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Tissue-specific modification of clock methylation in aging mice

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Abstract. – BACKGROUND: Circadian rhythms tend to change as animals age; however, the molecular mechanisms underlying these are not yet fully understood.

OBJECTIVE: To investigate whether the DNA methylation of clock genes changes with age and contributes to circadian dysfunction in aged animals.

METHODS: We examined the methylation of clock promoters in the stomach, kidney, striatum, and spleen by using a methylation-specific polymerase chain reaction (MSP) assay.

RESULTS: Our results show that different tissues exhibit specific patterns of clock methylation. Additionally, methylation frequency decreased significantly in older mice at the Per1 promoter in the stomach, but it was significantly increased in older mice at the Cry1, Bmal2, and Npas2 promoters in the spleen.

CONCLUSION: The findings from our study suggest that DNA methylation contribute to age-related changes in circadian rhythms in certain slave oscillators.

Key Words:
Aging, Promoter methylation, Clock genes.

Introduction

Circadian rhythms are daily oscillations at the behavioral, physiological and molecular levels with a period of around 24 h. The suprachiasmatic nucleus (SCN) of the hypothalamus is the central pacemaker of circadian rhythms in mammals. Other brain regions and peripheral tissues are synchronized by the SCN *via* a myriad of redundant cues, and are regarded as slave oscillators. The molecular clock machinery in the SCN and the slave oscillators are believed to be similar. In brief, a number of clock genes and their resultant proteins form interlocking transcription-translation feedback loops that generate and maintain the 24-h mRNA and protein oscillations and the resulting biological rhythms. Several clock genes have already been identified, including Bmal1, Bmal2, Clock, NPAS2, Per1, Per2, Cry1, and Cry2[1].

Circadian rhythms in humans, rodents and other animals tend to change as they age. Aging has been associated with both the loss of rhythmicity (lung and retrochiasmatic area) and phase-advance of rhythms (kidney, pineal) of Per1 expression[2]. Circadian rhythms of cell proliferation in the spleen are reduced in aged rats[3]. Daily oscillations of dopamine and serotonin are modified in the striatum of aged rats[4]. The activities of circadian food-entrainable oscillators, located in the stomach, are also altered with age[5,6]. The molecular mechanisms underlying these age-related changes in circadian rhythms remain to be elucidated. Recently, several lines of evidence have indicated that aging affects the entrainment of circadian signals to external stimuli for slave oscillators, raising the possibility that the molecular clock machinery in peripheral tissues changes with age[7,8].

DNA methylation is a chemical modification of DNA structure and an important epigenetic regulator of gene expression[9]. Differences in DNA methylation are known to be acquired with age in multiple tissues[10-12]. DNA methylation of clock genes has been studied in many tissues[13,14], cancer cells[15], tumor samples[16-18], and during perinatal development[19]. These studies indicated that DNA methylation contributes to the regulation of clock gene expression. In addition, the methylation status of Npas2 has been reported to

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be associated with aging[11]. To investigate whether the DNA methylation of clock genes changed with age and contributed to circadian dysfunction in aged animals, we examined the methylation of clock promoters in the stomach, kidney, striatum, and spleen by using a methylation-specific polymerase chain reaction (PCR) assay (MSP assay). MSP results indicated that different patterns of epigenetic deregulation occur in slave oscillators over time, and they possibly contribute to circadian changes associated with normal aging.

Materials and Methods

Mice

Young (3-months-old, n = 14) and old (24-months-old, n = 14), male C57BL/6 mice were housed at an animal facility on a 12-h light-dark cycle (lights on at 08:00; lights off at 20:00) and allowed to feed *ad libitum*. Animal treatment was in accordance with the National Institutes of Health (NIH) guidelines for animal experimentation and the Institutional Animal Care and Use Committee guidelines of Capital Medical University.

Genomic DNA Preparations and Bisulfite Treatment

Genomic DNA (gDNA) was prepared using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). For the bisulfite reaction, in which cytosine is converted to uracil and 5'-methylcytosine remains non-reactive, genomic DNAs were initially denatured with 0.3 mol/L (M) NaOH. Sodium metabisulfite solution (pH 5.0) and hydroquinone were then added at final concentrations of 3.0 M and 0.5 mM, respectively. The reaction mixtures were incubated under mineral oil at 50°C in the dark for 16 h. The denatured DNA was purified using Wizard DNA purification resin (Promega, Madison, WI, USA), and modification was then terminated by treatment with 0.3 M NaOH at 37°C for 15 min, followed by ethanol precipitation.

Promoter Analysis and Primer Design

Promoters for the clock genes were analyzed using online software (http://www.mspprimer.org/cgi-mspprimer/design.cgi). The primers used for the MSP of each clock promoter were designed using the same software; these have been reported previously[20].

Methylation-Specific Polymerase Chain Reaction (MSP)

Bisulfite-treated genomic DNA was amplified with HS Taq (Takara, Shiga, Japan). PCR products were then loaded and electrophoresed on 2% agarose gels, stained with GelRed (Biotium, Hayward, CA, USA). To ensure specificity of clock promoter methylation, genomic DNA treated with SssI, a CpG methylase (New England Biolab, Herts, UK), was used as a positive control, whereas genomic DNA without bisulfite treatment was used as negative control.

Statistical Analysis

The methylation frequencies of clock genes in different age groups were compared and analyzed using Pearson Chi-square tests. We compared 8 clock genes, using Bonferroni's correction for multiple comparisons. Significance levels were established at a p-value less than 0.00625.

Results

In this study, 28 mice were examined; 14 were sacrificed at 3 months of age and grouped as young mice, the other 14 mice were sacrificed at 24 months of age and grouped as old mice. Methylation frequencies of 8 clock promoters (Per1, Per2, Cry1, Cry2, Clock, Npas2, Bmal1 and Bmal2) were examined using MSP and the results were summarized in Table I-IV. Partial methylation was present at the Cry1 promoter in all tissues examined, at the Per1 and Bmal1 promoters in the stomach, and at the Bmal2 and Npas2 promoters in the spleen (Figure 1). Methylation frequency at the Bmal1 promoter was very high in the stomach, with 27 out of 28 mice showing methylation. With regard to the age related changes, methylation frequency was found to have decreased significantly in the old mice at the stomach Per1 promoter (p = 0.006), but increased significantly in old mice at the Cry1, Bmal2 and Npas2 promoters in the spleen (p = 0.001; p = 0.006; p = 0.002, respectively). Virtually no methylation was detected in the spleen of young mice, whereas 11/14 old mice demonstrated methylation in at least one locus. Some of the MSP amplicons were cloned and sequenced, which confirmed our MSP results (data not shown).

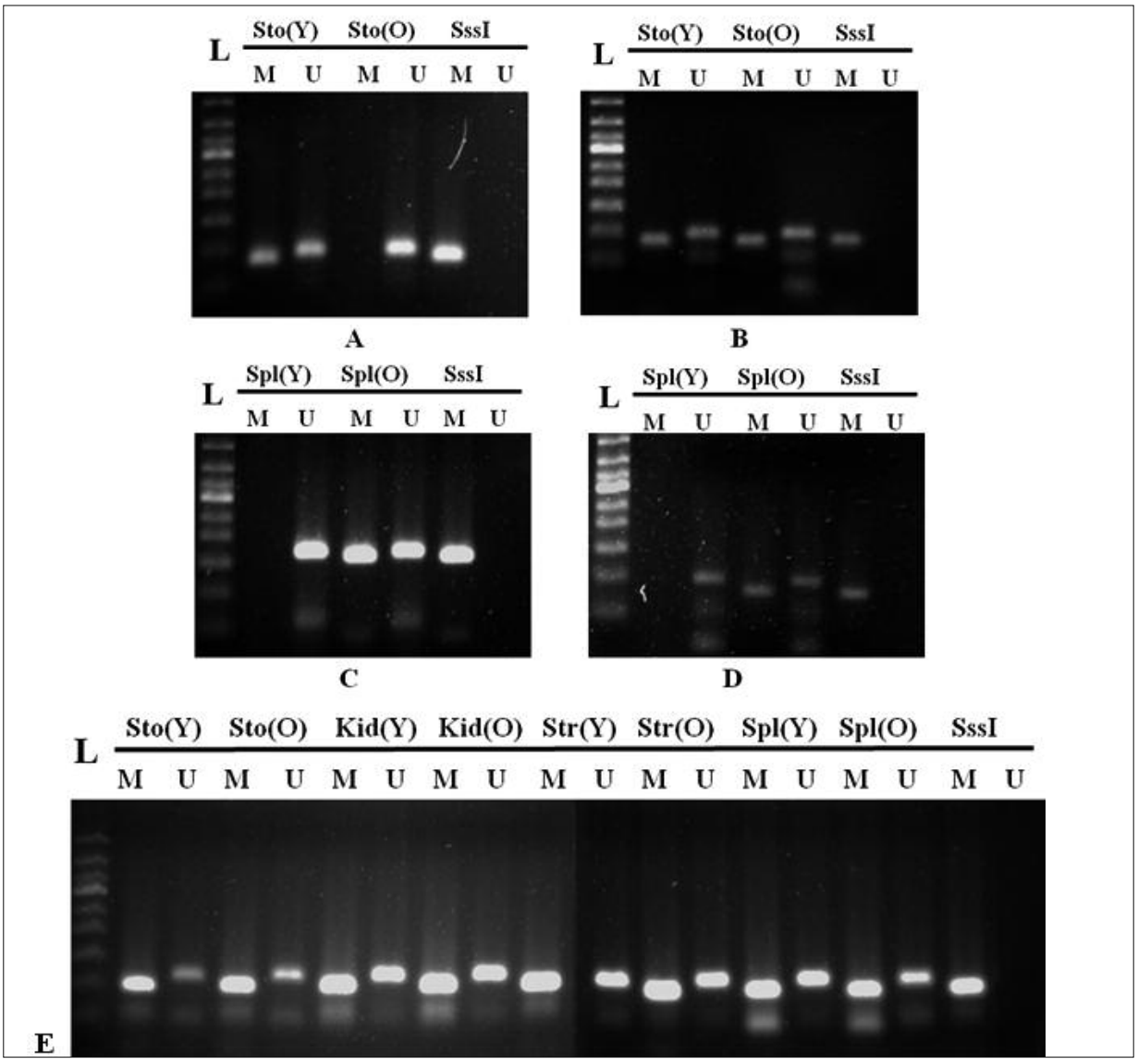

Figure 1. Methylation analysis of clock promoters. This figure shows the representative MSP analysis of Per1 ***(A)***, Bmal1 ***(B)***, Bmal2 ***(C)***, Npas2 ***(D)*** and Cry1 ***(E)*** in the Stomach (Sto), Kidney (Kid), Striatum (Str) and Spleen (Spl). SssI: methylase treated DNA positive control; L: DNA ladder; M: PCR using methylation specific PCR primers; U: PCR using unmethylation specific PCR primers.

Discussion

SCN is the primary pacemaker of circadian rhythm. Ablation of SCN abolishes most types of circadian rhythm at the behavioral, physiological and molecular levels. It would be of interest to examine the age related changes of DNA methylation therein. However, SCN is very tiny in mice. It is very difficult to dissect SCN precisely. Therefore, we waived the SCN in the present study and examined the stomach, kidney, spleen and striatum. Certain lines of evidence suggest that the DNA methylation of clock genes change with age in these tissues. Firstly, a functional molecular clock has been identified in these tissues[21-24]. Secondly, a phase-advance rhythm of Per1 expression was reported in the kidney of aged mice[2]. Expression of Bmal1 was repressed in the striatum of aged rodents[25]. Expression of Bmal1 and Per3 was also down-regulated in peripheral blood, which is closely connected to the spleen[26]. However, the mechanisms underlying age-related clock deregulation are unclear.

Table I. Amount and frequency (%) of methylation in the promoters of clock genes present in the stomach.

	Methylation status (%)		
	Young	Aged	*p* value for young vs aged
Total	14	14	
Per1			
PM	6 (42.9%)	0 (0.0%)	0.006
U	8 (57.1%)	14 (100.0%)	
Per2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Cry1			
PM	3 (21.4%)	2 (14.3%)	0.622
U	11 (78.6%)	12 (85.7%)	
Cry2			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Bmal1			
PM	14 (100.0%)	13 (92.9%)	0.309
U	0 (0.0%)	1 (7.1%)	
Bmal2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Clock			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Npas2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	

*U: Unmethylated sequence; PM, partially-methylated sequence. Eight clock genes were compared, the level of statistical significance was established at a *p*-value less than 0.00625.

Table II. Amount and frequency (%) of methylation in the promoters of clock genes in the kidney.

	Methylation status (%)		
	Young	Aged	*p* value for young vs aged
Total	14	14	
Per1			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Per2			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Cry1			
PM	3 (21.4%)	3 (21.4%)	1.000
U	11 (78.6%)	11 (78.6%)	
Cry2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Bmal1			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Bmal2			
PM	0 (0.0%)	0(0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Clock			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Npas2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	

*U: Unmethylated sequence; PM, partially-methylated sequence. Eight clock genes were compared, with significance levels established at a *p*-value less than 0.00625

Table III. Amount and frequency (%) of methylation in the promoters of clock genes of the striatum.

	Methylation status (%)		
	Young	Aged	*p* value for young vs aged
Total	14	14	
Per1			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Per2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Cry1			
PM	8 (57.1%)	3 (21.4%)	0.053
U	6 (42.9%)	11 (78.6%)	
Cry2			
PM	0(0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Bmal1			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Bmal2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Clock			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	
Npas2			
PM	0 (0.0%)	0 (0.0%)	–
U	14 (100.0%)	14 (100.0%)	

*U: Unmethylated sequence; PM, partially-methylated sequence. Eight clock genes were compared, and the level of statistical significance was established at a *p*-value less than 0.00625.

Table IV. Amount and frequency (%) of methylation in the promoters of splenic clock genes.

	Methylation status (%)		
	Young	Aged	*p* value for young vs aged
Total	14	14	
Per1			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Per2			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Cry1			
PM	0 (0.0%)	8 (57.1%)	0.001
U	14 (100.0%)	6 (42.9%)	
Cry2			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Bmal1			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Bmal2			
PM	0 (0.0%)	6 (42.9%)	0.006
U	14 (100.0%)	8 (57.1%)	
Clock			
PM	0 (0.0%)	0 (0.0%)	-
U	14 (100.0%)	14 (100.0%)	
Npas2			
PM	0 (0.0%)	7 (50.0%)	0.002
U	14 (100.0%)	7 (50.0%)	

*U: Unmethylated sequence; PM, partially-methylated sequence. Eight clock genes were compared, and the level of statistical significance was established at a *p*-value less than 0.00625.

Age-related changes in DNA methylation were found in the stomach and spleen. Because DNA methylation repressed clock expression efficiently[16-18], it is very likely that some clock expression and even molecular rhythms are modulated in the aged stomach and spleen. For example, DNA methylation of Npas2 may repress its expression in older spleens, Bmal1 activity would thus be affected, having less of its partner available, and transcription of Pers, Crys, Nr1d1, and probably other genes, would be modulated. However, there has been no study of the influence of age on the expression of clock genes in the stomach and spleen, and we did not examine clock expression in this study either. It is still hard to ascertain to what extent clock expression and molecular rhythms would be affected by DNA methylation, and this warrants further study. Of note, there are many sub-populations of cells in both the stomach and spleen. Molecular rhythms, and probably methylation patterns, are distinct in different sub-populations[23]. Hence, it would be of importance to examine clock expression and clock methylation, cell type by cell type, when trying to establish the role of DNA methylation in regulating molecular rhythms during aging.

Our study indicated that different tissues demonstrate specific patterns of clock methylation. In the kidney and striatum, methylation was detected only at the Cry1 locus. In the stomach and spleen, 3 clock loci were methylated. Additionally, the methylation frequency of Cry1 altered, with aging, in the spleen yet remained constant in other tissues. Similarly, methylation of Per1, Bmal2 and Npas2 was changed in only one tissue. These observations were in accordance with the notion of the tissue-specific effects of aging on the epigenome[10,12]. We also noticed that methylation of the clock promoters only occurred in some samples regardless of the tissue type. This intra-individual variation has actually been reported previously[27].

Almost all mice were methylated at the Bmal1 promoter in the stomach, indicating that methylation contributes to the regulation of Bmal1 expression. Bmal1 dysfunction leads to circadian arrhythmicity[28]. Moreover, Bmal1 can be silenced by promoter CpG island hypermethylation[18]. It is reasonable to speculate that Bmal1 is silenced in some stomach cells. It would be of interest to investigate which groups of cells were methylated at the Bmal1 locus and, therefore, lack functional molecular oscillations. Moreover, it would be helpful to quantify the methylation levels of Bmal1 promoters in both young and old mice, which would precisely answer the question of whether methylation of Bmal1 changes with age.

The spleen seems to represent a special case with regard to clock methylation. Three loci were methylated in the spleen (Cry1, Bmal2, and Npas2). We recently examined the methylation of 7 clock promoters in human peripheral lymphocytes[29]. Our results from that study indicate that both Cry1 and Npas2 were partially methylated, which is consistent with the present observations. All of these loci were methylated only in old mice, suggesting the spleen is particularly prone to age-related circadian dysfunction.

Conclusions

Our data supports the hypothesis that DNA methylation plays a role in age-related circadian deregulation.

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Conflict of Interest

The Authors declare no conflict of interest for this article.

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