification in this issue. Among the nine circadian genes studied, only *PER1* and *CRY1* were methylated. In the circadian gene regulation cascade, *PER1* and *CRY1* play as the first response to light in the morning and then start the circadian cycle [2]. This regulatory role of *PER1* and *CRY1* in the circadian gene regulation cycle might be the cause for their vulnerability for epigenetic methylation.

Taken together, our results suggested that circadian gene dysregulation might be more prevalent in DLB patients and circadian gene epigenetic regulation might be an interesting field for further research. This study also provided a piece of data linking between circadian gene dysregulation and behavior and psychiatry problems in dementia patients. It might be also warranted further researches on the treatment of entraining normal circadian rhythm for the dementia patients with behavior/sleep disorders [35,37].

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