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Brief communication

Methylation analysis of circadian clock gene promoters in forensic autopsy specimens

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ABSTRACT

DNA methylation in gene promoter regions influences gene expression. Circadian clock genes play an important role in the formation of a biological clock and aberrant methylation of these genes contributes to several disorders. In this study, we examined forensic autopsy specimens to determine whether DNA methylation status in the promoter regions of nine circadian clock genes (*Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Tim*, and *Ck1e*) is related to a change in acquired diathesis and/or causes of death. Methylation-specific PCR and direct sequencing methods revealed that the promoters of *Per1*, *Cry2*, *Bmal1*, *Clock*, and *Ck1e* were unmethylated in all the forensic autopsy specimens, while the promoters of *Per2*, *Per3*, *Cry1*, and *Tim* were partially methylated. Methylation status varied between individuals and between tissues in the same patient. A detailed analysis of methylation patterns in the *Cry1* promoter region revealed that the patterns also varied between individuals and the *Cry1* promoter had highly methylated patterns in two cases that had been exposed to methamphetamine. These results suggest that the methylation status of clock gene promoters varies between individuals. Methamphetamine use may influence methylation in the *Cry1* gene promoter region and disturb circadian rhythmicity.

1. Introduction

DNA methylation is an epigenetic event that affects cellular mechanisms by changing gene expression; the phenomenon is particularly important in cancer development [1–5]. Recent studies have revealed that various diseases are associated with abnormal changes in DNA methylation status [6,7]. Circadian clock genes play an important role in the formation of a biological clock; abnormal methylation of these genes reportedly contributes to increased morbidities such as sleep disturbance [8], senile cognitive impairment [9,10], heart disease [11], lifestyle-related disease [12], gastric cancer [13], breast cancer [14,15], and hepatocellular carcinoma [16]. Thus, disruption of the circadian rhythm may play an important role in the development of these diseases.

Pathomorphological abnormalities cannot be detected in cases of sudden unexpected death due to cardiac arrhythmia or the influence of long-term medication, which are encountered in the field of forensic medicine [17–19]. In some patients liable to sudden death, homeostasis or the balance in biological functions might be impaired by environmental factors, living conditions, and/or medical treatment. Although these persons show no evident pathomorphological abnormalities, their susceptibility to stress-induced damage may be heightened.

We hypothesize that an aberrant circadian rhythm is one of the abnormalities that are relatively difficult to detect. In this study, we focused on nine circadian clock genes: *Period1 (Per1)*, *Period2 (Per2)*, *Period3 (Per3)*, *Cryptochrome1 (Cry1)*, *Cryptochrome2 (Cry2)*, *Bmal1*, *Clock*, *Timeless (Tim)*, and *Casein kinase 1 epsilon (Ck1e)* [16,20]. Molecular components of the circadian oscillator have been found to be an autoregulatory transcription/translation-based feedback loop comprised of these nine genes [21,22]. *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, and *Tim* act as negative regulators, whereas *Bmal1* and *Clock* are positive regulators. *Ck1e* binds and phosphorylates PER proteins to regulate their stability. The CLOCK and BMAL1 proteins form heterodimers, bind to E-box enhancer sequences in promoter regions, and activate the transcription of target genes including *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2*. The nuclear accumulation of PER/CRY complexes suppresses transcription by interacting with CLOCK/BMAL1 complexes. The master clock of the circadian rhythm is located in the suprachiasmatic nucleus (SCN) of the hypothalamus [23,24]. Recent studies have shown that peripheral circadian rhythms may be driven or synchronized by the central pacemaker in the SCN, and circadian gene expression could be reflected in peripheral cells [25–27]. Hence, disturbance of these genes would have a profound influence on circadian rhythmicity.

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To determine whether DNA methylation status in these promoter regions is related to acquired diathesis and/or causes of death, we attempted to detect DNA methylation modification in forensic autopsy specimens.

2. Material and methods

2.1. Materials and DNA extraction

We used commercially available kits to extract genomic DNA from cardiac blood, hearts, lungs, livers, kidneys, and cerebral tissues (preserved at −30 °C), formalin-fixed organs (fixation lasting for more than 3 months), and paraffin-embedded tissue blocks (preserved for more than 3 months after preparation). Samples were obtained from cases (n = 32) that had undergone judicial autopsy at our forensic unit between 2009 and 2010. Samples were from patients who died from asphyxia (10 cases), fire fatality (7 cases), hemorrhagic shock (2 cases), lethal arrhythmia (3 cases), ischemic heart disease (3 cases), head injury (2 cases), hydrogen sulfide intoxication (2 cases), and unknown causes (3 cases). Formalin-fixed organs and paraffin-embedded tissue blocks including hearts, lungs, livers, kidneys, and cerebral tissues were selected each from 2 cases with lethal arrhythmia and ischemic heart disease, with postmortem intervals within 24 h.

This study was approved by the Research Ethics Committee for Epidemiological Clinical Studies of Fujita Health University School of Medicine.

2.2. Bisulfite treatment

When extracted genomic DNA is treated with bisulfite (sodium hydrogen sulfite), cytosine is replaced by uracil. However, the sensitivity of methylated cytosine is so low that it does not undergo substitution [28]. We performed bisulfite treatment with a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Australia) that is based on this principle.

2.3. Methylation-specific polymerase chain reaction (MSP)

We used bisulfite-treated DNA to prepare two types of primers based on the sequences of nine clock gene promoters (*Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Timeless*, and *Ck1e*): one specific to the methylated sequence and the other specific to the unmethylated sequence. We then performed methylation-specific PCR (MSP)

Table 1
Oligonucleotide primers used for methylation-specific PCR of the promoter regions.

Gene
Per1
Per1-M (amplicon size, 298 bp; AT, 54 °C)
Up: 5′-ATTTAGGTTTACGTGCGTTC-3′, D: 5′-CGACTCAAAAACGAAAATCG-3′
Per1-U (amplicon size, 318 bp; AT, 54 °C)
Up: 5′-TAGTATTAGTATTTAGGTTTATGTGTGTTT-3′, D: 5′-AACAACAATCCAACTCAAAAACAAAAATCA-3′
Per2
Per2-M (amplicon size, 140 bp, AT, 58 °C)
Up: 5′-GCGGTTTCGTTGCGGTTTAC-3′, D: 5′-GCCGACGCCGTTTCAACCCG-3′
Per2-U (amplicon size, 160 bp, AT, 58 °C)
Up: 5′-GTGGTGTGGTGTGGTTTTGTTGTGGTTTAT-3′, D: 5′-ACACCCCCACACCAACACCATTTCAAACCA-3′
Per3
Per3-M (amplicon size, 182 bp, AT, 57 °C)
Up: 5′-CGGGAGTTTTGGGTATTCGC-3′, D: 5′-CGACCCGACTAACTAAAACG-3′
Per3-U (amplicon size, 207 bp, AT, 57 °C)
Up: 5′-TGGGTGGTTGGGTGGGAGTTTTGGGTATTTGT-3′, D: 5′-AATCCAACACCAACAACCCAACTAACTAAAACA
Cry1
Cry1-M (amplicon size, 166 bp, AT, 59 °C)
Up: 5′-TCGTTTTGTTTTTAGGGGTC-3′, D: 5′-GCAACCGCCTAAAAACGACG-3′
Cry1-U (amplicon size, 187 bp, AT, 59 °C)
Up: 5′-ATTTTGGGTGGGTTGTTTTGTTTTTAGGGGTT-3′, D: 5′-ACACCCACCACAACCACCTAAAAACAACA-3′
Cry2
Cry2-M (amplicon size, 150 bp, AT, 56 °C)
Up: 5′-GTTTATTTTCGGTATTTCGC-3′, D: 5′-TAACGATTAACCCAAAAACG-3′
Cry2-U (amplicon size, 171 bp, AT, 56 °C)
Up: 5′-TTGAGATTTGGTTTATTTTTGGTATTTTGT-3′, D: 5′-TACCTTCCACCTAACAATTAACCCAAAAACA-3′
Bmal1
Bmal1-M (amplicon size, 138 bp, AT, 61 °C)
Up: 5′-GGAGGTGTTTGTTTATTCGC-3′, D: 5′-AAATACGCGAAATCGCGTCG-3′
Bmal1-U (amplicon size, 161 bp, AT, 58 °C)
Up: 5′-TAGGTTAGGGATGGAGGTGTTTGTTTATTTGT-3′, D: 5′-AACCCCCAACAAAATACACAAAATCACATCA-3′
Clock
Clock-M (amplicon size, 168 bp, AT, 52 °C)
Up: 5′-GTTTTTTATTCGATTAGGTTTC-3′, D: 5′-CGTTAAACAACACGAAACCG-3′
Clock-U (amplicon size, 188 bp, AT, 55 °C)
Up: 5′-GTTGGTTGGTTGTGTTTTTTATTTGATTAGGTTTT-3′, D: 5′-CAACTTACCCCATTAAACAACACAAAACCA-3′
Timeless
Tim-M (amplicon size, 144 bp, AT, 52 °C)
Up: 5′-AGCGTTGTGATTTTTAGATC, D: 5′-CCTACGCGAAAAACGAAACG-3′
Tim-U (amplicon size, 164 bp, AT, 50 °C)
Up: 5′-AGTAGAAAATTTATAGTGTTGTGATTTTTAGATT-3′, D: 5′-TCCCCACCTACACAAAAAACAAAACA-3′
Ck1e
Ck1e-M (amplicon size, 110 bp, AT, 54 °C)
Up: 5′-GTGTTTTTTTATTTTTACGC-3′, D: 5′-CGAAAAATTTAAAAAACCCG-3′
Ck1e-U (amplicon size, 128 bp, AT, 52 °C)
Up: 5′-GTTAGTATTTGTGTGTTTTTTTATTTTTATGT-3′, D: 5′-CCACCCCAAAAAATTTAAAAAACCCA-3′

M, methylation-specific primer sets; U, unmethylation-specific primer sets; Up, (+) strand primer; D, (−) strand primer; AT, annealing temperature.