Up-regulation of *PER3* Expression Is Correlated with Better Clinical Outcome in Acute Leukemia

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Abstract. *Background: Altered expression of circadian clock genes has been linked to various types of cancer. This study aimed to investigate whether these genes are also altered in acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). Materials and Methods: The expression profiles of nine circadian clock genes of peripheral blood (PB) leukocytes from patients with newly-diagnosed AML (n=41), ALL (n=23) and healthy individuals (n=51) were investigated. Results: In AML, the expression of period 1 (PER1), period 2 (PER2), period 3 (PER3), cryptochrome 1 (CRY1), cryptochrome 2 (CRY2), brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1), and timeless (TIM) was significantly down-regulated, while that of CK1ε was significantly up-regulated. In ALL, the expression of PER3 and CRY1 was significantly down-regulated, whereas those of CK1ε and TIM were significantly up-regulated. Recovery of PER3 expression was observed in both patients with AML and those with ALL who achieved remission but not in patients who relapsed after treatment. Conclusion: Circadian clock genes are altered in patients with acute leukemia and up-regulation of PER3 is correlated with a better clinical outcome.*

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Acute leukemia comprises about 20,000 cancer diagnoses and 10,000 deaths in the United States each year (1). Although most patients with acute leukemia achieve remission with advanced chemotherapy, relapse remains a major concern. The etiology of most acute leukemia remains unknown. Acute myeloid leukemia (AML) is a heterogeneous and complex hematological malignancy caused by de-regulation of multiple signaling pathways and is characterized by the clonal proliferation of myeloid precursors and reduced capacity of differentiation of these cells in the bone marrow and blood (2). The annual incidence of AML is three to five cases per 100,000 of the US and Taiwan populations (3, 4). Acute lymphoblastic leukemia (ALL) is an intractable hematological malignancy of T- or B-lymphoblasts characterized by the over-production and accumulation of lymphoblasts (5). ALL is the most common pediatric malignancy, accounting for about 25% of cancer cases occurring before the age of 15 years (6). The annual incidence of ALL is about one case per 100,000 of the Taiwan population (4). Overall survival for children with ALL exceeds 85%, however, for adults with ALL, it is very low (approximately 30% to 40%) (6). When treated with intensive chemotherapy or autologous hematopoietic stem cell transplantation, the incidence of relapse is high and the outcomes are unsatisfactory (7).

The circadian clock is an autonomous biological

pacemaker in organisms that drives a nearly 24-hour cycle of behavior and physiology. Many biochemical and physiological processes in humans exhibit daily rhythmic changes, such as sleep activity, body temperature, hormone and neurotransmitter release, metabolism and gene expression. Circadian rhythms are organized in a hierarchical fashion. The master pacemaker, the suprachiasmatic nucleus, is located in the anterior hypothalamus. Core circadian clock genes, period 1-3 *(PER1-3),* cryptochrome 1-2 *(CRY1-2),* clock *(CLOCK),* brain and muscle aryl hydrocarbon receptor nuclear translocator *(ARNT)*-like 1 *(BMAL1),* casein kinase 1 epsilon *(CK1E),* timeless *(TIM),* reverse strand of erb alpha *(REV-ERBA)*, and retinoic acid-related orphan receptor A (*RORA*), and the proteins encoded by these genes constitute the circadian oscillator and circadian rhythms (8). Every peripheral cell is also equipped with a molecular clock which regulates daily oscillations of gene expression (9). A recent genome-wide study of the mouse has demonstrated that 43% of all protein-coding genes showed circadian rhythms in transcription in a largely organ-specific manner (10). It is believed that the cell cycle, DNA repair, apoptosis, angiogenesis and immune functions are controlled by the molecular clock (9, 11). Maintaining a proper circadian clock function is crucial for an organism to respond to light and dark cycles properly.

Many epidemiological studies have linked altered circadian rhythms to cancer susceptibility, including to breast (12), ovarian (13), colorectal (14) and prostate (15) cancer, but the exact underlying mechanisms are still not fully understood. Altered expression of circadian clock genes has also been linked to various types of cancer, such as breast (16), ovarian (17), non-small cell lung (18), colorectal (19) and kidney cancer (20), and chronic lymphoid leukemia (21,22). We have also reported deregulation of circadian clock genes in chronic myeloid leukemia (CML) (23, 24), head and neck squamous cell carcinoma (HNSCC) (25, 26) and gastric cancer (27).

In the present study, we further analyzed the expression levels of the nine circadian clock genes in patients with acute leukemia to explore the possible role of these genes in leukemogenesis.

Materials and Methods

Patients, healthy individuals and samples. Peripheral blood (PB) samples were collected from 51 healthy adult volunteers (19 men and 32 women) aged 23 to 57 years (mean±SD=33.88 ± 6.75 years), 44 patients newly-diagnosed with AML and 23 newly-diagnosed with ALL from 1999 through 2003 from the Division of Hematology-Oncology, Department of Internal Medicine, Kaohsiung Medical University Hospital. Clinical characteristics including patients' sex, age, percentages of bone marrow blasts and French-American-British classification subtypes are listed in Table I. Collection of all PB samples was carried out between 8:00 AM and 11:00 AM and all the PB samples were processed within one hour 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 from PB. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to isolated PB leukocytes and the samples were frozen at −80˚C in a deep freezer until analysis. Informed consent was obtained from all patients and healthy adult volunteers prior to PB acquisition and this study was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital (approval number KMUH-IRB-990483).

Table I. *Characteristics of patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL).*

	AML	ALL
Characteristic	Number	Number
Gender		
Male	24	14
Female	20	9
Median age (range), years	44 (16-85)	43 (15-70)
Bone marrow blast cells (%), mean±SE	66.54±3.22	87.19±2.33
FAB subtype	M0:1	L1:8
	M1:3	L2:11
	M2:19	L3:4
	M3:6	
	M4:9	
	M5:4	
	M6:2	

FAB: French-American-British classification.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of circadian clock genes. Total RNA was extracted from PB total leukocytes using TRIzol reagent (Invitrogen). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to generate cDNA. The expression of the nine circadian clock genes were analyzed using the TaqMan® system and the sequences of the forward and reverse primers and TaqMan® MGB probes are as previously described (23). Expression of β-actin (*ACTB*) gene was used for normalizing expression of circadian clock genes in qRT-PCR. All reactions were carried out in a 20 μl final volume containing 50 ng cDNA (as total input RNA), 400 nM each primer, 200 nM probe, and 10 μl 2X TaqMan® Universal PCR Master Mix (Applied Biosystems). Real-time qPCR was performed in an ABI 7700 sequence detector (Applied Biosystems) and the PCR cycling parameters were 95˚C for 10 min followed by 40 cycles of PCR reactions at 95˚C for 30 sec and 60˚C for 1 min. The relative expression levels of the circadian clock genes were calculated by the comparative threshold cycle Ct (ΔΔCt) method. The Ct of each circadian clock gene was first normalized to the Ct of *ACTB* to obtain the relative threshold cycle (ΔCt). The relative fold change in expression between patients with acute leukemia and healthy individuals was then evaluated by calculating $2^{-\Delta\Delta Ct}$.

Statistical analysis. SPSS for Windows Release 15.0 software

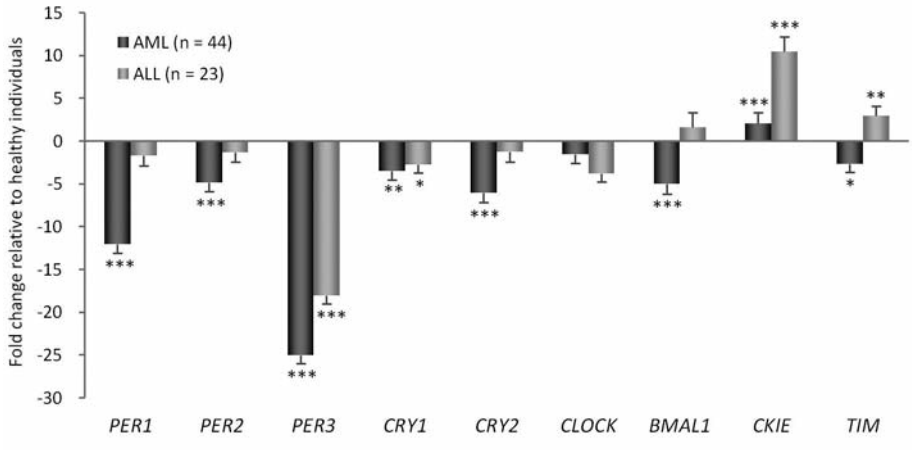

Figure 1. *Expression of circadian clock genes in peripheral blood (PB) leukocytes of patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) determined by real-time quantitative reverse-transcriptase polymerase chain reaction. Expression of the nine circadian clock genes in PB leukocytes from 44 patients newly diagnosed with AML, 23 patients newly diagnosed with ALL, and 51 healthy individuals were determined. Compared to the PB leukocytes of healthy individuals, the expression of period 1 (PER1), PER2, PER3, cryptochrome 1 (CRY1), CRY2, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1), and timeless (TIM) was significantly down-regulated ($p<0.05$), while that for casein kinase 1 epsilon (CK1E) was significantly up-regulated ($p<0.001$) in patients with AML. In patients with ALL, the expression of PER3, CRY1 was significantly down-regulated ($p<0.05$), whereas that of CK1E and TIM was significantly up-regulated ($p<0.01$). The y-axis represents the fold change of mRNA expression level of patients relative to healthy individuals. The relative expression in patients is calculated by the comparative Ct ($\Delta\Delta$Ct method). The mean mRNA expression in healthy individuals (n=51) was designated with a value of 1, whereas the level of mRNA expression in patients with AML or ALL is calibrated to obtain the fold change in patients with AML or ALL. Statistically significant at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.*

(SPSS, Chicago, IL, USA) was used to compute statistical analyses and the values of ΔCt were used for all the statistical analysis computation. The Student paired *t*-test was used to detect the differences between the expression of each circadian clock gene in PB leukocytes from patients before and after treatment. Pearson correlation analysis was used to detect the correlation between percentages of blast cells and expression of circadian clock genes. The tests were two-sided and a value of $p<0.05$ was considered statistical significant.

Results

Analysis of circadian clock gene expression in PB leukocytes using qRT-PCR. PB samples from 41 patients with AML, 23 patients with ALL and 51 healthy individuals were examined for the expression of the nine circadian clock genes using qRT-PCR to elucidate whether the expression levels of circadian clock genes were altered in patients with acute leukemia. Our data demonstrated that the expression of *PER1* ($p<0.001$), *PER2* ($p<0.001$), *PER3* ($p<0.001$), *CRY1* ($p<0.01$), *CRY2* ($p<0.001$), *BMAL1* ($p<0.001$), and *TIM* ($p<0.05$) was significantly down-regulated, while that for *CK1E* was significantly up-regulated ($p<0.001$) in patients with AML when compared to those in healthy individuals (Figure 1). In patients with ALL, the expression of *PER3* ($p<0.001$) and *CRY1* ($p<0.05$) was significantly down-regulated, whereas that of *CK1E* ($p<0.001$) and *TIM* ($p<0.01$) were significantly up-regulated when compared to those in healthy individuals (Figure 1).

Correlations between percentages of blast cells in bone marrow and expression of circadian clock genes in PB leukocytes of patients with AML and ALL. Over-production and accumulation of leukemic blast cells in bone marrow are characteristics of leukemia. We performed correlation analysis to investigate whether the numbers of blast cells in bone marrow correlated with the expression of circadian clock genes. We found that in patients with AML, increased blast cells in bone marrow correlated with decreased expression of *PER1* ($p=0.001$), *PER3* ($p=0.001$), *BMAL1* ($p=0.008$), and *CK1E* ($p=0.005$) (Table II). However, in patients with ALL, the number of blast cells in bone marrow was not correlated with expression of any of the genes (Table II).

Expression of circadian clock genes in PB leukocyte pre- and post-treatment of patients with AML and ALL. To investigate whether the altered expression of circadian clock genes in PB leukocytes of patients with AML or ALL recovers after treatment, we analyzed 20 and 16 paired pre- and post-treatment PB samples from patients with AML and ALL,

Table II. *Correlation between the percentage of blast cells in bone marrow and expression of circadian clock genes in peripheral blood leukocytes of patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL).*

Blasts in bone marrow (%) *vs*. expression of	AML	ALL
	Pearson correlation coefficient (*p*-Value)	Pearson correlation coefficient (*p*-Value)
Period 1 (PER1)	–0.354 (0.001)	0.067 (0.675)
Period 2 (PER2)	–0.151 (0.169)	0.053 (0.742)
Period 3 (PER3)	–0.367 (0.001)	–0.196 (0.219)
Cryptochrome 1 (CRY1)	0.038 (0.731)	0.144 (0.370)
Cryptochrome 2 (CRY2)	–0.061 (0.580)	0.097 (0.548)
Clock (CLOCK)	–0.214 (0.050)	–0.031 (0.845)
Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1)	–0.287 (0.008)	–0.085 (0.598)
Casein kinase 1 (CK1E)	–0.302 (0.005)	0.238 (0.133)
Timeless (TIM)	–0.203 (0.062)	0.179 (0.263)

respectively. Among the 20 patients with AML, 13 patients achieved remission and seven patients experienced relapse after treatment. Among the 16 patients with ALL, eight patients achieved remission and eight experienced relapse after treatment. In patients with AML, the expression of *PER1* (Figure 2A), *PER3* (Figure 2C) and *TIM* (Figure 2I) was significantly up-regulated in patients who achieved remission but remained low in patients whose disease relapsed after treatment. In patients with ALL, the expression of *PER3* (Figure 3C), *CRY2* (Figure 3E) and *BMAL1* (Figure 3G) was significantly up-regulated in patients with ALL who achieved remission but remained low in patients whose disease relapsed after treatment.

Discussion

Studies of human cancer and animal models have revealed that the disruption of circadian rhythms is an essential endogenous factor contributing to development of mammalian cancer (28). Our previous studies also confirm this issue in CML, HNSCC and gastric cancer (23-27). Only two studies have reported altered expression of circadian clock genes in acute leukemia, one reported down-regulation of *BMAL1* expression by methylation in patients with AML and ALL (29), and the other reported down-regulation of *PER2* expression in patients with AML (30); therefore in the present study, we further investigated the expression of the nine circadian clock genes in acute leukemia. We found the expression of *PER1, PER2, PER3, CRY1, CRY2, BMAL1* and *TIM* was significantly down-regulated and *CK1E* was significantly up-regulated in PB leukocytes in patients with AML, when compared to those from healthy individuals (Figure 1). In patients with ALL, the expression levels of *PER3* and *CRY1* were down-regulated and *CK1E* and *TIM* were significantly up-regulated in PB leukocytes when compared to those of healthy individuals (Figure 1). Our results suggest that the disturbance of circadian clock gene expression may play a role in the development of human acute leukemia.

PER1, *PER2* and *PER3* genes are members of the same PERIOD gene family but *PER3* was thought to be less important in regulating circadian clock. However, *PER1, PER3, PER3* and *deleted in esophageal cancer 1 (DEC1)* genes were found to be expressed in a similar circadian oscillation pattern (31), suggesting that the oscillation of *PER3* may also be essential in maintaining circadian rhythm. Moreover, down-regulation of *PER3* has also been observed in various types of cancer, including CML (23), breast cancer (16), HNSCC (25), lung cancer (32) and colorectal cancer (33, 34). Our previous study also observed that in patients with CML, the daily pattern expression of *PER3* was disrupted in patients with newly-diagnosed pre-imatinib mesylate-treated and crisis-phase patients and partial recovery was observed when patients achieved complete cytogenetic response or major molecular response (24). The fact that in patients with acute leukemia, *PER3* was the most down-regulated gene and recovery of *PER3* was correlated with better clinical outcome demonstrates the possibility that de-regulation of multiple molecular pathways play a role in the development of acute leukemia and at least one of them is caused by the tissue-specific inactivation of the *PER3* gene. In a mouse model, deficiency of *PER3* gene was shown to increase susceptibility to breast cancer induced by carcinogen treatment or by overexpression of erythroblastosis oncogene B2 *(Erbb2)* (35). A functional polymorphism in *PER3* gene (rs2640908) was shown to be associated with overall survival of patients with hepatocellular carcinoma (36). *PER3* has also been showed to be required for *checkpoint kinase 2* activation in human cells and *PER3* overexpression led to inhibition of cell

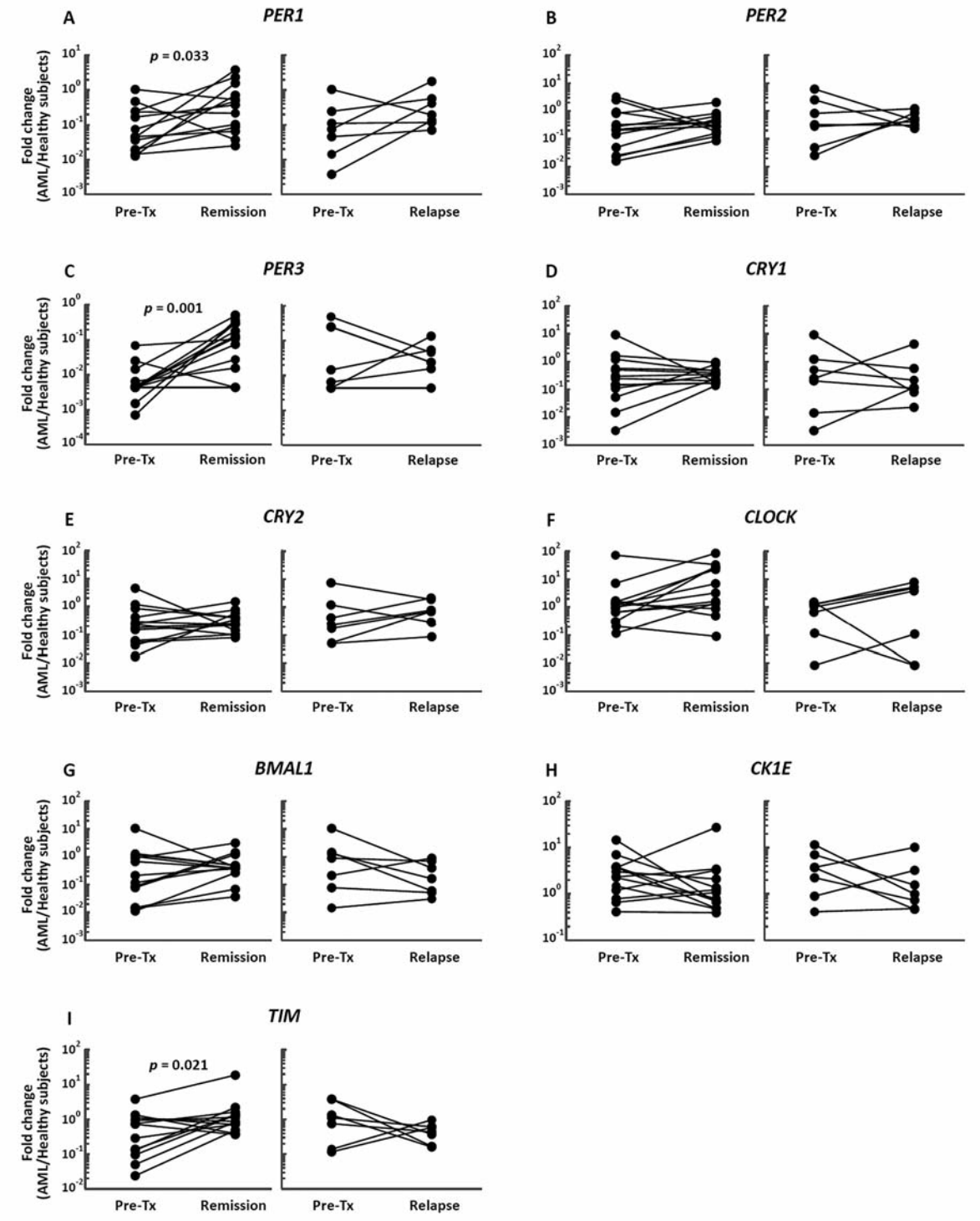

Figure 2. *Up-regulation of period 1 (PER1), period 3 (PER3) and timeless (TIM) expression is correlated with better clinical outcome in patients with acute myeloid leukemia (AML). Expression of the nine circadian clock genes in peripheral blood leukocytes from 13 patients with AML who achieved remission and seven patients whose disease relapsed after treatment was analyzed. The expression of PER1 (A), PER3 (C) and TIM (I) was significantly up-regulated in patients with AML who achieved remission but remained low in patients whose disease relapsed after treatment. The y-axis represents the fold change of mRNA expression level of patients relative to healthy subjects. The relative expression in patients with AML was calculated by the comparative Ct (ΔΔCt method). The mean mRNA expression in healthy individuals was designated a value of 1, whereas the level of mRNA expression in patients with AML was calibrated to obtain the fold change in patients with AML. The p-values indicated are the statistical significance evaluated between the means of ΔCt of pre-treatment (Pre-Tx) and remission states using paired t-test.*

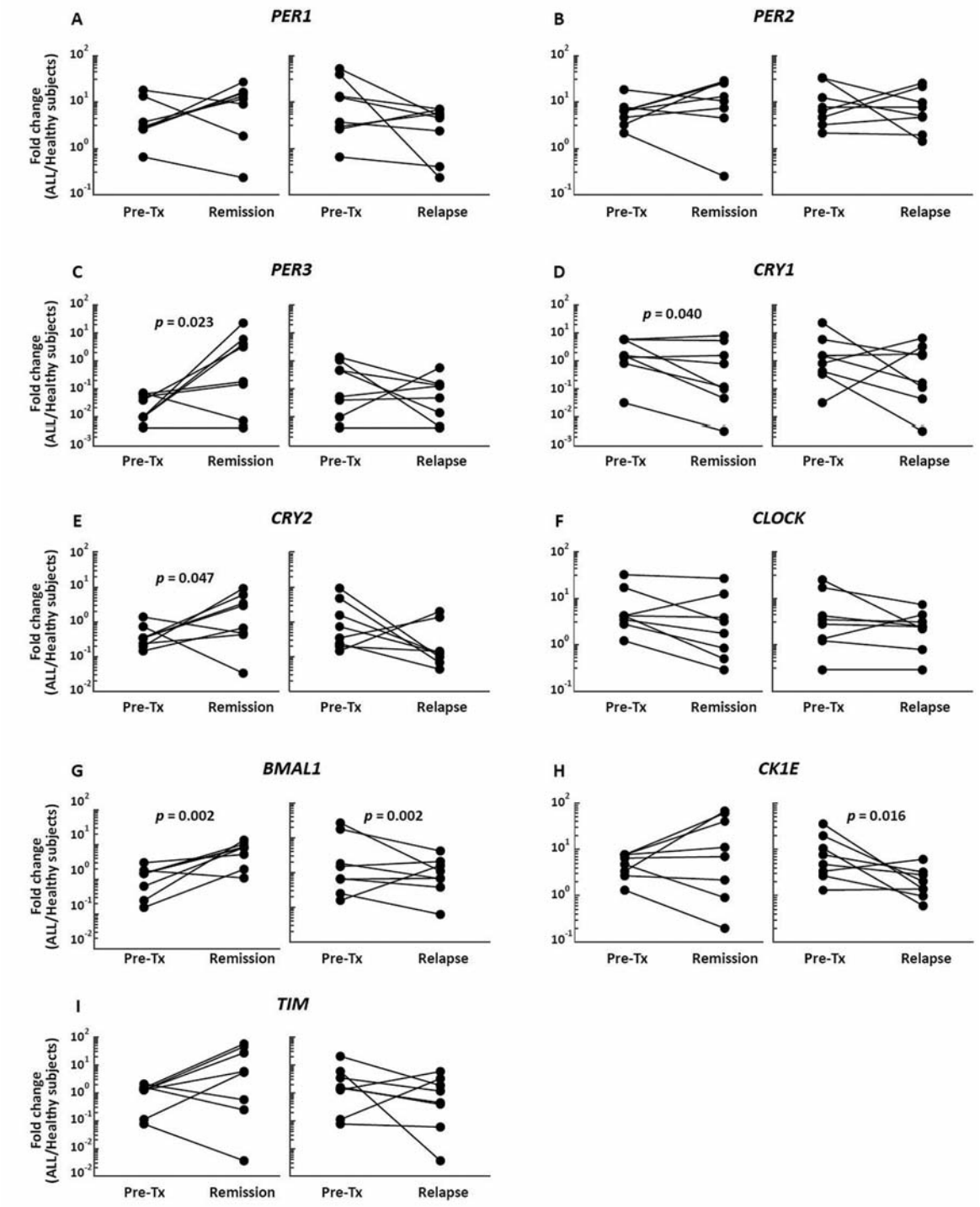

Figure 3. *Up-regulation of period 3 (PER3), cryptochrome 2 (CRY2) and brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1) expression is correlated with better clinical outcome in patients with acute lymphoid leukemia (ALL). Expression of the nine circadian clock genes in peripheral blood leukocytes from eight patients with ALL who achieved remission and eight patients whose disease relapsed after treatment was analyzed. The expression levels of PER3 (C), CRY2 (E) and BMAL1 (G) were significantly up-regulated in patients with ALL who achieved remission but remained low in patients whose disease relapsed after treatment. The y-axis represents the fold change of mRNA expression level of patients relative to healthy subjects. The relative expression in patients with ALL was calculated by the comparative Ct (ΔΔCt method). The mean mRNA expression in healthy individuals was designated a value of 1, whereas the level of mRNA expression in patients with ALL was calibrated to obtain the fold change in patients with ALL. The p-values indicated are the statistical significance evaluated between the means of ΔCt of pre-treatment (Pre-Tx) and remission or relapse states using paired t-test.*

proliferation and increased apoptosis (37). However, these studies are still not sufficient to clarify the definitive significance of *PER3* in tumor development.

In spite of the stereotype of being redundant within the circadian system, many recent studies have associated *PER3* with sleep homeostasis and mental disorders in humans. For example, polymorphisms of the *PER3* gene have been associated with diurnal preference (38, 39), morningness-eveningness preference and circadian rhythm sleep disorder (40), and bipolar I disorder and schizophrenia (41). In *PER3*-knockout mice, the direct response to light and the synchronization activity was suppressed (42), their running wheel activity was increased, and rapid eye movement (REM) sleep and non-REM sleep were reduced in the middle of the dark phase, and delta activity was enhanced at the end of the dark phase (43). The function of *PER3* may not be as prominent as that of *PER1* and *PER2* but more and more evidence supports its crucial function in the time-keeping system by fine adjustment of response to light and environmental stimuli.

In conclusion, we examined the expression of the nine core circadian clock genes in patients with acute leukemia and our data indicate that different genes are de-regulated in AML and ALL. In both diseases, *PER3* was the most down-regulated gene and recovery of *PER3* expression was correlated with better clinical outcomes. It is possible that down-regulation of circadian clock genes, especially *PER3*, may result in de-regulation of the cell cycle, favoring proliferation of blastic cells. Our next goal will be to seek the missing link between de-regulation of *PER3*, sleep disturbance and development of acute leukemia.

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