# Altered Expression of Circadian Clock Genes in Human Chronic Myeloid Leukemia

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*Abstract* Circadian clock genes use transcriptional-translational feedback loops to control circadian rhythms. Recent studies have demonstrated that expression of some circadian clock genes displays daily oscillation in peripheral tissues including peripheral blood and bone marrow. Circadian rhythms regulate various functions of human body, and the disruption of circadian rhythm has been associated with cancer development and tumor progression. However, the direct links between aberrant circadian clock gene expression and human disorders remain largely unknown. In this study, comparisons were made between the expression profiles of 9 circadian clock genes from peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) from 18 healthy volunteers. Peripheral blood (PB) total leukocytes from 54 healthy volunteers and 95 patients with chronic myeloid leukemia (CML) were also investigated. Similar expression profiles of all 9 circadian clock genes were observed in PBMCs and PMNs of healthy individuals. In PB total leukocytes of healthy individuals, the daily pattern of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *CKIε* expression level peaked at 0800 h, and *BMAL1* peaked at 2000 h. Daily pattern expression of these 7 genes was disrupted in newly diagnosed pre–imatinib mesylate–treated and blast crisis–phase patients with CML. Partial daily pattern gene expression recoveries were observed in patients with CML with complete cytogenetic response and major molecular response. The expression of *CLOCK* and *TIM* did not show a time-dependent variation among the healthy and patients with CML. These results indicate a possible association of the disrupted daily patterns of circadian clock gene expression with the pathogenesis of CML.

*Key words* circadian clock genes, circadian rhythm, chronic myeloid leukemia (CML), imatinib mesylate, complete cytogenetic response (CCyR), major molecular response (MMR)

Circadian rhythm is present in humans and previously studied eukaryotes within a 24-h cycle. The daily rhythmic changes are exhibited in various physiological processes, such as sleep-activity, appetite, hormone levels, metabolism, and gene expression (Young and Kay, 2001; Strayer and Kay, 1999; Reppert

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and Weaver, 2002). The mammalian circadian system is composed of a central pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus and peripheral oscillators (Reppert and Weaver, 2002; Balsalobre, 2002; Shearman et al., 2000). The SCN clock is entrained to a 24-h day by the daily light-dark cycle through the retina-to-SCN neural pathways (Weaver, 1998). To date, at least 9 mammalian core circadian clock genes have been identified: *PERIOD1* (*PER1*), *PERIOD2* (*PER2*), *PERIOD3* (*PER3*), *CLOCK*, *CRYPTOCHROME1* (*CRY1*), *CRYPTOCHROME2* (*CRY2*), *BMAL1*, *CASEIN KINASE 1ε* (*CK1ε*), and *TIMLESS* (*TIM*) (Young and Kay, 2001; Strayer and Kay, 1999; Reppert and Weaver, 2002; Balsalobre, 2002). The transcriptional-translational feedback loops were used by the molecular components of the central and peripheral circadian oscillators. The loops rely on positively regulating genes (such as *CLOCK* or *BMAL1*) and negatively regulating genes (such as *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *TIM*) in the oscillators. Therefore, the marked characteristic of circadian systems is the prominent daily cycling of circadian clock gene mRNA and protein expression and downstream circadian clock-controlled gene RNA and protein expression (Reppert and Weaver, 2001).

In rodents, the oscillated expression of mammalian circadian clock genes is found not only in the SCN central pacemaker but also in peripheral tissues, such as liver (Takata et al., 2002), eye (Park and Kang, 2006), lung (Park and Kang, 2006), heart (Herichová et al., 2007), spleen (Park and Kang, 2006), and kidney (Herichová et al., 2007). Recently, the rhythmic expression of circadian clock genes in human peripheral tissues has been reported. The expression of *CLOCK*, *TIM*, *PER1*, *CRY1*, and *BMAL1* was shown in human oral mucosa and skin. The expression of *CLOCK* and *TIM* was not rhythmic, while peak rhythmic expression of *PER1*, *CRY1*, and *BMAL1* was displayed in the morning, late afternoon, and at night, respectively (Bjarnason et al., 2001). In human peripheral blood mononuclear cells (PBMCs), the circadian gene expressions of *PER1*, *PER2*, *PER3*, and *DEC1* peaked levels during the daytime (Takata et al., 2002; Boivin et al., 2003; Fukuya et al., 2007; Kusanagi et al., 2008; Archer et al., 2008; Hida et al., 2009), while *CLOCK* exhibited no daily variation (Takata et al., 2002; Kusanagi et al., 2008). The peak expression levels of *PER2* and *BMAL1* in the oral mucosa and skin occurred in the morning and–at night, respectively (Bjarnason et al., 2001). In PBMCs, in contrast, *PER2* and *BMAL1* had a synchronized pattern. Teboul and colleagues (20005) observed 2 different patterns of expression in *BMAL1*, with the peak and nadir levels occurring at habitual times of activity. The expression of *PER1* gene displayed a similar profile in both PBMCs and polymorphonuclear cells (PMNs), with peak expression found in the morning (Kusanagi et al., 2004). In human $CD34^+$ cells, circadian rhythms of *PER1*, *PER2*, and *CRY2* were also observed (Tsinkalovsky et al., 2007).

Disruption of circadian rhythms has been associated with cancer development and tumor progression (Hastings et al., 2003; Filipski et al., 2002; Fu and Lee, 2003). In SCN-destructed mice, implanted osteosarcoma and pancreatic adenocarcinoma grew 2 to 3 times faster than in sham-operated mice (Filipski et al., 2002). A poor survival rate was associated with patients with damped rhythmic rest-activity cycle in metastatic colorectal cancer (Innominato et al., 2009; Mormont et al., 2000). Epidemiologic studies also suggested that circadian disruption is a critical risk factor for the tumorigenesis of breast cancer (Stevens, 2005; Schernhammer et al., 2003; Davis et al., 2001), colorectal cancer (Schernhammer et al., 2003), and prostate cancer (Zhu et al., 2006).

Chronic myeloid leukemia (CML) is a malignant hematopoietic stem cells disorder characterized by excessive proliferation of myeloid cells with differentiation in the bone marrow and peripheral blood (Sawyers, 1999). The incidence of CML is 1 to 2 cases/100 000/year, and the median age at diagnosis is close to 60 years (Baccarani and Dreyling, 2009). The natural history of CML begins from a benign chronic phase, but it eventually progresses into an accelerated phase and then a rapid fetal blastic crisis within 3 to 5 years. CML is characterized by Philadelphia (Ph) chromosome, designated t(9;22)(q34;q11), which generates a unique *BCR-ABL* fusion gene from the fusion of the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (Shtivelman et al., 1985). In the *BCR-ABL* fusion gene, the normally regulated tyrosine kinase located on the ABL protein was constitutively activated by the fused BCR gene. The activation of the tyrosine kinase activity is required for its oncogenic activity. Therefore, the identification and quantification of Ph-positive metaphases and *BCR-ABL* transcripts are valuable for diagnostic confirmation and for monitoring the response to therapy. Imatinib mesylate (Gleevec, STI571), a tyrosine kinase inhibitor, was the first molecularly targeted therapy used for Ph-positive CML. It selectively inhibits tyrosine kinase activity of BCR-ABL protein by occupying

the ATP-binding site of ABL domain (Schindler et al., 2000). Imatinib has proven to be very effective for treating patients with CML in chronic phase. With advances in technology, using real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) to monitor *BCR-ABL* fusion transcript provides more precise assessment of response to therapies as compared to bone marrow cytogenetics or fluorescence in situ hybridization (Hughes et al., 2006; Baccarani and Dreyling, 2009). With serial *BCR-ABL* transcript monitoring, the responses to imatinib treatment for individual patients with CML can be monitored and the risk of disease progression can be predicted. The ideal responses to imatinib treatments are remissions from the initial complete hematological response (CHR) to major cytogenetic response (MCR) and finally to the disappearance of Ph chromosome (complete cytogenetic response; CCyR) and marked reduction of *BCR-ABL* (major molecular response; MMR).

Therefore, in this study we investigated the daily patterns of circadian clock gene expression in human chronic myeloid leukemia (CML) to elucidate the association between the disturbed daily pattern of circadian clock gene expression and this particular disease. Real-time quantitative RT-PCR was used to analyze the daily expression pattern of the 9 circadian clock genes in the peripheral blood total leukocytes from 54 healthy volunteers and 95 patients with CML.

## MATERIALS AND METHODS

### Healthy Subjects, Patients, and Samples

From September 2004 throughout July 2007, 54 healthy volunteers (41 men and 13 women) aged 22 to 69 years (mean ± SD, 32.15 ± 11.02) and 95 hospitalized patients with CML were investigated in this study. For the comparison of circadian clock gene expression between PBMCs and PMNs, another group of 18 healthy volunteers (10 men and 8 women) aged 26 to 36 years (mean ± SD, 29.39 ± 2.62) were also investigated between February 2007 and September 2009. Subjects included in this study did not experience jet lag or shift work 1 week before the experiment. No female individuals enrolled in this study were in menstrual phases during the experiment. No hospitalization, psychiatric disease, or medications were taken by the healthy individuals 6 months before this experiment took place. The criteria for diagnosing chronic phase (CP), accelerated phase (AP), and blast crisis (BC) were as previously described (Kantarjian et al., 1988) and response and relapse criteria were as previously reported (Talpaz et al., 2002; Druker et al., 2006). CP was defined as less than 5% of blast cells in peripheral blood (PB) or bone marrow (BM). The criteria for AP was either 10% to 19% blasts in PB or BM or more than 20% blasts plus promyelocytes in PB or BM. CHR was defined as a leukocyte count less than $10 \times 10^9$/L, a platelet count of less than $450 \times 10^9$/L, fewer than 5% myelocytes and metamyelocytes, no blasts or promyelocytes, no extramedullary involvement, and no signs of the AP or BC. MCR was defined as 1% to 35% Ph-positive metaphases in at least 20 cells in metaphase per bone marrow sample. The criteria for CCyR and MMR were no Ph-positive metaphases and 3-log of greater reduction of BCR-ABL transcript as detected by real-time RT-PCR, respectively. Patients with CML enrolled in this study were categorized into 4 major groups: (1) newly diagnosed, pre–imatinib mesylate–treated CP patients ($n$ = 15), (2) AP and BC patients ($n$ = 9), (3) CHR and MCR patients ($n$ = 16), and (4) CCyR and MMR ($n$ = 55), and their clinical characteristics are listed in Table 1. The time schedule and the daily activity of healthy volunteers and patients with CML were not restricted but they were asked to have breakfast between 0700 and 0800, lunch between 1130 and 1330 h, and dinner between 1730 and 1930 h; they were also asked to go to sleep before 2400 h and wake up at around 0700 h. Before the experiment, all participants were asked to follow the prearranged schedule as described above strictly for 1 week before the experiment. Healthy volunteers continued their usual daily activities during the day and were hospitalized at night from 2000 to 0800 h in the single-bed room at the ward of Division of Hematology-Oncology, Department of Internal Medicine, Kaohsiung Medical University Hospital. Collection of PB was carried out 4 time daily at 2000, 0200, 0800 (after breakfast), and 1400 h, respectively. For the PB collection at 0200 h, subjects were reclined and blood was drawn under minimal (~20 lux) light density. All individuals fell asleep immediately after blood was drawn. All of the heparinized PB samples were processed within 4 h of collection. Ammonium chloride lysis buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA) was used to deplete red blood cells (RBC) from PB. Total leukocytes of PB from both the healthy volunteers and patients with CML were used for the analysis of circadian clock gene expression. For comparison of-circadian

**Table 1. Characteristics of patients with chronic myeloid leukemia (CML).**

| Characteristics | Onset[a] (*n* = 15) | AP/BC[b] (*n* = 9) | CHR/MCR[c] (*n* = 16) | CCyR/MMR[d] (*n* = 55) |
|---|---|---|---|---|
| **Sex** | | | | |
| **Male** | 8 | 2 | 9 | 29 |
| **Female** | 7 | 7 | 7 | 26 |
| **Median age, year (range)** | 35.0 (25–72) | 43.0 (21–80) | 42.0 (26–83) | 38.0 (16–74) |
| **No. of Ph-chromosome–positive patients (%)** | 15/15 (100) | 9/9 (100) | 15/16 (93.75) | 0/55 (0.0) |
| **No. of *BCR-ABL* fusion gene–positive patients (%)** | 15/15 (100) | 9/9 (100) | 16/16 (100) | 24/55 (43.6) |
| **Blast cells, %, mean ± SE** | 13.61 ± 1.38 | 52.40 ± 11.12 | 7.91 ± 1.04 | 3.97 ± 0.41 |
| **Complete blood count, mean ± SE** | | | | |
| **WBC, $10^9$/L** | 114.51 ± 29.82 | 96.95 ± 52.57 | 7.15 ± 1.71 | 4.99 ± 0.29 |
| **RBC, $10^9$/L** | 3.63 ± 0.27 | 3.14 ± 0.16 | 4.15 ± 0.20 | 3.98 ± 0.08 |
| **Platelet, $10^9$/L** | 537.87 ± 86.91 | 276.00 ± 113.89 | 197.88 ± 34.62 | 184.26 ± 8.56 |
| **Hemoglobin, g/dL** | 10.74 ± 0.61 | 9.36 ± 0.43 | 11.88 ± 0.51 | 12.23 ± 0.21 |

a. Onset: newly diagnosed pre–imatinib mesylate–treated patients with CML.
b. CML-AP/BC: accelerated-phase and blast crisis–phase patients with CML.
c. CML-CHR/MCR: patients with CML with complete hematologic response and major cytogenetic response.
d. CML-CCyR/MMR: patients with CML with complete cytogenetic response and major molecular response.

clock gene expression between PBMCs and PMNs, PB from another group of 18 healthy volunteers was separated into white blood cells (WBC) and RBC by dextran (GE Healthcare, Uppsala, Sweden) sedimentation and WBC was further separated into PBMCs and PMNs by Ficoll-Paque (GE Healthcare) density gradient centrifugation. This study was approved by the Institute Review Board of the Kaohsiung Medical University Hospital, and informed consents were obtained from all participants.

### Real-Time Quantitative RT-PCR Analysis of Circadian Clock Genes

Total RNA of each sample was extracted from total leukocytes, PBMCs, or PMNs using TRIzol reagent (Invitrogen, Carlsbad, CA). The 2 μg RNA input for cDNA synthesis was determined by spectrophotometric $OD_{260}$ measurement, and cDNA was generated with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI). The cDNA sequence of the 9 circadian clock genes was evaluated, and the specific forward and reverse primers and TaqMan probe were designed using Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). TaqMan MGB probes designed by the software were synthesized and labeled with appropriate fluorescent dyes by Applied Biosystems. Sequences of the forward and reverse primers and probes are listed in Supplementary Table S1. Expression of human housekeeping genes, *ACTB* (β-actin), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *HPRT* (hypoxanthine phosphoribosyltransferase), *18S* (18S ribosomal RNA), *TBP* (TATA box binding protein) and *POLR2A* (RNA polymerase II polypeptide A) were evaluated and validated for normalizing RNA expression in real-time quantitative RT-PCR of circadian clock genes. All 6 TaqMan endogenous controls were purchased from Applied Biosystems. All reactions were carried out in a 25-μL final volume containing 50 ng of cDNA (as total input RNA), 400 nM each primer, 200 nM probe, and 12.5 μL 2x TaqMan Universal PCR Master Mix (Applied Biosystems). Real-time quantitative PCR was performed in an ABI 7700 Sequence Detector (Applied Biosystems). and the PCR cycling parameters were set as follows: 95 °C for 10 min followed by 40 cycles of PCR reactions at 95 °C for 20 sec and 60 °C for 1 min. Expression of validated endogenous control gene was also examined by real-time RT-PCR as the internal control for normalization of target gene expression. The amount of circadian clock gene was normalized to the endogenous reference gene to obtain the relative threshold cycle ($\Delta C_T$). The relative gene expression was calculated by equalizing differences to the $\Delta C_T$ of 0800 h in healthy individuals (i.e., $2^{-(\Delta C_T \text{ of X} - \Delta C_T \text{ of 0800 h})}$), and values were normalized so that the expression level of healthy individuals at 0800 h equaled 1.0.

### Statistical Analysis

Repeated-measures ANOVA was used to detect the differences among 4 different time points in each circadian clock gene expression; the comparison of daily patterns between different groups of individuals in each circadian clock gene expression and the comparison of gene expression of the same gene at the same time point between 2 different groups were evaluated with post hoc comparison under repeated-measures ANOVA. The values of $\Delta C_T$ were used for all the statistical analysis. All tests were 2-sided with statistical significance set at 0.05, and all computations were made using SPSS for Windows Release 13.0 software (SPSS, Chicago, IL).

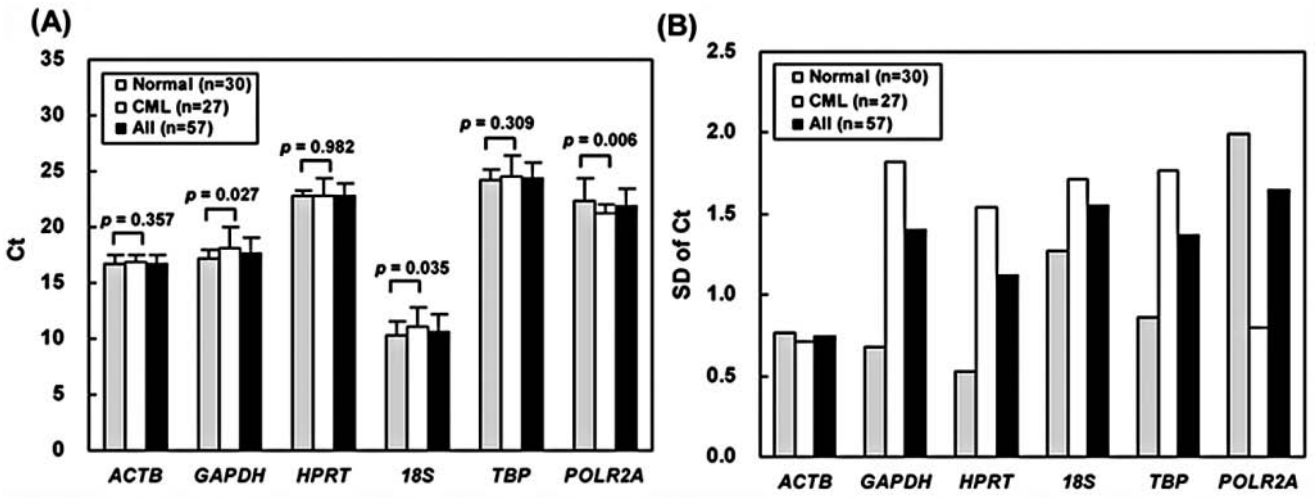


**Figure 1. mRNA expression of 6 endogenous control genes in peripheral blood samples measured by real-time quantitative RT-PCR. (A) The average $C_T$ with standard deviation (SD). Normal: healthy individuals; CML: patients with chronic myeloid leukemia; all: sum of healthy individuals and patients with CML. *ACTB* = β-actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *HPRT* = hypoxanthine phosphoribosyl-transferase; *18S* = 18S ribosomal RNA; *TBP* = TATA box binding protein; POLR2A = RNA polymerase II polypeptide A. Error bars are SD. (B) Variation of 6 human endogenous controls as measured by SD of $C_T$. (Annotation as for Figure 1A).**

## RESULTS

### Evaluation and Validation of Housekeeping Genes for Real-Time Quantitative RT-PCR of Circadian Clock Genes

To identify the best candidate gene to be use as an endogenous control for normalization of circadian clock genes, real-time quantitative RT-PCR analysis of 6 human housekeeping genes, *ACTB*, *GAPDH*, *HPRT1*, *18S*, *TBP*, and *POLR2A*, was performed on PB total leukocytes from 30 healthy individuals and 27 patients with CML. As shown in Figure 1A, the expression of genes showed less difference between healthy individuals and patients with CML: *HPRT* ($p = 0.982$), *ACTB* ($p = 0.357$), and *TBP* ($p = 0.309$). The SD was used to identify the gene with the least variation among the 6 endogenous control genes. The endogenous control genes with the lowest standard deviation across all the samples were *ACTB* (0.74), *HPRT* (1.12), and *TBP* (1.37) (Figure 1B). *ACTB*, *HPRT*, and *TBP* were shown to be suitable candidate genes for this study. Since circadian clock genes displayed a wide expression range with the $C_T$ values between 20 and 30, to obtain a positive value of normalized relative threshold cycle ($\Delta C_T$) for the expression of all the circadian clock genes, *ACTB* ($C_T$ values fell between 15.19 and 18.88) was chosen to be the most appropriate endogenous control gene for this study.

### Similar Circadian Pattern of Clock Gene Expression in Peripheral PBMCs and PMNs

PBMCs and PMNs from 18 healthy volunteers were examined for the expression of the 9 circadian clock genes using real-time quantitative RT-PCR to elucidate if their expression profiles of circadian clock genes are similar. Our data demonstrated that the daily patterns of the 9 circadian clock genes were similar in PBMCs and PMNs ($p > 0.05$) (Figure 2, A-I).

### Circadian Patterns of Circadian Clock Gene expression in Healthy Individuals

To investigate whether the circadian clock gene expression show a time-dependent variation in human PB total leukocytes, we analyzed PB total leukocytes from 54 healthy individuals using real-time quantitative RT-PCR. Repeated-measures ANOVA analyses indicated that in healthy individuals, among the 9 circadian clock genes analyzed, transcripts of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *CKIε* displayed a time-dependent variation pattern with a peak level at 0800 h (Figure 3, A-E, G) and *BMAL1* with peak expression level at 2000 PM (Figure 3H). Although the mean expression level of *BMAL1* was highest at 2000 h, we found that in 17 of the 54 healthy individuals, the peak *BMAL1* expression levels were at 0800 h. The transcripts of other 2 genes, *CLOCK* and *TIM*, did not display a time-dependent variation pattern (Figure 3F, 3I).

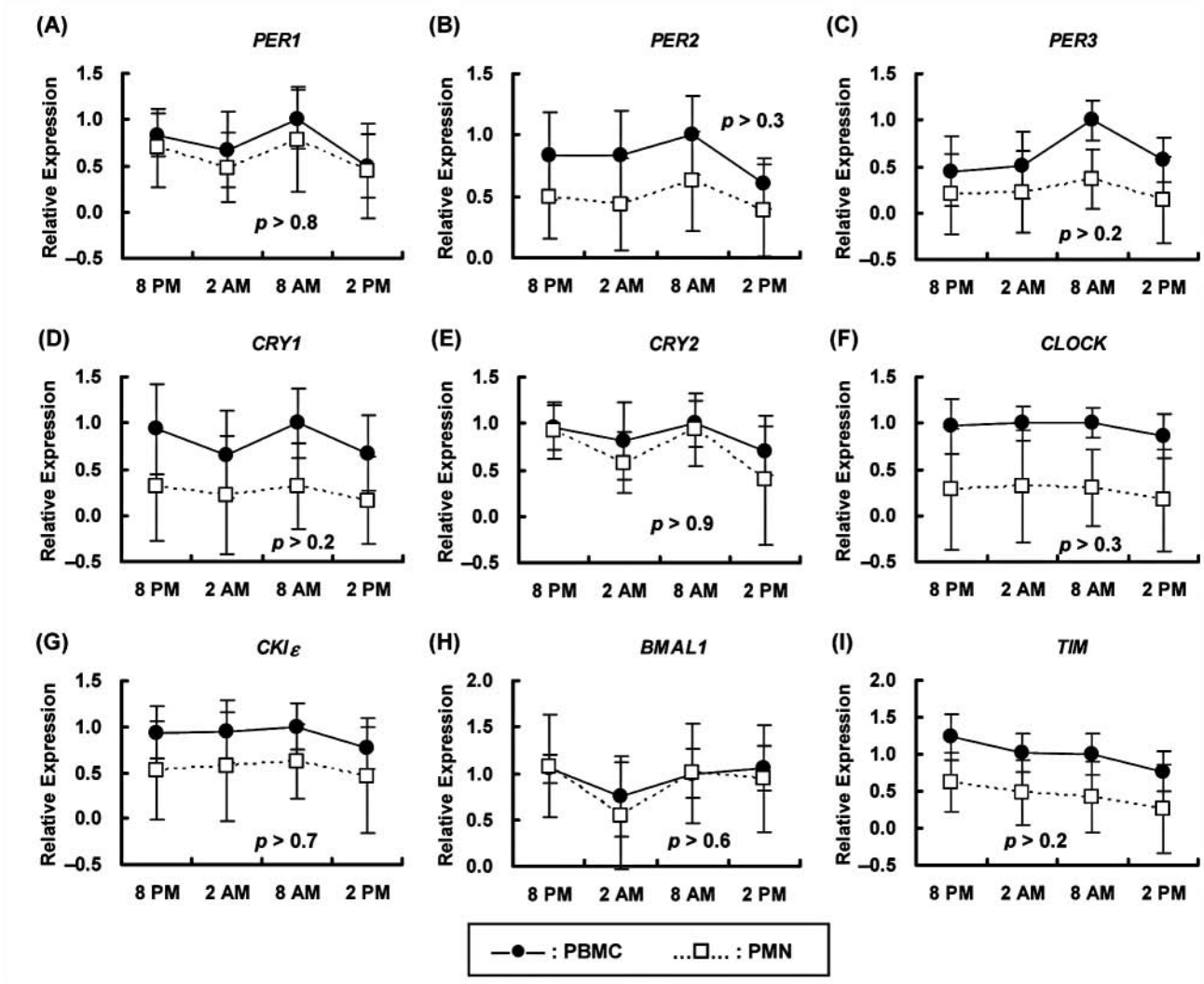


**Figure 2. Circadian patterns of 9 circadian clock genes in human PBMCs and PMNs of 18 healthy individuals. The x-axis indicates the time points that PB was collected. The y-axis represents the relative mRNA expression level. The value of the mRNA expression at 0800 h in PBMC is designated 1, and the levels of all other mRNA expression are calibrated to this value. Solid circles and open squares represent PBMCs and PMNs, respectively. The *p* values indicated are the statistical significance evaluated with repeated-measures ANOVA for comparison of the daily patterns between PBMCs and PMNs.**

### Abolished Daily Patterns of Circadian Clock Gene Expression in Patients with CML

The circadian patterns of the 9 circadian clock genes in patients with CML were also examined to determine whether the daily patterns were abolished in patients with CML. We analyzed PB total leukocytes from 95 patients with CML: 15 newly diagnosed pre–imatinib mesylate–treated, 9 AP/BC, 16 CHR/MCR, and 55 CCyR/MMR. Repeated-measures ANOVA analyses showed that the daily patterns of the 7 circadian clock genes that changed over time in healthy individuals were abolished in newly diagnosed, pre–imatinib mesylate–treated and AP/BC patients with CML (Figure 3, panels A-E, G, H). In CHR/MCR patients with CML, only *PER1*, *PER3*, and *CRY2* showed slight time-dependent variations (Figure 3, A, C, E). In CCyR/MMR patients with CML, transcripts of *PER1*, *PER2*, *PER3*, *CRY2*, and *CKI*ε also displayed time-dependent variations; however, the daily patterns were different from those in healthy individuals (Figure 3, A, B, C, E, G). The peak expression levels for *PER1*, *PER2*, *CRY2*, and *CKI*ε shifted from 0800 h to 2000 h. *PER3* shifted from 0800 h to 0200 h, all with insignificant changes. The daily patterns of expression showed no recovery for *CRY1* and *BMAL1*

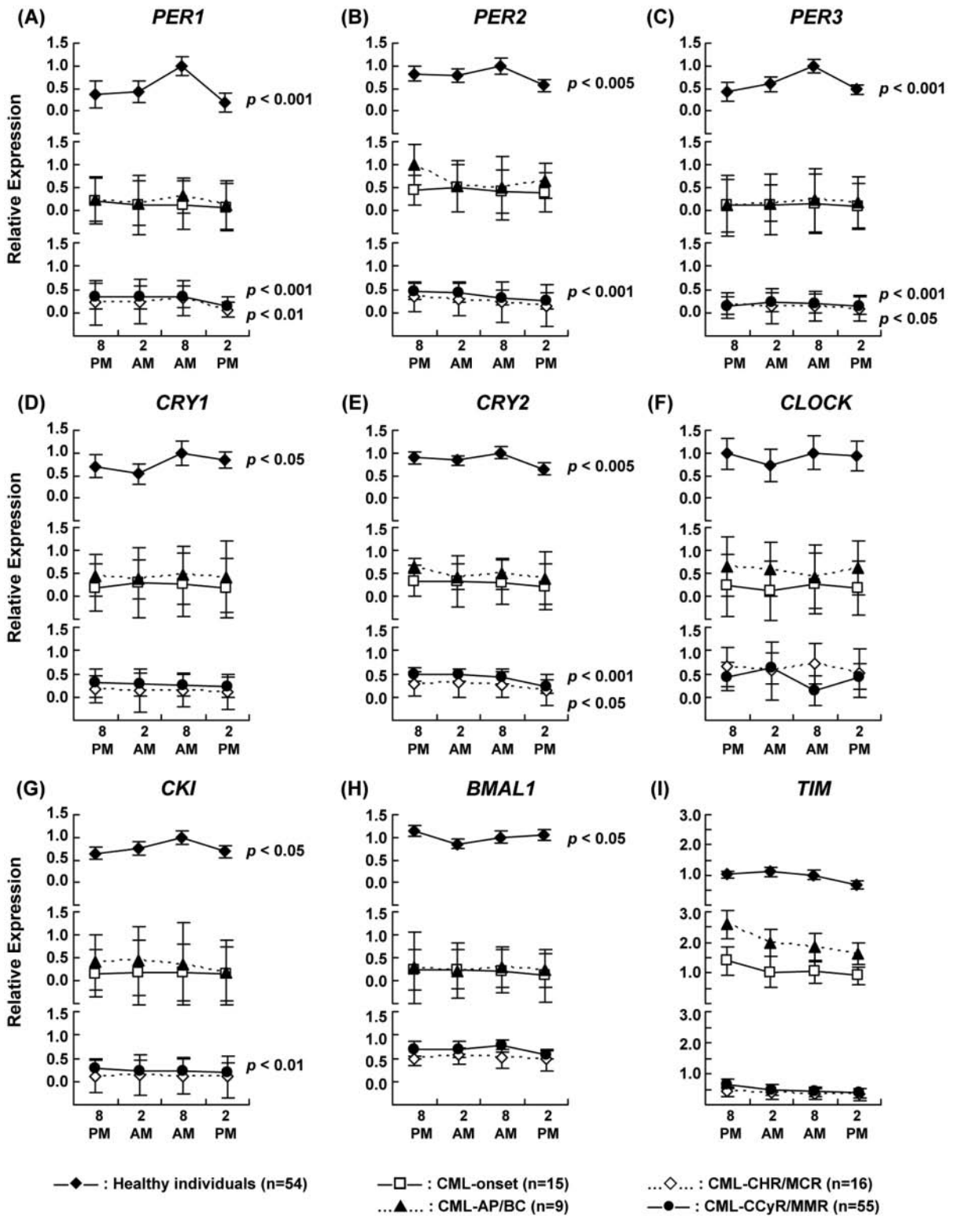


**Figure 3.** Circadian patterns of 9 circadian clock genes in human PB of 54 healthy individuals and 95 patients with CML. The *x*-axis indicates the time points that PB was collected. The y-axis represents the relative mRNA expression level. The value of the mRNA expression at 0800 h in healthy individuals is designated 1, and the levels of all other mRNA expression are calibrated to this value. Solid diamonds represent healthy individuals; open squares (CML onset) represent newly diagnosed, pre–imatinib mesylate–treated patients with CML; solid triangles (CML-AP/BC) represent accelerated-phase and blast crisis-phase patients with CML; open diamonds (CML-CHR/MCR) represent patients with CML with complete hematologic response and major cytogenetic response; solid circles (CML-CCyR/MMR) represent patients with CML with complete cytogenetic response and complete molecular response. $p < 0.05$, $p < 0.01$, $p < 0.005$, and $p < 0.001$ indicate the statistical significance evaluated with repeated-measures ANOVA.

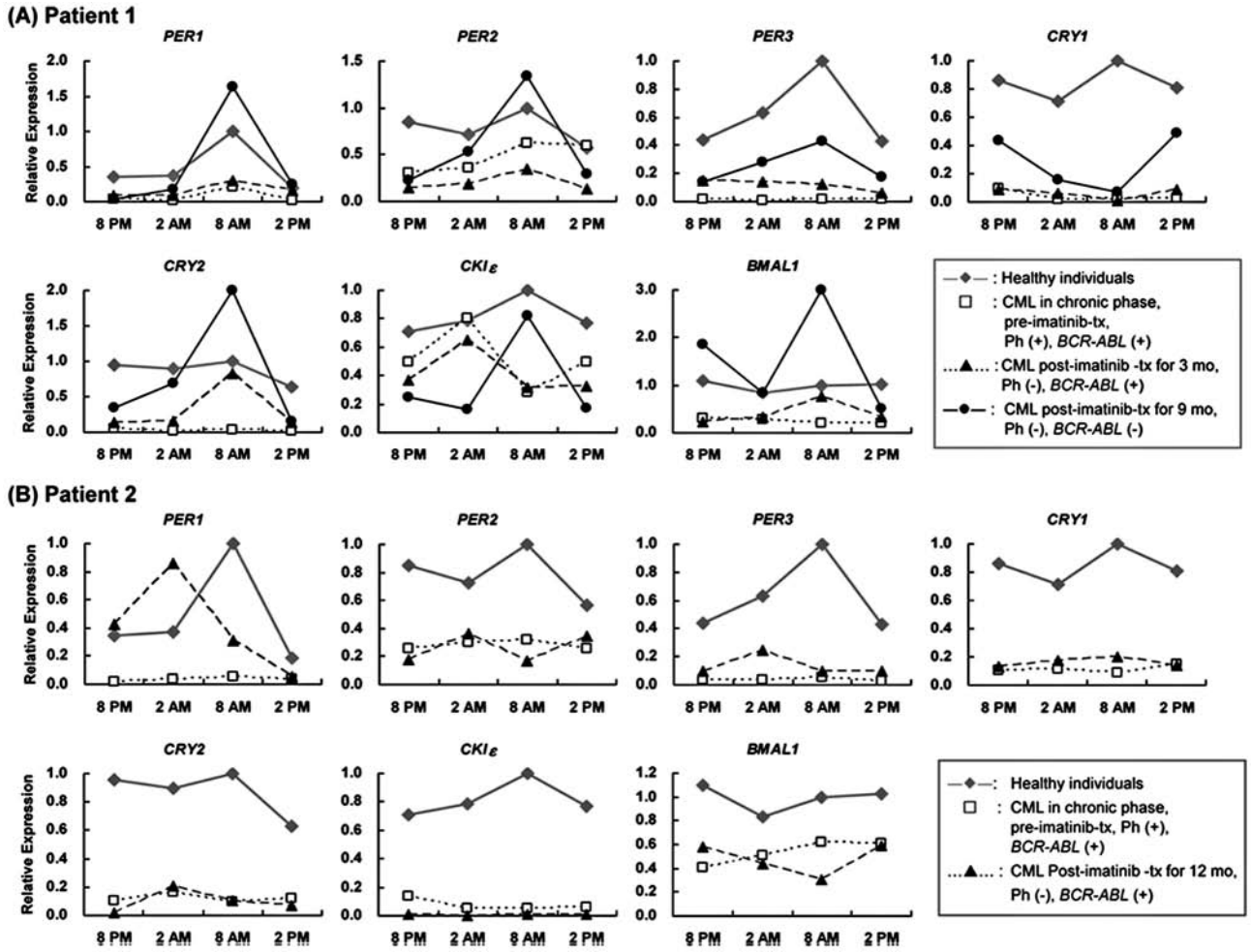


**Figure 4. Expression profiles of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CKIε*, and *BMAL1* of 2 representative patients with CML. The y-axis represents the relative mRNA expression level. The value of the mRNA expression at 0800 h in healthy individuals is designated 1, and the levels of all other mRNA expression are calibrated to this value. The x-axis indicates the time points that PB was collected. The expression profiles of healthy individuals (solid diamonds) are listed as references. Ph = t(9,22) Philadelphia chromosome; *BCR-ABL*: *BCR-ABL* fusion transcripts. In patient 1, the expression daily patterns of the 4 circadian clock genes have recovered to those similar in healthy individuals after imatinib mesylate treatment and achieved complete cytogenetic response and molecular response. In patient 2, the expression daily patterns similar to those in healthy individuals remain abolished if the patient did not achieve complete cytogenetic response and molecular response.**

(Figure 3D, 3H). In both healthy individuals and patients with CML, the expression nadirs of *PER1*, *PER2*, *PER3*, and *CRY2* genes were at 1400 h. The 2 genes that did not change over time in healthy individuals also did not display time-dependent variations in patients with CML.

Changes of Circadian Patterns of Circadian Clock Gene Expression After Imatinib Mesylate Treatment

The circadian patterns of circadian clock gene expression in CML patients during their treatments were monitored to elucidate the relationship between the maintenance of circadian clock gene expression rhythms and disease status. We found, in most cases, that the CCyR/MMR of disease was accompanied by recoveries of the expressed daily patterns and upregulations of these circadian clock genes. Figures 4 and 5 show 4 cases. In patients 1 and 3, the expressed daily patterns and expression levels of *PER1*, *PER2*, *PER3*, *CRY2*, *CKIε*, and *BMAL1* genes recovered to similar levels as compared to healthy individuals after imatinib mesylate treatment and achieved CCyR. In contrast, the expression daily patterns did not recover to normal in patients 2 and 4, in whom CCyR was not achieved. The expression levels of these 4 genes stayed low in patients 2 and 4. Two more cases are listed in Supplementary Figure S1.

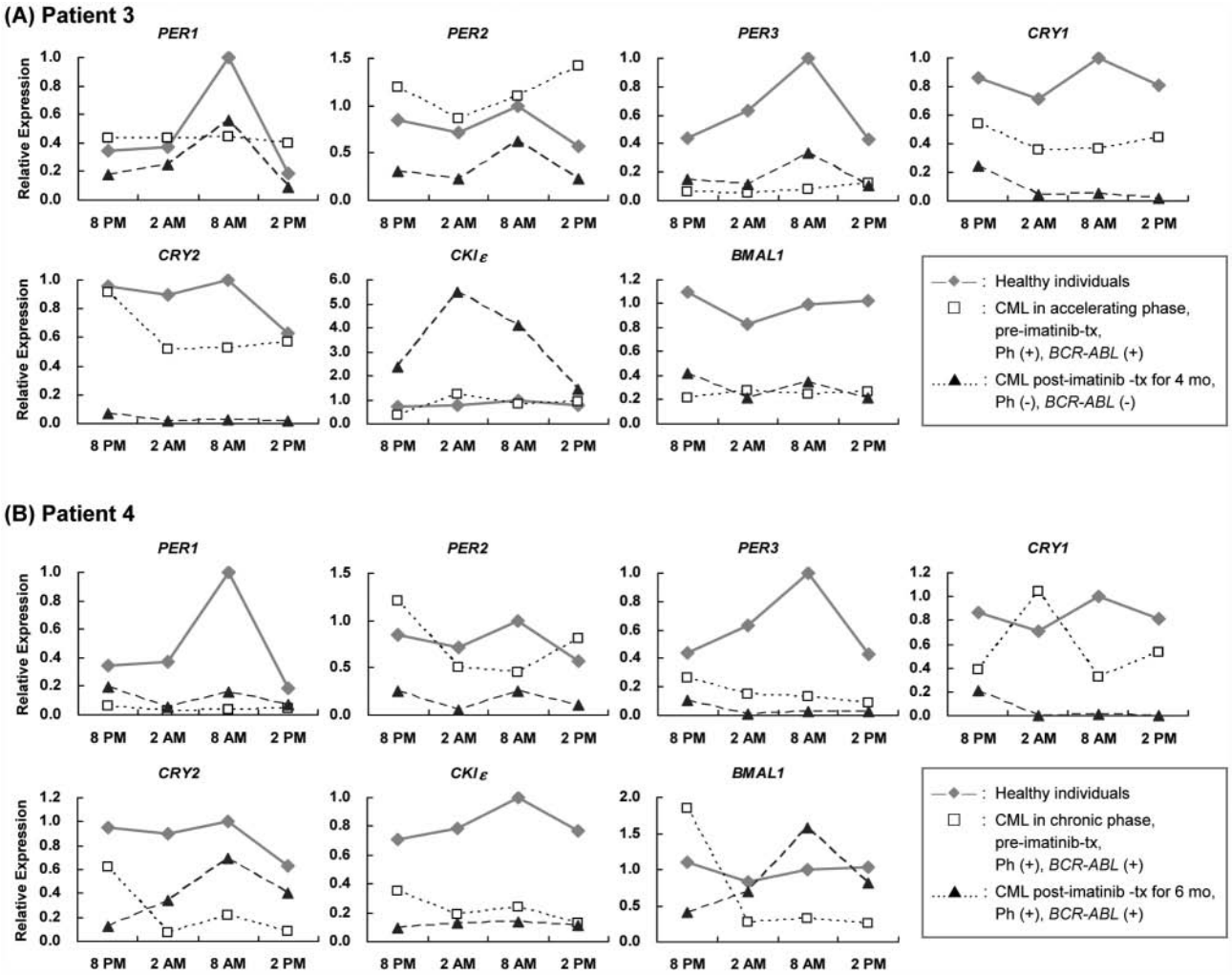


**Figure 5. The expression profiles of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CKIε*, and *BMAL1* of 2 post–imatinib mesylate–treated patients with CML, with expression daily patterns of the 4 circadian clock genes not exactly the same as those in healthy individuals. The y-axis represents the relative mRNA expression level. The value of the mRNA expression at 0800 h in healthy individuals is designated 1, and the levels of all other mRNA expression are calibrated to this value. The x-axis indicates the time points that PB was collected. The expression profiles of healthy individuals (diamonds) are listed as references. Ph = t(9,22) Philadelphia chromosome; *BCR-ABL* = *BCR-ABL* fusion transcripts. In patient 3, the expression daily patterns of *PER1*, *PER2*, and *PER3* recovered to the point they are similar to those in healthy individuals after imatinib mesylate treatment and CCyR, but the expression daily patterns of *CRY2* remained abolished. In patient 4, while still *BCR-ABL* positive after imatinib mesylate treatment, only the expression daily patterns of *PER3* remained abolished.**

### Downregulation of Circadian Clock Genes in Patients with CML

Transcripts of *PER2*, *PER3*, *CRY1*, *CRY2*, *CK1ε*, and *TIM* were significantly downregulated at 4 time points in CHR/MCR and CCyR/MMR patients with CML ($p < 0.001$). The expression of *PER1* in CHR/MCR and CCyR/MMR patients with CML at 2000 h and 0200 h were similar to healthy individuals but was significantly lowered at 0800 h ($p < 0.001$). In AP/BC patients with CML, transcripts of *CLOCK* were slightly decreased compared with healthy individuals but showed no differences in other patients with CML. At 0800 h, the expression levels of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CK1ε*, and *TIM* were significantly downregulated in CHR/MCR and CCyR/MMR patients with CML ($p < 0.001$).

## DISCUSSION

Circadian rhythms regulate various functions of human body (Hastings et al., 2003; Mormont et al., 2000). The mammalian circadian system is composed

of a central pacemaker in the SCN and peripheral oscillators (Reppert and Weaver, 2002; Balsalobre, 2002; Shearman et al., 2000). Both central and peripheral circadian oscillators use transcriptional-translational feedback loops that rely on PER, CLOCK, BMAL1, CRY, and TIM proteins. Since many genes involved in proliferation are under the control of circadian clock, maintaining the circadian clock rhythms can be a critical control point for cancer development and tumor progression (Lee, 2006).

In this study, we have analyzed the daily patterns of circadian clock gene expression in healthy individuals and patients with CML. Transcripts of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *CKIε* displayed daily patterns peaking at 0800 h and *BMAL1* had that peak expression level at 2000 PM in healthy individuals. After entering CCyR/MMR, partial recoveries of the daily patterns of *PER1*, *PER2*, *PER3*, *CRY2*, and *CKIε* genes were noted; however, the daily patterns were different from those in healthy individuals. Our results are consistent with previous reports demonstrating that in human PBMCs, expressions of *PER1*, *PER2*, *PER3*, and *DEC1* were rhythmic with the peak level in habitual time of activity (Boivin et al., 2003; Fukuya et al., 2007; Kusanagi et al., 2008; Archer et al., 2008); expressions of *PER1*, *PER2*, and *PER3* showed daily variation and expression levels were high in the morning (Takata et al., 2002; Kusanagi et al., 2004; Fukuya et al., 2007; Archer et al., 2008; Hida et al., 2009); and in bone marrow $CD34^+$ cells, expression of *PER1*, *PER2*, and *CRY2* showed circadian rhythms (Tsinkalovsky et al., 2007). The expression of *CLOCK* was arrhythmic in human PBMC (Kusanagi et al., 2008), bone marrow $CD34^+$ cells (Tsinkalovsky et al., 2007), and oral mucosa (Bjarnason et al., 2001). This coincided with our finding that the expression of *CLOCK* did not display time-dependent variations in PB total leukocytes of either healthy individuals and patients with CML. Our results also agreed with the finding in oral mucosa and skin that the expression levels of *PER2* peaked in the morning and *BMAL1* peaked at night (Bjarnason et al., 2001). However, in contrast to our results, Teboul and colleagues (2005) observed 2 patterns of *BMAL1* expression in PBMCs, with peak expression levels in the morning and at night, respectively. In fact, in our study, 17 of the 54 healthy individuals demonstrated peak *BMAL1* expression levels at 0800 h. This may also explain why the range of change of *BMAL1* daily pattern is smaller than other circadian clock genes that also showed daily patterns.

Similar *PER1* expression profiles have been demonstrated both in PBMCs and PMNs (Hida et al., 2009), and it was suggested that circadian clock gene activity could be preserved across different PB cell types. However, no further study supports the hypothesis that all of the circadian clock genes display similar expression profiles in PBMCs and PMNs. In this study, we have provided evidence for this hypothesis. It can be concluded that the expression patterns of circadian clock genes in either PBMCs or PMNs are compatible with those in total leukocytes.

The aberrant expression of circadian clock genes has recently been associated with many human cancers, such as CML (Yang et al., 2006), acute myeloid leukemia (Murga et al., 2003), chronic lymphocytic leukemia (Lewintre et al., 2008; Eisele et al., 2009), breast cancer (Chen et al., 2005; Winter et al., 2007), endometrial cancer (Shih et al., 2005), cervical cancer (Hsu et al., 2007), colon cancer (Krugluger et al., 2007), and non–small cell lung cancer (Gery et al., 2007). Large epidemiological studies also have suggested a link between circadian disruption and breast cancer (Stevens, 2005; Schernhammer et al., 2003; Davis et al., 2001), colorectal cancer (Schernhammer et al., 2003), and prostate cancer (Zhu et al., 2006). In this study, we have demonstrated that the expression levels of circadian clock genes were downregulated in the PB total leukocytes of patients with CML as well.

Imatinib mesylate is an effective tyrosine kinase inhibitor (Schindler et al., 2000) that can inhibit autophosphorylation of ABL, c-Kit, and platelet-derived growth factor receptor-β (Joensuu et al., 2001; Apperley et al., 2002). Imatinib mesylate has almost replaced allogeneic stem-cell transplantation and interferon-α treatments and has become the major treatment option for CML. Imatinib mesylate was very effective against newly diagnosed chronic-phase CML, in which the CHR rate and the CCyR rate were shown to be greater than 90% and 70% to 80%, respectively (Druker et al., 2006). AP and BC patients with CML were less sensitive to imatinib mesylate treatment. The majority of patients did not achieve CCyR after treatment (Baccarani et al., 2009). Imatinib mesylate resistance has hindered its useful application in treating CML, advanced CML in particular. In this study, we also observed the recoveries of downregulated expression and abolished daily patterns in patients with CML treated with imatinib mesylate and achieved CCyR/MMR (Figure 4, patient 1). The expression of circadian clock genes remained low and arrhythmic if the CML patient became resistant to imatinib

mesylate (Figure 4, patient 2). In some of the patients with CML studied, the circadian clock genes showed a time-dependent variation after imatinib mesylate treatment, but the patterns were not exactly the same as compared to healthy individuals (Figure 5). Therefore, we can hypothesize that the recovery of circadian clock gene daily patterns reflects the effectiveness of imatinib mesylate in treating patients with CML. The t(9,22) chromosomal aberration, which gives rise to fusion of the *ABL* and *BCR* genes and synthesis of a chimeric BCR-ABL p210 protein with deregulated tyrosine kinase activity, was believed to be the initiating event in human CML. Since it was shown that the circadian clock controls the expression of cell cycle–related genes (Matsuo et al., 2003), it has been assumed that the function of circadian clock genes is located upstream of the activity of BCR-ABL. Thus, when the circadian clock genes are functional, the activities of BCR-ABL can be inhibited. However, our results may provide an alternative hypothesis. Because patients with CML received imatinib mesylate treatment and achieved remission, which means they had a reduction in *BCR-ABL* genes, those patients displayed a recovery of time-dependent variation in circadian clock gene; in contrast, imatinib mesylate–refractory patients with CML (i.e., their *BCR-ABL* genes remained high) showed no recovery in circadian clock gene daily patterns. Therefore, it is also possible that the function of BCR-ABL is located upstream of the functions of circadian clock genes, which in turn control the cell cycle. The regulation network is too complicated to be explained by the limited case numbers (Figures 4, 5, and S1) in our study. Yet our observation reveals an import link between the level of *BCR-ABL* and the functions of circadian clock genes, and the regulatory mechanisms is worth elucidating.

It is still controversial whether the circadian rhythm disturbance is a carcinogenic effect itself. Since asynchrony of cell proliferation between normal and malignant tissues was commonly observed (Hrushesky et al., 1998; Bjarnason and Jordan, 2000; Granda and Lévi, 2002) and loss of circadian rhythmicity was commonly seen in patients with advanced cancers, the circadian rhythm disturbance may be both a cause and an effect of the disease. Disruption of the circadian clock gene expression rhythms may alter the balance that restrains and promote cell division, resulting in prosurvival and proliferation of tumor cells. Tumor cells accelerate their own growth by disrupting circadian rhythms, so restoring circadian rhythms in cancer patients should improve their prognoses. In this study, we provide evidence for these hypotheses, although the exact mechanisms remain to be elucidated.

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## NOTE

Supplementary online material for this article is available on the *Journal of Biological Rhythms* website: http://jbr.sagepub.com/supplemental.

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