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ORIGINAL ARTICLE

Estimating time of death based on the biological clock

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Abstract The biological clock may stop at the time of death in a dead body. Therefore, the biological clock seems useful for estimating the time of death. In this study, we tried to read the biological clock in tissues from dead bodies to estimate the time of death using molecular biological techniques. At first, we examined real-time RT-PCR analysis of gene expression for mPer2 and mBmal1, which constitutes a feedback loop in the oscillation system, in the kidney, liver, and heart of mice. We could detect circadian oscillation of these gene expressions in mouse tissues even at <48 h after death. Thus, the ratio of mPer2/mBmal1 was found to be useful for estimating the time of death. We next applied this method to the liver, kidney, and heart obtained from forensic autopsy cases with less than 72 h of postmortem interval. Significant circadian oscillation of hPer2/hBmal1 ratio could be detected in these autopsy samples. We further examined gene expression for hRev-Erbα, a component of another feedback loop. The ratios of hRev-Erbα/hBmal1 showed higher amplitude of oscillation than those of hPer2/hBmal1 and are considered more suitable for estimating the time of death. In particular, a hRev/hBmal1 ratio of >50 indicated the time of death as 0200–0900 hours, and a hRev/hBmal1 ratio that considerably exceeded 75 indicated the time of death as 0200–0800 hours. On the other hand, a hRev/hBmal1 ratio of less than 25 strongly indicated the time of death as 1000–2300 hours. Taken together, these findings indicate that gene expression analyses of the biological clock could be powerful methods for estimation of the time of death.

Keywords Time of death · Biological clock · Real-time RT-PCR

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Introduction

In forensic practice, estimating the time of death is one of the most important procedures but is often extremely difficult. There are many methods for estimating the time of death, which can be divided into two categories (rate methods and concurrence methods). In rate methods, which are mainly used in forensic practice, the time of death is estimated on the basis of postmortem changes, such as the amount and distribution of rigor mortis [1, 2], the change in body temperature [3], hypostasis [4], changes of potassium concentration in vitreous humor [5, 6], development and growth of insects in the corpse [7], and the degree of putrefaction of the body. However, these changes are strongly influenced by unpredictable endogenous and environmental factors. Examples of concurrence methods include reading the time on a wristwatch stopped by a traffic accident or determining the extent of digestion of the last known meal, which may contribute to estimating the time of death [8].

Accumulated evidence indicates that biological clock systems work in most cells in most tissues (peripheral clock) in addition to the core clock in the hypothalamic suprachiasmatic nucleus (SCN), and several genes have been identified as associated with the biological clock [9–11]. If the biological clock stops at death, reading the biological clock

might be valuable for estimating the time of death. In this study, we investigated real-time RT-PCR analysis of the gene expression of molecules involved in circadian clock oscillation systems in the kidney, liver, and heart from mouse and human dead bodies and discussed its applicability for estimating the time of death in forensic practice.

Materials and methods

Mouse samples

Specific pathogen-free 8- to 30-week-old male and female mice (BALB/c and C57BL/6) were obtained from Sankyo Laboratories (Tokyo, Japan). All mice were bred and housed at a constant temperature (23±2°C), with a 12-h light/dark cycle (light on at 0800 hours and off at 2000 hours). They were fed with standard feed and given water ad libitum. For fresh samples, mice were sacrificed at intervals of 2 or 3 h by the inhalation of diethyl ether, and then kidney, liver, and heart were removed immediately and stored at −80°C until RNA extraction. In another series, obtained tissue samples were left for 24 or 48 h at room temperature in order to examine the influence of postmortem interval on clock gene expression. All animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University.

Autopsy samples

Kidney, liver, and heart samples were obtained from 38 forensic autopsy cases with known time of death (25 men and 13 women). The age of autopsied subjects ranged from 8 months to 85 years (average, 60.7 years), and postmortem intervals in all cases were less than 72 h (average, 27.3 h). The causes of death of the subjects were asphyxia (two cases), drowning (six cases), fire fatality (three cases), traumatic shock (eight cases), acute respiratory failure (two cases), ischemic heart disease (eight cases), brain injury (two cases), aortic rupture (two cases), sepsis (two cases), and others (three cases—hemorrhagic shock, carbon monoxide poisoning, and hypothermia). Tissue samples were taken during autopsy, immediately frozen in liquid nitrogen, and stored at −80°C until use.

Extraction of total RNA and real-time RT-PCR

Total RNA was extracted from tissue samples (100 μg) using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions, and 5 μg of total RNA was reverse transcribed into cDNA at 42°C for 1 h in 20 μl reaction mixture containing mouse Moloney leukemia virus reverse transcriptase (PrimeScript, TAKARA BIO INC., Otsu, Japan) with six random primers (TAKARA BIO INC.). Thereafter, generated cDNA was subjected to real-time PCR analysis using SYBR® Premix Ex Taq™ II kit (TAKARA BIO INC.) with specific primer sets (Table 1). The relative quantity of target gene expression to *β-actin* gene was measured by a comparative CT method.

Results

Biological clock in mouse tissues

We selected two main oscillator genes, period 2 (*mPer2*) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*mBmal1*), in circadian clock system to examine the biological clock in mouse tissues. Significant circadian oscillation of mPer2 and mBmal1 gene expression could be detected in the kidneys, livers, and hearts removed from mice immediately after sacrifice (Fig. 1a). Consistent with many previous reports (12), gene expression levels of *mPer2* and *mBmal1* oscillated in the anti-phase (Fig. 1a). We selected mPer2/mBmal1 ratio as a parameter for reading the biological clock in mice. As shown in Fig. 1b, mPer2/mBmal1 ratio was low from 0000 to 1200 hours, and started to increase after 1500 hours, eventually peaking around 2000 hours. Next, we examined the effects of postmortem interval on the detection level of *mPer2* and *mBmal1* gene expression in mouse kidney samples. In accordance with the increase of postmortem intervals, the detection level of *mPer2* and *mBmal1* gene expression gradually decreased (Fig. 2a and b). However, circadian oscillation of each gene

Table 1 Sequences of the primers used for real-time PCR

Transcript	Sequence
mBmal1	(F) 5′-TCAGATGACGAACTGAAACACCTAA-3′
	(R) 5′-TTTGGATGCAGGTAGTCAAACAAG-3′
mPer2	(F) 5′-ATCAGCCATGTTGCCGTGTC-3′
	(R) 5′-CGTGCTCAGTGGCTGCTTTC-3′
mβ-actin	(F) 5′-CATCCGTAAAGACCTCTATGCCAAC-3′
	(R) 5′-ATGGAGCCACCCATCCACA-3′
hBmal1	(F) 5′-GCCTACTATCAGGCCAGGCTCA-3′
	(R) 5′-AGCCATTGCTGCCTCATCATTAC-3′
hPer2	(F) 5′-CGTTGGAACCACCCAGACATC-3′
	(R) 5′-ATGCAGTCGCAAGCTGTCAGA-3′
hRev-Erba	(F) 5′-TCAGCTGGTGAAGACATGACGAC-3′
	(R) 5′-GGAGCCACTGGAGCCAATGTA-3′
hβ-actin	(F) 5′-TGGCACCCAGCACAATGAA-3′
	(R) 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

(F) forward primer, *(R)* reverse primer

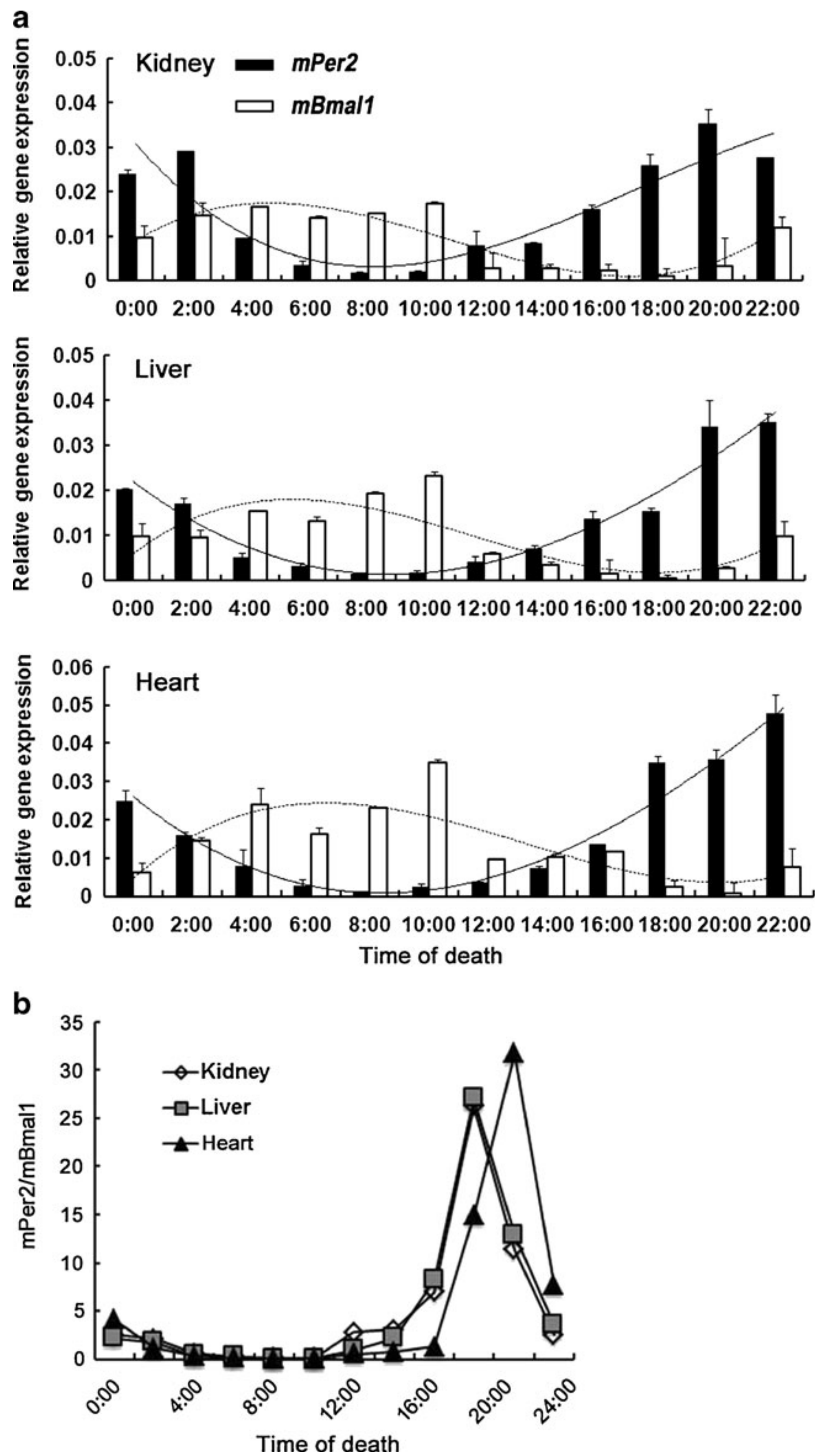

Fig. 1 Circadian expression profiles of *mPer2* and *mBmal1* in mouse tissue. **a** Gene expression for mPer2 and mBmal1 in mouse fresh tissues was analyzed by real-time PCR as described in the "Materials and methods" section. The results indicate the mean ± SEM (n=6). **b** Circadian oscillation profiles of mPer2/mBmal 1 ratio in fresh mouse tissues. The results indicate the mean (n=6)

expression was similarly detectable at 48 h after sacrifice. Since *mBmal1* mRNA was more stable than *mPer2*, mPer2/mBmal1 ratio gradually decreased according to postmortem interval. However, circadian oscillation of the mPer2/mBmal1 ratio could still be observed in samples with 48 h postmortem interval (Fig. 2c). Similar observations were obtained in analyses of mPer2/mBmal1 ratios in the liver and heart samples (data not shown). These observations implied that mPer2/mBmal1 ratios might be useful for the estimation of the time of death.

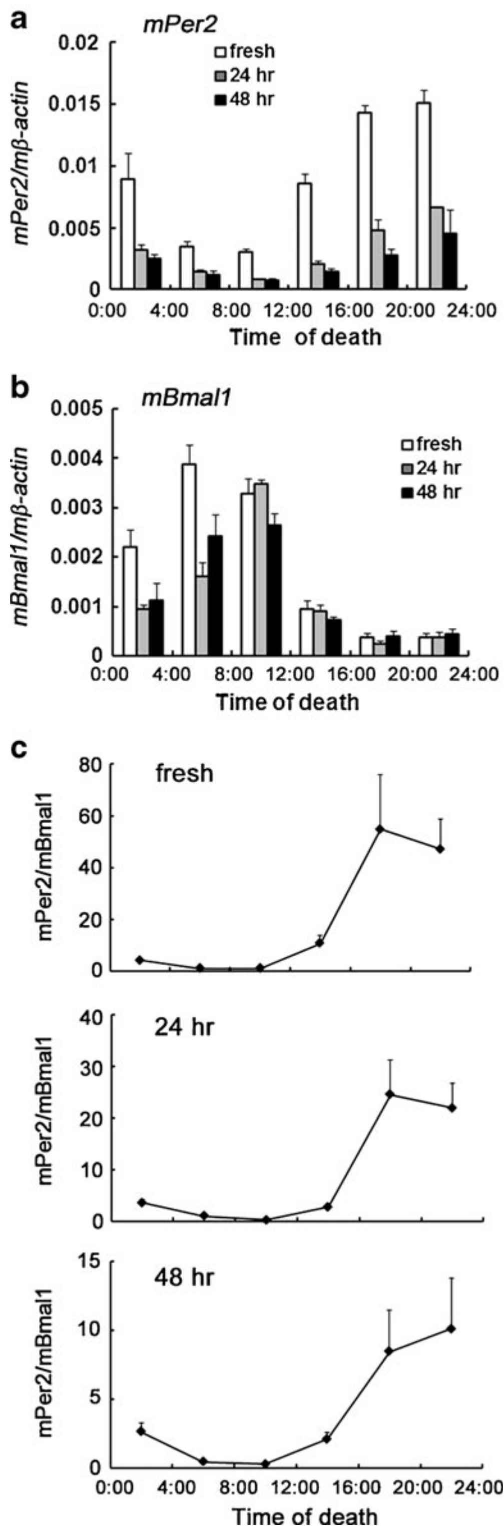

Fig. 2 Effects of postmortem interval on detection levels of *mPer2* and *mBmal1* gene expression in mouse kidney. Changes of detection levels of gene expression for **a** mPer2 and **b** mBmal1 during postmortem interval. The results indicate the mean ± SEM (n=6). **c** Changes of mPer2/mBmal1 ratio during postmortem interval. The results indicate the mean ± SEM (n=6)

Biological clock in tissues from autopsy cases

Gene expression levels for hBmal1, hPer2, and retinoic acid-related orphan nuclear receptor (hRev-Erbα) in the kidneys, livers, and hearts obtained from autopsy cases with defined time of death (less than 72 h) were analyzed by real-time PCR (Fig. 3). Circadian oscillation of *hBmal1* gene expression was clearly observed in these tissues. On the other hand, *hPer2* gene expression showed somewhat irregular patterns in these tissues, especially in the kidney (Fig. 3a–c). Therefore, we further examined another oscillator gene, *hRev-Erb*α in circadian clock system. The gene expression pattern for hRev-Erbα showed a clearer circadian rhythm in these tissues (Fig. 3d–f).

Circadian oscillation could be detected in both hPer2/hBmal1 (Fig. 4a) and hRev-Erbα/hBmal1 (hRev/hBmal1) ratios (Fig. 4b) in the kidneys, livers, and hearts from autopsy samples, which were high in the morning and low in the night as mirror images of the mPer2/mBmal1 ratio in mouse samples. These observations were not influenced by gender, age, postmortem interval, or the cause of death (data not shown).

The mean values of hPer2/hBmal1 ratio and hRev/hBmal1 ratio in the three organs showed more distinct circadian oscillation (Fig. 4c and d). However, the amplitude of circadian oscillation of hRev/hBmal1 ratio was much higher than that of hPer2/hBmal1 ratio. Thus, from the aspects of forensic safety, the hRev/hBmal1 ratio was more useful for estimating the time of death in forensic practice. Out of 38 cases, 11 cases had a hRev/hBmal1 ratio of more than 50. Among the 11 cases, ten cases had a time of death of 0200–0800 hours, together with a hRev/hBmal1 ratio of more than 75. The remaining one had a time of death of 0900 hours with a hRev/hBmal1 ratio of about 75. These observations implied that a hRev/hBmal1 ratio of >50 indicates a time of death of 0200–0900 hours and that a hRev/hBmal1 ratio that considerably exceeded 75 indicates a time of death of 0200–0800 hours. In contrast, 27 cases with a hRev/hBmal1 ratio of less than 50 had a time of death of 0000–0100 hours or 1000–2300 hours. In particular, a hRev/hBmal1 ratio of less than 25 strongly indicated a time of death of 1000–2300 hours.

Discussion

The circadian rhythm in biological, physiological, and behavioral processes has been found in organisms from cyanobacteria to humans [12, 13]. The molecular mechanisms of the biological clock that controls the circadian rhythm are essentially conserved from drosophila to mammals, including humans [14, 15]. The circadian clock system in mammals consists of a feedback loop with

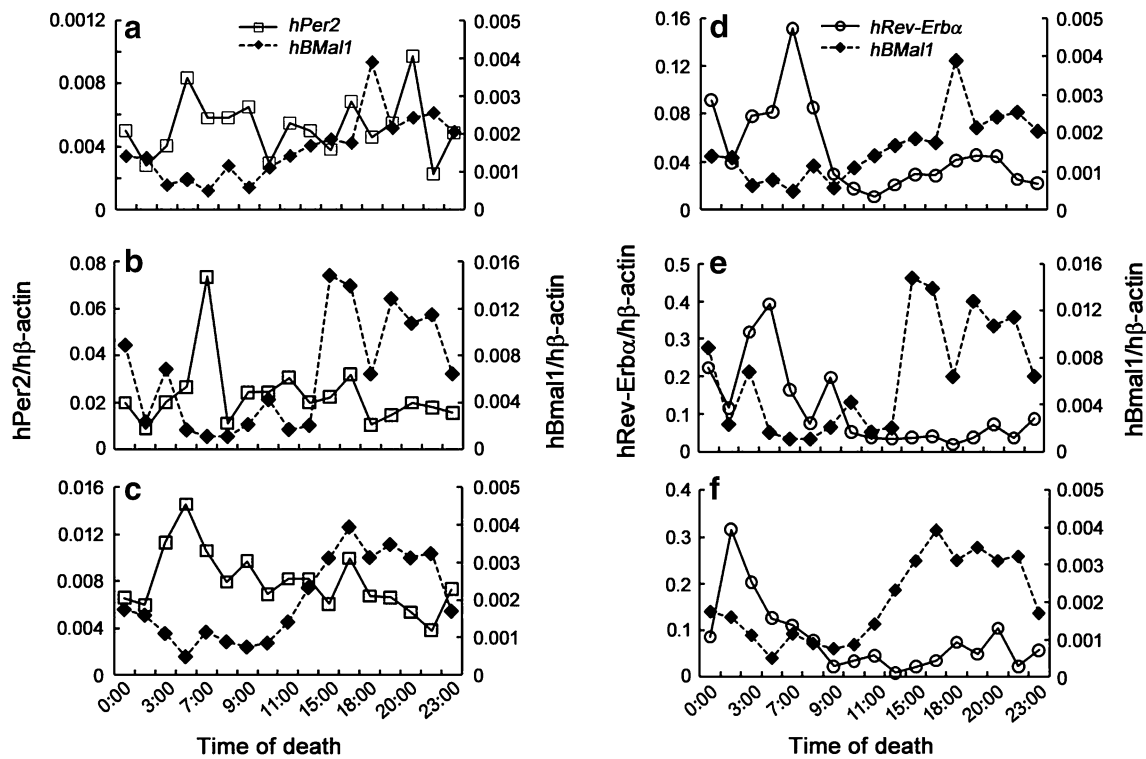

Fig. 3 Circadian gene expression profile for hPer2, hRev-Erbα, and hBmal1 in autopsy cases. Gene expression for **a**–**c** hPer2, **d**–**f** hRev-Erbα, and **a**–**f** *hBmal1* in the kidneys (**a** and **d**), livers (**b** and **e**), and hearts (**c** and **f**) from autopsy cases was analyzed by real-time PCR as described in the "Materials and methods" section. The results indicate the mean (n=1–4; numbers of samples are ≤4 at each time point)

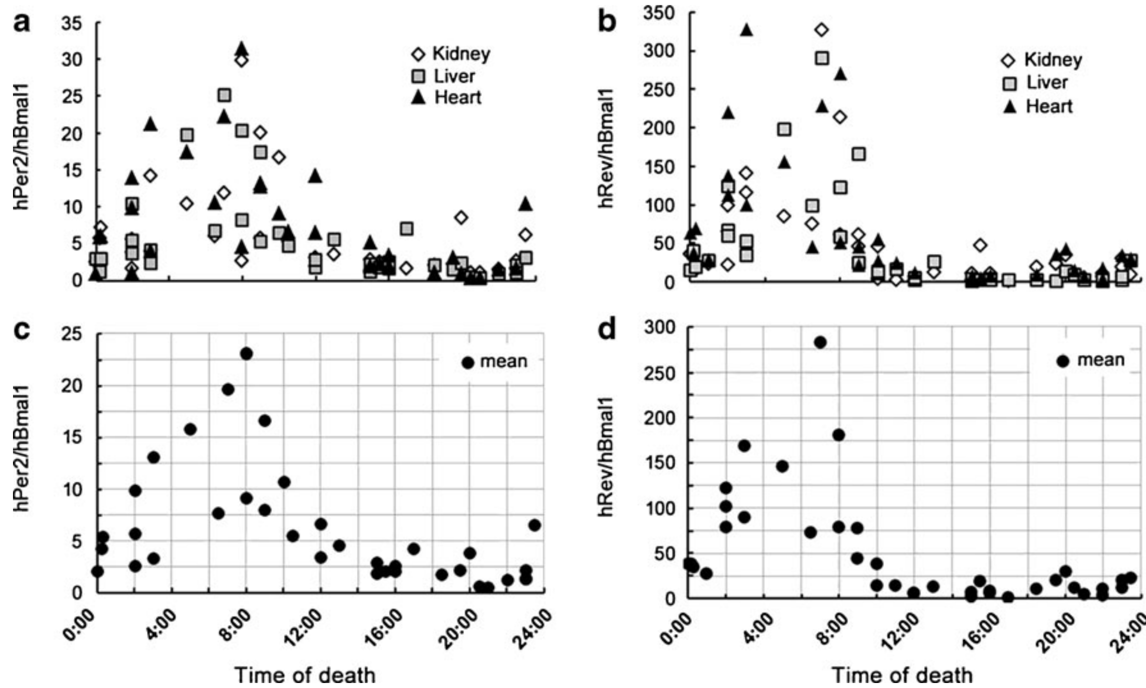

Fig. 4 Circadian oscillation profiles of **a** hPer2/hBmal1 ratio and **b** hRev/hBmal1 ratio in the kidney, liver, and heart from autopsy cases (n=38). The results indicate mean values of **c** hPer2/hBmal1 ratio and **d** hRevh/hBmal1 ratio of three tissues (kidney, liver, and heart)

Bmal1–Clock and Period–Cryptochrome (Per–Cry) complexes. Bmal1–Clock complex acts as a transcriptional activator for Per and Cry. In contrast, Per forms a complex with Cry and inhibits the activity of the Bmal1–Clock complex, which drives the circadian biological clock [16, 17]. Thus, if the biological clock could be read in corpses, it might be useful for estimating the time of death in forensic practice. In this study, we tried to read the biological clock using gene expression levels for three oscillator molecules, Bmal1, Per2, and Rev-Erbα1 by real-time RT-PCR.

We found clear and rigid oscillation of *mBmal1* and *mPer2* gene expression in mouse fresh kidney, liver, and heart, implying that the strictly controlled breeding condition (light/dark cycle) of mice produces a rigid circadian rhythm. Since *mBmal1* and *mPer2* gene expression oscillates in the anti-phase, mPer2/mBmal1 ratio oscillates at a higher amplitude. Therefore, the mPer2/mBmal1 ratio seems to be a suitable parameter for reading the biological clock.

Accumulated evidence indicates that mRNA in a dead body without advanced postmortem changes is stable [18, 19] and can be used for forensic purposes [20, 21]. However, mRNA for inducible genes is less stable than that for housekeeping genes such as *β-actin* [22]. In this study, we used mRNA for β-actin as an internal standard to quantify mRNA for circadian oscillators. Therefore, although the detection level of mRNA for Bmal1 and Per2 gradually decreased in a manner dependent on postmortem interval, circadian oscillation of *mBmal1* and *mPer2* gene expression and mPer2/mBmal1 ratio could be detected at least 48 h after death in mouse experiments.

In contrast to the mouse experiments, the gene expression pattern of *hPer2* did not show a rigid circadian rhythm in autopsy cases. This is explainable by the different lifestyles that affect the gene expression pattern of *hPer2*. On the other hand, *hBmal1* and *hRev-Erbα* gene expressions showed a clearer circadian rhythm in the tissues, indicating that individual lifestyle has less of an effect on *hBmal1* and *hRev-Erbα* gene expression patterns. Therefore, hRev/hBmal1 ratio was considered to be more useful for reading the biological clock in human tissues.

In the present study, we could not find a significant effect of sex, age, cause of death, and postmortem interval on the hRev/hBmal1 ratio in the tissues from autopsy cases. However, several lines of evidence indicate that some diseases, work style, and feeding pattern affect the biological clock in peripheral tissues. Attenuated gene expression for components of the circadian clock system was observed in diabetic mice [23] and patients with type 2 diabetes [24]. Disruption of clock gene expression was observed in pineal body of patients with Alzheimer's disease [25]. Entrainment of the clock in blood cells was observed in human subjects undergoing a stimulated night shift protocol with bright light treatment [26], indicating that the expression pattern of biological clock genes may shift in night workers. Significant alteration of the expression pattern of biological clock genes in peripheral tissues but not SCN was observed in mice bred with a restricted period of feeding [27, 28]. Upon the application of the present methods to forensic practices, information on disease and lifestyle of cadavers should be taken into consideration.

A method for estimating the time of death based on the amount of melatonin in pineal body, serum, and urine was reported [29]. It is well-known that the amount of melatonin shows a significant circadian rhythm and is high in nighttime and low in daytime in pineal body, serum, and urine. Since the biological clock controls gene expression of the enzymes for melatonin synthesis [30], the measurement of melatonin can be used to indirectly read the biological clock. Thus, the combination of clock gene expression with melatonin measurement would make the estimation of the time of death more accurate.

Finally, it is needless to say that uncertainty inevitably exists in the estimation of the time of death in forensic practice. To minimize the uncertainty in estimating the time of death, we should conduct it on the basis of a lot of parameters. Our present method for estimating the time of death is a concurrence method and is quite different from conventional methods based on postmortem changes. Thus, we emphasized that this method may be helpful in forensic practices, but that more investigations have to be performed. Especially, our method combined with others such as body temperature method of Henssge [31, 32] will contribute to increasing the accuracy of estimating the time of death in forensic practices.

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References

1. Mallach HJ (1964) Zur Frage der Todeszeitbestimmung. Berl Med 18:577–582
2. Kobayashi M, Takatori T, Nakajima M, Sakurada K, Hatanaka K, Ikegaya H, Matsuda Y, Iwase H (2000) Onset of rigor mortis is earlier in red muscle than in white muscle. Int J Leg Med 113:240–243
3. Marshall TK, Hoare FE (1962) The use of the body temperature in estimating the time of death. J Forensic Sci 7:211–221
4. Vanezis P, Trujillo O (1996) Evaluation of hypostasis using a colorimeter measuring system and its application to assessment of the post-mortem interval (time of death). Forensic Sci Int 78:19–28
5. Coe JI (1989) Vitreous potassium as a measure of the postmortem interval: an historical review and critical evaluation. Forensic Sci Int 42:201–213

6. Muñoz JI, Suárez-Peñaranda JM, Otero XL, Rodríguez-Calvo MS, Costas E, Miguéns X, Concheiro L (2001) A new perspective in the estimation of postmorteminterval (PMI) based on vitreous. J Forensic Sci 46:209–214
7. Matoba K, Terazawa K (2008) Estimation of the time of death of decomposed or skeletonized bodies found outdoors in cold season in Sapporo city, located in the northern district of Japan. Leg Med (Tokyo) 10:78–82
8. Horowitz PV, Pounder DJ (1985) Gastric emptying-forensic implications of current concepts. Med Sci Law 25:201–214
9. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109:307–320
10. Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. Nature 417:78–83
11. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci USA 101:5339–5346
12. Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290
13. Golden SS, Ishiura M, Johnson CH, Kondo T (1997) Cyanobacterial circadian rhythms. Annu Rev Plant Physiol Plant Mol Biol 48:327–354
14. Helfrich-Förster C, Edwards T, Yasuyama K, Wisotzki B, Schneuwly S, Stanewsky R, Meinertzhagen IA, Hofbauer A (2002) The extraretinal eyelet of Drosophila: development, ultrastructure, and putative circadian function. J Neurosci 22:9255–9266
15. Stanewsky R (2003) Genetic analysis of the circadian system in Drosophila melanogaster and mammals. J Neurobiol 54:111–147
16. Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Hum Mol Genet 15:R271–R277
17. DeBruyne JP, Weaver DR, Reppert SM (2007) CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat Neurosci 10:543–545
18. Johnson SA, Morgan DG, Finch CE (1986) Extensive postmortem stability of RNA from rat and human brain. J Neurosci Res 16:267–280
19. Marchuk L, Sciore P, Reno C, Frank CB, Hart DA (1998) Postmortem stability of total RNA isolated from rabbit ligament, tendon and cartilage. Biochim Biophys Acta 1379:171–177
20. Hayashi T, Ishida Y, Mizunuma S, Kimura A, Kondo T (2009) Differential diagnosis between freshwater drowning and saltwater drowning based on intrapulmonary aquaporin-5 expression. Int J Leg Med 123:7–13
21. Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U (2007) Successful RNA extraction from various human postmortem tissues. Int J Leg Med 121:136–142
22. Inoue H, Kimura A, Tsuji T (2002) Degradation profile of mRNA in a dead rat body: basic semi-quantification study. Forensic Sci Int 130:127–132
23. Ando H, Oshima Y, Yanagihara H, Hayashi Y, Takamura T, Kaneko S, Fujimura A (2006) Profile of rhythmic gene expression in the livers of obese diabetic KK-A(y) mice. Biochem Biophys Res Commun 346:1297–1302
24. Ando H, Takamura T, Matsuzawa-Nagata N, Shima KR, Eto T, Misu H, Shiramoto M, Tsuru T, Irie S, Fujimura A, Kaneko S (2009) Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. Diabetologia 52(2):329–335
25. Wu YH, Fischer DF, Kalsbeek A, Garidou-Boof ML, van der Vliet J, van Heijningen C, Liu RY, Zhou JN, Swaab DF (2006) Pineal clock gene oscillation is disturbed in Alzheimer's disease, due to functional disconnection from the "master clock". FASEB J 20:1874–1876
26. Cermakian N, Boivin DB (2006) The regulation of central and peripheral circadian clocks in humans. Obes Rev 10(Suppl 2):25–36
27. Hara R, Wan K, Wakamatsu H, Aida R, Moriya T, Akiyama M, Shibata S (2001) Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells 6:269–278
28. Schibler U, Ripperger J, Brown SA (2003) Peripheral circadian oscillators in mammals: time and food. J Biol Rhythm 18:250–260
29. Mikami H, Terazawa K, Takatori T, Tokudome S, Tsukamoto T, Haga K (1994) Estimation of time of death by quantification of melatonin in corpses. Int J Leg Med 107:42–51
30. Chong NW, Bernard M, Klein DC (2000) Characterization of the chicken serotonin N-acetyltransferase gene. Activation via clock gene heterodimer/E box interaction. J Biol Chem 275:32991–33299
31. Henssge C (1992) Rectal temperature time of death nomogram: dependence of corrective factors on the body weight under stronger thermic insulation conditions. Forensic Sci Int 54:51–66
32. Henssge C, Althaus L, Bolt J, Freislederer A, Haffner HT, Henssge CA, Hoppe B, Schneider V (2000) Experiences with a compound method for estimating the time since death. I. Rectal temperature nomogram for time since death. Int J Leg Med 113:303–319