Downregulation of circadian clock genes in chronic myeloid leukemia: Alternative methylation pattern of *hPER3*

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Disruption of circadian rhythm is believed to play a critical role in cancer development. To gain further insights into the roles of circadian genes in chronic myeloid leukemia (CML), we analyzed peripheral blood from 53 healthy individuals and 35 CML patients for the expression of the nine circadian genes. The expression levels of *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1* were significantly impaired in both chronic phase and blastic crisis of CML cases compared with those in healthy individuals ($P < 0.001$). Methylation studies in the promoter areas of these six genes revealed that only the CpG sites of the *hPER3* gene were methylated in all of the CML patients, and the methylated CpG frequencies differed significantly in patients at blastic crisis (8.24 ± 0.73) or at chronic phase (4.48 ± 0.48). The CpG sites of the *hPER2* gene were also methylated in 40% of the CML patients. No mutation was found within the coding region of *hPER3* in CML cases. Our results suggest that the downregulated *hPER3* expression in CML is correlated with the inactivation of *hPER3* by methylation. (*Cancer Sci* 2006; 97: 1298–1307)

Circadian rhythm is present in all eukaryotic and some prokaryotic life forms. These organisms exhibit daily rhythmic changes in various physiological processes, such as gene expression, metabolism and behavior.[1–6] In mammals, the central pacemaker is located at the suprachiasmatic nuclei (SCN) of the anterior hypothalamus and the SCN clock is entrained to the 24 h day by the daily light–dark cycle through the retina-to-SCN neural pathways.[7] Recently, mammalian peripheral oscillators in body cells similar to those operating in the SCN have been found, and these peripheral circadian rhythms are possibly driven or synchronized by the SCN.[3,8] All known circadian oscillators use transcriptional–translational feedback loops that rely on positive and negative elements in oscillators. Therefore, the strong daily cycling of clock gene mRNA,[9,10] clock proteins,[11,12] and clock-controlled gene RNA and proteins,[13,14] is characteristic of circadian systems. To date, at least nine mammalian core circadian genes have been identified: *Period1* (*Per1*), *Period2* (*Per2*), *Period3* (*Per3*), *Clock*, *Cryptochrome1* (*Cry1*), *Cryptochrome2* (*Cry2*), *Bmal1*, *Casein kinase 1ε* (*CK1ε*) and *Timless* (*TIM*).[1–6,8,15] Circadian genes and the proteins produced by these genes constitute the molecular components of the circadian oscillator and generate circadian rhythms.[16] The negative feedback loop of circadian oscillators was proposed to involve the dynamic regulation of *Per1*, *Per2*, *Per3*, *Cry1* and *Cry2* genes.

Among these circadian genes, the *period* (*PER*) genes occupy a predominant position in the current model of the autoregulatory transcriptional–translational feedback loops.[1–6,8,15] Recent studies have shown that mice deficient in *mPer2* are cancer prone (salivary gland hyperplasia and lymphoma) and display deregulated temporal expression of genes involved in cell cycle regulation, such as c-*Myc*, *Cyclin D1*, *Cyclin A* and *Mdm-2*.[17] The results revealed that *Per2* is an essential circadian gene and it is associated with proliferation control in mammals. In $mPer1^{-/-}mCry2^{-/-}$ double mutants, an age-dependent decay of the circadian clock both at the behavioral and molecular levels was observed.[18] In addition, *mPer1* has been suggested to play a role in synchronizing the circadian clock to environmental stimuli.[19,20]

Compared to the *Per1* and *Per2* genes, fewer studies have been carried out to demonstrate the specific functional role of *Per3* in the circadian system. Mouse *Per3* RNA levels have been shown to oscillate in the SCN and eyes but are not altered acutely by light exposure at different times during subjective night in SCN.[20] In *mPer3* targeted disrupted mice, the *mPer1*, *mPer2*, *mCry1* and *Bmal1* RNA rhythms in the SCN did not differ from those in wild-type mice.[21] In *Per1*/*Per3* and *Per2*/*Per3* double-mutant mice, the behavioral rhythms of these mice were similar to the rhythms of mice with disruption of *Per1* and *Per2* alone, respectively.[21] Although the *mPer3* gene seems to play a less critical role in the generation of circadian oscillation in mice, it appears that it is involved in the process of adjusting the phase of the oscillator.[20] In humans, the exact functions of *PER3* remain unknown.

Disruption of circadian rhythms has a profound influence on human health and has been associated with cancer.[22–25] Because asynchrony of cell proliferation between normal and malignant tissues is commonly observed, it may imply that maintaining the circadian clock is important in tumor suppression. In the present study, we explore the possible roles of circadian genes in the development of human chronic myeloid leukemia (CML).

Materials and Methods

Samples and patients. Peripheral blood (PB) samples were collected from healthy adults and CML patients from July 1999 through August 2003. In order to rule out the bias of time, collection of all PB samples was carried out between 8:00 AM and 11:00 AM. All of the PB samples were processed within 1 h of collection. Ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetracetic acid) was used to deplete red blood cells (RBC) from PB. Total RNA and genomic DNA were extracted from total leukocytes by standard procedures, as described previously.[26,27] Patients who were included in the chronic-phase group carried less than 30% of blast cells in the bone marrow (10.39 ± 1.39%, $n = 19$) and those who were included in the blastic crisis group carried over 30% of blast

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Table 1. Oligonucleotide primers and probes for real-time quantitative reverse transcription–polymerase chain reaction analysis of the nine circadian genes

Gene	GenBank accession no.	Amplicon size (bp)	Direction	Sequence	Location
PER1	AF022991	102	F	5′-CAT CAC GTC TGA GTA CAC ACT TCA GA-3′	808–833
			P	5′-CGA ATC GTC TAC ATT TC-3′	875–891
			R	5′-AGG ACG GCT GCC TGC TC-3′	893–909
PER2	NM_003894	98	F	5′-TCC AGA TAC CTT TAG CCT GAT GA-3′	353–375
			P	5′-CCA TCT ACA AGT GGC TGC-3′	396–413
			R	5′-TTT GTG TGT GTC CAC TTT CGA-3′	429–449
PER3	NM_016831	94	F	5′-ACA AAC AGA ACC ACA AGG CA-3′	1893–1912
			P	5′-CTT ACA AGC TGG TTT GC-3′	1927–1943
			R	5′-CGT CCA TTT GTT GGC ATT T-3′	1986–1968
CRY1	NM_004075	91	F	5′-AGA ACA GAT CCC AAT GGA GAC T-3′	1847–1868
			P	5′-TAT CAG GCG TTA TTT GCC TGT-3′	1830–1830
			R	5′-GTG CAT TCC AGG GAT CAT AGA-3′	1917–1937
CRY2	XM_051030	86	F	5′-CCT CCT CAG TCG GGA TCA A-3′	231–249
			P	5′-CGA TGG AGG TTC CTA C-3′	251–266
			R	5′-GCG GGA GTT CAG TTT CCT TA-3′	297–316
CLOCK	AF011568	107	F	5′-CAA CGC ACA CAT AGG CCA T-3′	1069–1087
			P	5′-CAG GTT AGC TAC ACC TCA G-3′	1122–1140
			R	5′-TTG GGT TCT TCA ACA GTG CA-3′	1156–1175
BMAL1	D89722	97	F	5′-CAT GCA ACG CAA TGT CCA G-3′	318–336
			P	5′-CTG TGC TAA GGA TGG CT-3′	354–370
			R	5′-GTG TAT GGA TTG GTG GCA CCT-3′	394–414
CKI∈	NM_152221	110	F	5′-AGG TCC TCT GCA AAG GCT ATC C-3′	945–966
			P	5′-CGA ATT CTC AAC ATA CCT CAA-3′	970–990
			R	5′-GCG GAA GAG CTG ACG TAG GTA A-3′	1033–1054
TIM	NM_003920	97	F	5′-CAG CAC CAC CAG GAC AAG C-3′	353–371
			P	5′-CTG ATG GTG AAC TTG ACA C-3′	395–413
			R	5′-GCA GAT TGC CAA AAC AGA GCA-3′	423–443

F, forward; P, probe; R, reverse.

cells in the bone marrow ($44.89 \pm 6.66\%$, $n = 16$). This study was approved by the Institute Review Board of the Kaohsiung Medical University Hospital.

Real-time quantitative reverse transcription–polymerase chain reaction analysis. The cDNA sequences of the nine circadian genes were 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, USA). Probes designed by the software were synthesized and labeled with appropriate fluorescent dyes (Applied Biosystems). Sequences of the forward and reverse primers and probe are listed in Table 1. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also examined by real-time reverse transcription–polymerase chain reaction (RT-PCR) as the RNA internal control. The amount of circadian gene was normalized to the endogenous reference GAPDH to obtain the relative threshold cycle (ΔC_T), and then related to the ΔC_T of healthy adult cases to obtain the relative expression level ($2^{-\Delta\Delta CT}$) of circadian genes.

Reverse transcription was carried out in a final volume of 25 μL containing 2 μg RNA, 0.5 μg random primers (10-mers), 2 mM dNTPs, 25 IU RNasin (Promega, Madison, WI, USA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 200 IU Moloney Murine Leukemia Virus reverse transcriptase (Promega). The reaction was first denatured for 5 min at 70°C and incubated at 37°C for 60 min, then stopped by heat inactivation at 95°C for 5 min

Real-time quantitative PCR was carried out in an ABI 7700 Sequence Detector (Applied Biosystems) using the TaqMan Universal PCR Master Mix protocol (Applied Biosystems). All reactions were carried out in a 25-μL final volume containing 400 nM each primer, 200 nM probe and 12.5 μL 2× TaqMan Universal PCR Master Mix. 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 s and 60°C for 1 min.

To determine the interassay precision, four replicates of cDNA of each sample were run on four separate days. Intra-assay (within-run) precision was determined by calculating mean, standard deviation (SD) and coefficient of variance (CV) of the C_T values for each sample and for each set of primers and probe on each day.

Methylation-specific PCR. Bisulfite treatment of genomic DNA was carried out using the CpGenome DNA modification kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, genomic DNA (1 μg in a volume of 100 μL) was denatured in 0.2 M NaOH at 37°C for 10 min and incubated with 3 M sodium bisulfite at 50°C for 16 h. After bisulfate treatment, DNA was desulfonated, ethanol-precipitated, washed, and resuspended in 50 μL of water and used immediately or stored at −20°C until use.

Bisulfite-modified DNA was amplified by PCR using primer sets designed specific for the promoter regions of *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *BMAL1*, as listed in Table 2. PCR was carried out in a 50-μL final volume containing 200 nM each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 2 IU *Taq* DNA polymerase (Promega) and 1× PCR buffer. The amplification procedure was carried out as follows: 95°C for 10 min followed by 40 cycles of PCR reactions at 95°C for 1 min, ~55°C–65°C [depending on the melting temperature (T_m) of each primer set] for 30 s and 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining with a 100-bp ladder as a reference.

CpG methylase (*Sss*I)-treated and sodium bisulfite-modified genomic DNA was used as a positive control for methylation-specific

Table 2. Oligonucleotide primers used for methylation-specific polymerase chain reaction for the promoter regions of *hPER1*, *hPER2*, *hPER3*, *hCRY1* and *hCRY2*

Gene	Location	Annealing temperature (°C)
PER1		
PER2-M (amplicon size, 298 bp; location, −1879 to −1582)		
Forward: 5′-ATT TAG GTT TAC GTG CGT TC-3′	−1879 to −1860	56
Reverse: 5′-CGA CTC AAA AAC GAA AAT CG-3′	−1582 to −1601	
PER1-U (amplicon size, 318 bp; location, −1889 to −1572)		
Forward: 5′-TAG TAT TAG TAT TTA GGT TTA TGT GTG TTT-3′	−1889 to −1860	56
Reverse: 5′-AAC AAC AAT CCA ACT CAA AAA CAA AAA TCA-3′	−1572 to −1601	
PER2		
PER2-M (amplicon size, 140 bp; location, −178 to −39)		
Forward: 5′-GCG GTT TCG TTG CGG TTT AT-3′	−178 to −159	62
Reverse: 5′-GCC GAC GCC GTT TCA AAC CG-3′	−39 to −58	
PER2-U (amplicon size, 160 bp; location, −188 to −29)		
Forward: 5′-GTG GTG TGG TGT GGT TTT GTT GTG GTT TAT-3′	−188 to −159	58
Reverse: 5′-ACA CCC CCA CAC CAA CAC CAT TTC AAA CCA-3′	−29 to −58	
PER3		
PER3-M (amplicon size, 182 bp; location, −281 to −453)		
Forward: 5′-CGG GAG TTT TGG GTA TTC GC-3′	−453 to −444	56
Reverse: 5′-CGA CCC GAC TAA CTA AAA CG-3′	−281 to −301	
PER3-U (amplicon size, 207, location, −269 to −465)		
Forward: 5′-TGG GTG GTT GGG TGG GAG TTT TGG GTA TTT GT-3′	−465 to −444	56
Reverse: 5′-AAT CCA ACA CCA ACA ACC CAA CTA ACT AAA ACA-3′	−269 to −301	
CRY1		
CRY1-M (amplicon size, 166 bp; location, −420 to −255)		
Forward: 5′-TCG TTT TGT TTT TAG GGG TC-3′	−420 to −401	56
Reverse: 5′-GCA ACC GCC TAA AAA CGA CG-3′	−255 to −274	
CRY1-U (amplicon size, 187 bp; location, −432 to −246)		
Forward: 5′-ATT TTG GGT GGG TTG TTT TGT TTT TAG GGG TT-3′	−432 to −401	65
Reverse: 5′-ACA CCC ACC ACA ACC ACC TAA AAA CAA CA-3′	−246 to −274	
CRY2		
CRY2-M (amplicon size, 150 bp; location, −308 to −159)		
Forward: 5′-GTT TAT TTT CGG TAT TTC GC-3′	−308 to −289	55
Reverse: 5′-TAA CGA TTA ACC CAA AAA CG-3′	−159 to −178	
CRY2-U (amplicon size, 171 bp; location, −318 to −148)		
Forward: 5′-TTG AGA TTT GGT TTA TTT TTG GTA TTT TGT-3′	−318 to −289	56
Reverse: 5′-TAC CTT CCA CCT AAC AAT TAA CCC AAA AAC A-3′	−149 to −178	
BMAL1		
BMAL1-M (amplicon size, 138 bp; location, 30–167)		
Forward: 5′-GGA GGT GTT TGT TTA TTC GC-3′	30 to 39	58
Reverse: 5′-AAA TAC GCG AAA TCG CGT CG-3′	153 to 167	
BMAL1-U (amplicon size, 161 bp; location, 18–178)		
Forward: 5′-TAG GTT AGG GAT GGA GGT GTT TGT TTA TTT GT-3′	18 to 39	60
Reverse: 5′-AAC CCC CAA CAA AAT ACA CAA AAT CAC ATC A-3′	153 to 178	

The locations indicated are base numbers corresponding to the transcriptional start sites of the circadian genes.

primers M (PER1-M, PER2-M, PER3-M, CRY1-M, CRY2-M, BMAL1-M), as CpG methylase methylates all cytosines within the double-stranded dinucleotide CG. DNA samples from healthy individuals that were negative for the M primer set and positive for the U primer set (PER1-U, PER2-U, PER3-U, CRY1-U, CRY2-U, BMAL1-U) were used as positive controls for U primers. To ensure the specificity of M and U primer sets for bisulfite-modified DNA, amplification using unmodified genomic DNA samples from healthy individuals and CML patients was also carried out.

To calculate the numbers of methylated CpG sites, direct sequencing using both forward and reverse primers was carried out for each sample. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer and BigDye Terminator Cycle Sequencing Kits Version 1.1 (Applied Biosystems) and the manufacturer's protocols were followed for the reactions.

Analysis of *hPER3* mutation. Polymerase chain reaction was carried out using primers for amplification of the 21 exons of *hPER3* as listed in Table 3. PCR was carried out in a 25-μL final volume containing 200 nM each primer, 200 μM each dNTP, 3.5 mM $MgCl_2$, 2 IU *Taq* DNA polymerase (Promega), and 1× PCR buffer. The amplification procedure was as follows: 35 cycles of PCR reactions at 95°C for 1 min, 58–65°C (depending on the T_m of each primer set) for 1 min and 72°C for 2 min. Direct sequencing of the PCR products of each amplicon was carried out using an ABI Prism 310 Genetic Analyzer and BigDye Terminator Cycle Sequencing Kit Version 1.1 (Applied Biosystems) and the manufacturer's protocols were followed for the reactions.

Subcloning of methylation-specific PCR products. Bisulfite-modifed DNA was amplified by PCR using a universal primer set designed for detection of both methylated and unmethylated promoter regions of *hPER3* as follows: PER3-MU (forward), 5′-GTT

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Table 3. Oligonucleotide primers for amplification of the human *PER3* gene

Region	Forward primer (5′→3′)	Location	Reverse primer (5′→3′)	Location	Size (bp)	Annealing temperature (°C)
Promoter	tgtcacccttgtctcctccc	506–525	catctcgaggtctccgcg	740–757	252	58
Exon 1	aagtgagcgagaagcaggct	691–710	gatttaggtccccctttccct	939–959	269	60
Exon 2	tgttccctaagccgcaaga	1311–1329	ggtgcattcgtaggatgaaatttc	1480–1503	253	60
Exon 3	agatactgttgtcactggactacctgtt	2550–2577	cacccaggttgatatggcaaa	2730–2750	201	60
Exon 4	actggtcatgttagagatccagaaga	3826–3851	catctgaacattggtcctgttacct	4122–4146	321	60
Exon 5	gagggagtgactgaccaggc	9800–9819	ccactgttgctggcttcca	9903–9921	122	60
Exon 6	gtagggtgcgtcaggaccag	14358–14377	tgattgctgacatttctgcatct	14566–14588	231	60
Exon 7	aatatgttaagtggttagttaggtgga	16912–16938	tcaaataggcaaatggacagat	17171–17192	281	60
Exon 8	acctgatgagtatgccacctgtg	18882–18904	tgcctctctggttaaaggttgc	19111–19132	251	60
Exon 9	aggacatttgtaatcagtatctgtgttaa	19506–19534	gctaaggaatattatttgatacaagagatg	19757–19786	281	60
Exon 10	tgactcagtctctcactgggc	24740–27760	tgtgaagtgaatttactgagcattaa	24925–24950	211	60
Exon 11	agccagcaattctggacttgtt	25731–25752	cattcagaaccgaggtagtacagaaa	25939–25964	234	58
Exon 12	tatcattcctttccctaagatgacct	26296–26321	cagagagtgacagcccatgga	26497–26517	222	56
Exon 13	aattgcacatccctttattctgtttc	35134–35159	agtctctacagtggcagtgtatgctt	35340–35365	232	60
Exon 14	gcacaattagggaacaggtaagaat	35942–35966	aggtaatatgaaaccagaattttatgc	36241–36266	287	60
Exon 15	agtttctcttacaccaccagctg	36324–36346	cgagcgaatggtattcatgact	36584–36605	282	60
Exon 16	tatgtcttcttccacctcagcc	42337–42358	tcctagtccttagatacagtttgtaacattta	42626–42657	321	58
Exon 17–1	ctctacctttatccttccaggagat	42975–42999	cgacaacagaggacagacagg	43396–43416	442	60
Exon 17–2	ccctttttccttacttggatactttt	43347–43371	actttagtaagatattcactttctcacttga	43717–43747	401	56
Exon 18	ctgtattttgtgataagaagattaaagtgtc	45656–45686	ggcattggagtttgaaacattagt	46055–46078	423	56
Exon 19	gactgtccgaggcaagagtc	51546–51565	ggcagaagaatcgcttgaac	51971–51989	444	65
Exon 20	gctattcctagatgacgggaaa	52802–52824	ctacctggccaaaatacatgagt	53055–53077	276	60
Exon 21	tttagaaacatgtgaccagcctt	58458–58480	gtctgtgtaacacgtcttggaaag	58660–58683	226	58

The locations indicated are base numbers according to the human *PER3* gene sequence (GenBank accession number NT_021937, region 1971241.2032500).

AAG GGC GGG GGT AGT AGG T-3′ (−520 to −510 to the transcriptional start site of the *hPER3* gene); and PER3-MU (reverse), 5′-CTC GCT CGA CTT TCA AAA CTC-3′ (−200 to −220 to the transcriptional start site of the human *hPER3* gene). The amplification area corresponded to −220 to −520 to the transcriptional start site of the *hPER3* gene. PCR was carried out as described for methylation-specific PCR. Cloning reactions were used the TOPO TA cloning kit version K2 (Invitrogen, Groningen, the Netherlands). Briefly, PER3-MU products were first purified before the addition of 3′ A-overhangs by incubation in 1 IU *Taq* and 2.5 mM dATP at 72°C for 10 min. They were then put on ice. PCR products with A-overhangs were incubated with vectors at room temperature for 30 min and then put on ice. The reaction mixture was added to TOPO-competent cells and incubated on ice for 20 min and then at 42°C for 30 s to heat shock the competent cells. They were then put on ice immediately. The mixture was added to 250 μL SOC (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl, and 20 mM glucose) medium and shaken horizontally at 37°C for 1 h. After shaking, the mixture was plated out on agar plates containing X-gal and 50 μg/mL ampicillin. After overnight incubation, the plasmid DNA was extracted from competent cells using QIApre Spin Miniprep Kit (Qiagen, Hilden, Germany) and digested with *Eco*RI to examine whether the plasmid DNA contained the correct insert. The plasmid DNA containing the correct insert was sequenced using SP6, T7 or PER3-MU primers on an ABI prism 310 Genetic Analyzer.

Tissue culture and demethylation studies. K562 cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 IU/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco), and grown at 37°C with 5% CO_2. Cells were plated at 10^6 cells/100-mm dish and treated the next day with 5-aza-2′-deoxycytidine (Sigma, St Louis, MO, USA) at a final concentration of 10^{-6} M, and/or Trichostatin A (Sigma) at a final concentration of 10^{-7} M for 5 days. The treated cells were harvested after 1, 2, 3, 4 and 5 days of treatment for real-time quantitative RT-PCR and methylation-specific PCR analysis as described above.

Statistics. Comparisons were made with the *t*-test using SPSS for Windows Release 9.0 (SPSS, Chicago, IL, USA). *P*-values <0.01 was regarded as being significantly different.

Results

Determination of circadian gene expression level by real-time quantitative RT-PCR. To investigate whether the expression of the nine circadian genes was downregulated in CML patients, we analyzed PB from 53 healthy individuals and 35 CML patients (19 in blastic crisis and 16 in chronic phase) using real-time quantitative RT-PCR. The results showed that the expression levels of the *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1* genes in CML patients were significantly impaired compared with those in healthy individuals. Furthermore, the degrees of downregulated expression of these six genes were correlated to these CML patients' clinical phases, that is, blastic crisis < chronic phase < healthy individuals (Table 4).

***hPER3*, but not *hPER2* or *hCRY1*, was methylated at the promoter region in CML.** To elucidate the possible mechanism for the downregulation of *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1* in CML, we first analyzed the methylation status of the promoter area of these six genes. For each gene, we designed two pairs of methylation-specific primers designated M and U to discriminate between methylated and unmethylated alleles and to discriminate between sodium bisulfite-modified and unmodified DNA. The M and U primer sets were specific for methylated and unmethylated alleles, respectively; both primer sets were specific for bisulfite-modified DNA but not for unmodified genomic DNA (Fig. 1). The results of methylation-specific PCR demonstrated that CML patients were positive for all of the six U primers at both chronic phase and blastic crisis, but were only positive for PER2-M and PER3-M, not for PER1-M,

Table 4. The expression levels of the nine circadian genes were determined by real-time quantitative reverse trasncription–polymerase chain reaction

Variable	Healthy individuals	CML	
		Chronic phase	Blastic phase
n	53	19	16
PER1			
ΔC_T (PER1-GAPDH)	4.19 ± 0.36	7.48 ± 0.67†	9.95 ± 0.70†
Relative expression	1	1/9.7	1/27.0
PER2			
ΔC_T (PER2-GAPDH)	4.39 ± 0.18	6.79 ± 0.47†	8.87 ± 1.02†
Relative expression	1	1/5.3	1/22.3
PER3			
ΔC_T (PER3-GAPDH)	7.16 ± 0.27	12.34 ± 0.87†	19.60 ± 1.26†
Relative expression	1	1/36.2	1/5544.7
CRY1			
ΔC_T (CRY1-GAPDH)	6.75 ± 0.26	9.33 ± 0.56†	12.15 ± 1.60†
Relative expression	1	1/6.0	1/42.2
CRY2			
ΔC_T (CRY2-GAPDH)	3.67 ± 0.33	6.79 ± 0.53†	7.80 ± 0.90†
Relative expression	1	1/8.7	1/17.6
CLOCK			
ΔC_T (CLOCK-GAPDH)	9.38 ± 0.89	10.57 ± 1.26	13.65 ± 1.52
Relative expression	1	1/2.3	1/19.3
BMAL1			
ΔC_T (BMAL1-GAPDH)	2.12 ± 0.20	3.73 ± 0.44†	5.00 ± 0.62†
Relative expression	1	1/3.1	1/7.4
CK1ε			
ΔC_T (CK1ε-GAPDH)	4.49 ± 0.19	2.69 ± 0.80	4.46 ± 0.63
Relative expression	1	1/0.29	1/0.98
TIM			
ΔC_T (TIM-GAPDH)	6.89 ± 0.29	5.07 ± 0.84	7.36 ± 0.63
Relative expression	1	1/0.28	1/1.38

†$P < 0.001$ compared with normal. Results are the mean ± SE. The level of circadian gene was normalized to the endogenous reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to obtain the normalized circadian gene expression (ΔC_T) value for each sample. The ΔC_T of chronic myeloid leukemia cases was first related to the ΔC_T of healthy individuals to obtain the relative threshold cycle ($\Delta\Delta C_T$) and then the relative expression levels ($2^{-\Delta\Delta CT}$) were calculated.

CRY1-M, CRY2-M or BMAL1-M (Fig. 1). The CpG sites at the promoter area of the *hPER3* gene were methylated in all of the CML cases, whereas the *hPER2* gene was methylated in only 40% (14/35) of CML cases and most of the PER2-M methylation-specific PCR results were weak positive. As the promoter of the *hPER3* gene was methylated in all of the CML cases, we further investigated the association between the downregulation and methylation status of the *hPER3* gene. In Fig. 2, we show three representative cases with *hPER3* expression levels higher at chronic phase and lower at blastic crisis (Fig. 2a). The methylation-specific PCR results demonstrated that patients 1 and 2 were positive for PER3-M and PER3-U at both chronic phase and blastic crisis, whereas patient 3, with lower *hPER* expression levels, was positive for PER3-M at both chronic phase and blastic crisis, and for PER3-U at chronic phase, but negative for PER3-U at blastic crises (Fig. 2b). The PER3-U primer set covers 29 CpG sites and the PER3-M primer set covers 25 CpG sites. PCR products of these primer sets were direct sequenced to calculate the number of methylated CpG sites (examples in Fig. 3). As shown in Table 5, no methylation was observed in normal individuals, whereas methylation was observed in all CML cases. Furthermore, the methylated CpG frequencies of the *PER3* gene did differ in patients at blastic crisis and at chronic phase of CML (CpG, 8.24 ± 0.73 vs 4.48 ± 0.48, $P < 0.001$; T/CpG, 10.47 ± 0.67 vs 14.67 ± 0.46, $P < 0.001$). Figure 4 shows the allelic patterns of CpG island methylation of chronic phase and blastic crisis in a representative CML case (patient 3 in Fig. 2). In this representative case, clones from chronic phase were methylated at only some of the CpG sites (Fig. 4a), whereas clones from blastic crisis were methylated at most CpG sites (Fig. 4b). Interestingly, we found that two CML cases with nearly undetectable *hPER3* expression were strongly positive for PER3-M but negative for PER3-U (Fig. 4c). The allelic patterns of CpG site methylation in these two patients also showed that most of the clones were fully methylated at all of the CpG sites (Fig. 4d).

***hPER3* expression was not downregulated by mutations within the coding region of *hPER3* in CML.** To investigate whether the genetic aberration is also a possible mechanism responsible for the downregulation of *hPER3* in CML, we carried out mutational analysis on the coding region of *hPER3* using PCR and direct sequencing. However, no mutation was found in any of the CML cases (data not shown). The results of gene mutational analysis suggested that mutations in the coding regions were not responsible for the downregulation of *hPER3* gene expression in CML.

Demethylation-associated reactivation of *hPER3* expression in CML cell line K562. As reversibility is a hallmark of epigenetic transcriptional repression, demethylation studies were carried out. We treated hypermethylated CML cells (K562) with 5-aza-2′-deoxycytidine and/or Trichostatin A to attempt demethylation of the *hPER3* gene over a 5-day exposure period. As shown in Fig. 5a, *hPER3* expression was increased after a 2-day incubation with 1 μM 5-aza-2′-deoxycytidine, 100 nM Trichostatin A, or combined treatment and persisted to 5 days after treatment, as determined by real-time quantitative RT-PCR. Methylation-specific PCR results also showed a time-dependent decrease in the intensities of PER3-M PCR products and a increase of intensities of PER3-U PCR products (Fig. 5b). However, the increase in *hPER3* expression was insignificant and was not restored to the levels of normal healthy individuals. The results demonstrated that 5-aza-2′-deoxycytidine, Trichostatin A, or combined treatment partially reactivate *PER*3 expression in K562 cells.

Discussion

In the present study, we analyzed the expression levels of the nine circadian genes. We found that only six genes (*hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1*) were expressed at lower levels in cells from CML patients compared with those from healthy individuals (Table 1). In addition, the downregulated expression of these six genes in cells from CML patients was also correlated with the patients' clinical phases, that is, blastic crisis < chronic phase < normal. These results suggested that the disturbance of circadian gene expression may play a role in the development of human CML.

CpG methylation has been shown to inactivate promoter function, leading to abolishment of gene expression.[28] In human hematological malignancies, aberrant methylation of normally unmethylated CpG islands is associated with transcriptional inactivation of defined tumor suppressor genes, such as DAP-kinase and *SOCS1* (suppressor of cytokine signaling-1) in acute myeloid leukemia,[29] Kruppel-like factor 4 (*KLF4*) gene and early growth response 3 (*EGR3*) gene in adult T-cell leukemia,[30] *SOCS-1*, *p16* and *E-cadherin* in multiple myeloma,[31] *SOCS1* in CML,[32] and *p21* (CIP1/WAF1/SDI1) in acute lymphoblastic leukemia.[33] Interestingly, our results showed that among the six downregulated circadian genes in CML patients, promoter CpG-site hypermethylation was found only in *hPER2* and *hPER3* (Fig. 1). In addition, there was a strong association between the methylation frequency of *hPER3* and the clinical phases of CML (Table 5). Because no genetic alterations were

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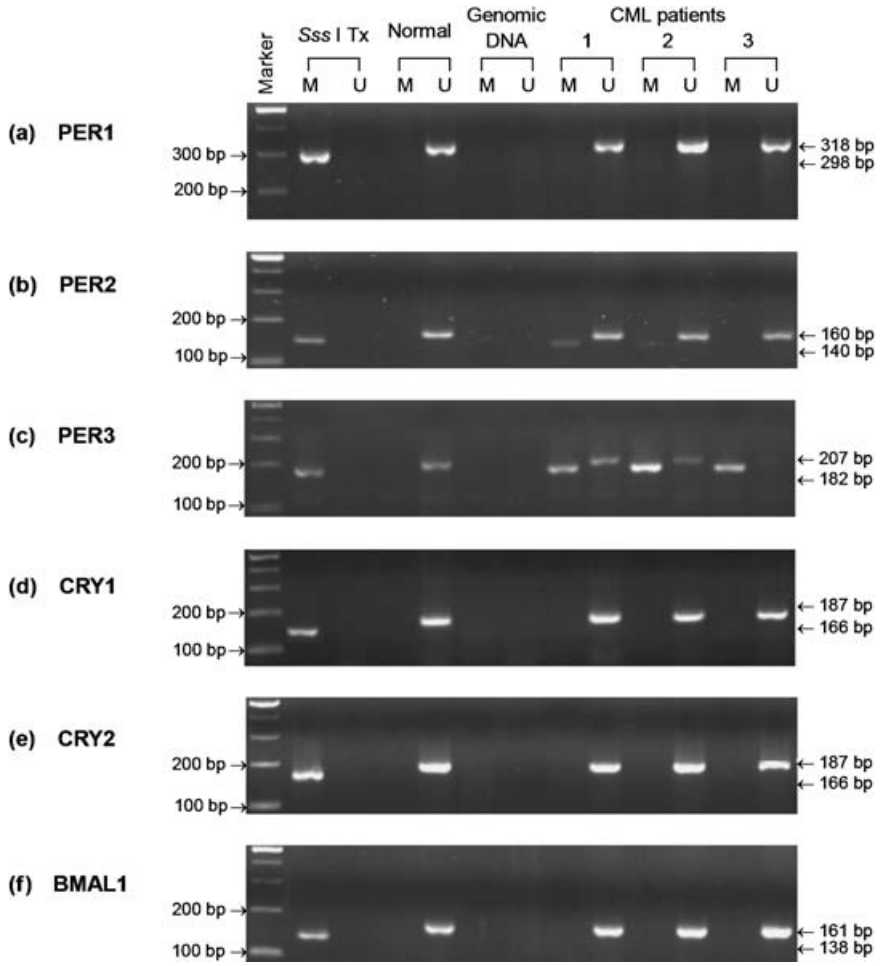
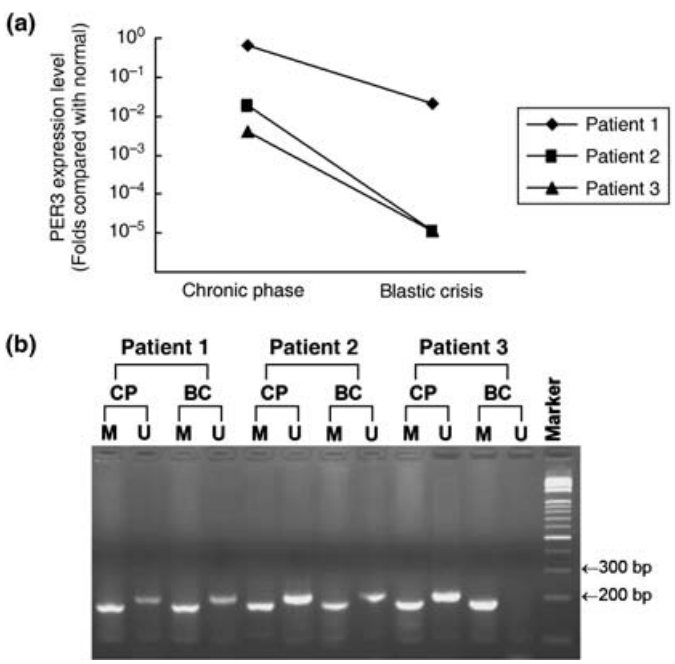


Fig 1. Methylation-specific PCR of *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1* genes. Methylation-specific PCR of *hPER1* (a), *hPER2* (b), *hPER3* (c), *hCRY1* (d), *hCRY2* (e), and *hBMAL1* (f). *Sss* I Tx is *Sss I*-treated, bisulphite-modified DNA of a normal individual. This DNA sample was used as a positive control for methyltaion-specific primers. Normal is bisulphite-modifed DNA of the same normal individual as in lane *Sss* I Tx. Genomic DNA is unmodified genomic DNA of a CML patient. M and U indicate methylation-specific PCR using Methylation-specific and Unmethylation-specific primer sets, respectively. CP and BC indicate chronic phase and blastic crisis of CML, respectively. Marker represents the 100-bp ladder DNA marker.



found within the coding regions of the *hPER3* genes in any of the CML patients, our data suggested that mutation or deletion was irrelevant to the downregulation of *hPER3*. Instead, CpG-site methylation at the promoter area contributed to the *hPER3* gene downregulation in CML patients. Furthermore, the promoter CpG-site hypermethylation of *hPER3* found in CML patients was absent in normal individuals, suggesting that it may be useful as a cancer-specific marker. Therefore, we suggest that downregulation of the *hPER3* gene in CML is partly due to promoter methylation, or as a result of deregulation of other circadian proteins, or disruption of the signal transduction pathway or the cell cycle influencing *hPER3* expression. It was reported that mice deficient in the *mPer2* gene were cancer prone, and these mice showed a markedly increased rate of tumor development, with the author suggesting that *mPer2* is a tumor suppressor gene.[17] In the present study we found that only 40% of the CML cases were hypermethylated at the promoter CpG site of *hPER2* and the methylation-specific PCR results were quite weak. Taken together, we suggest that *hPER3* inactivation may play a more important role in the development of CML.

Fig. 2. *hPER3* gene expression and methylation-specific PCR. (a) *hPER3* gene expression levels of three representative CML cases as measured by real-time quantitative RT-PCR. In the same CML patient, *hPER3* expression level was higher at chronic phase and was lower at blastic crisis. (b) Methylation-specific PCR of *PER3*. M and U indicate methylation-specific PCR using PER3-M and PER3-U primer sets, respectively. CP and BC indicate chronic phase and blastic crisis of CML, respectively. Marker represents the 100-bp ladder DNA marker. Patients 1, 2, and 3 correspond to the same patients in (a).

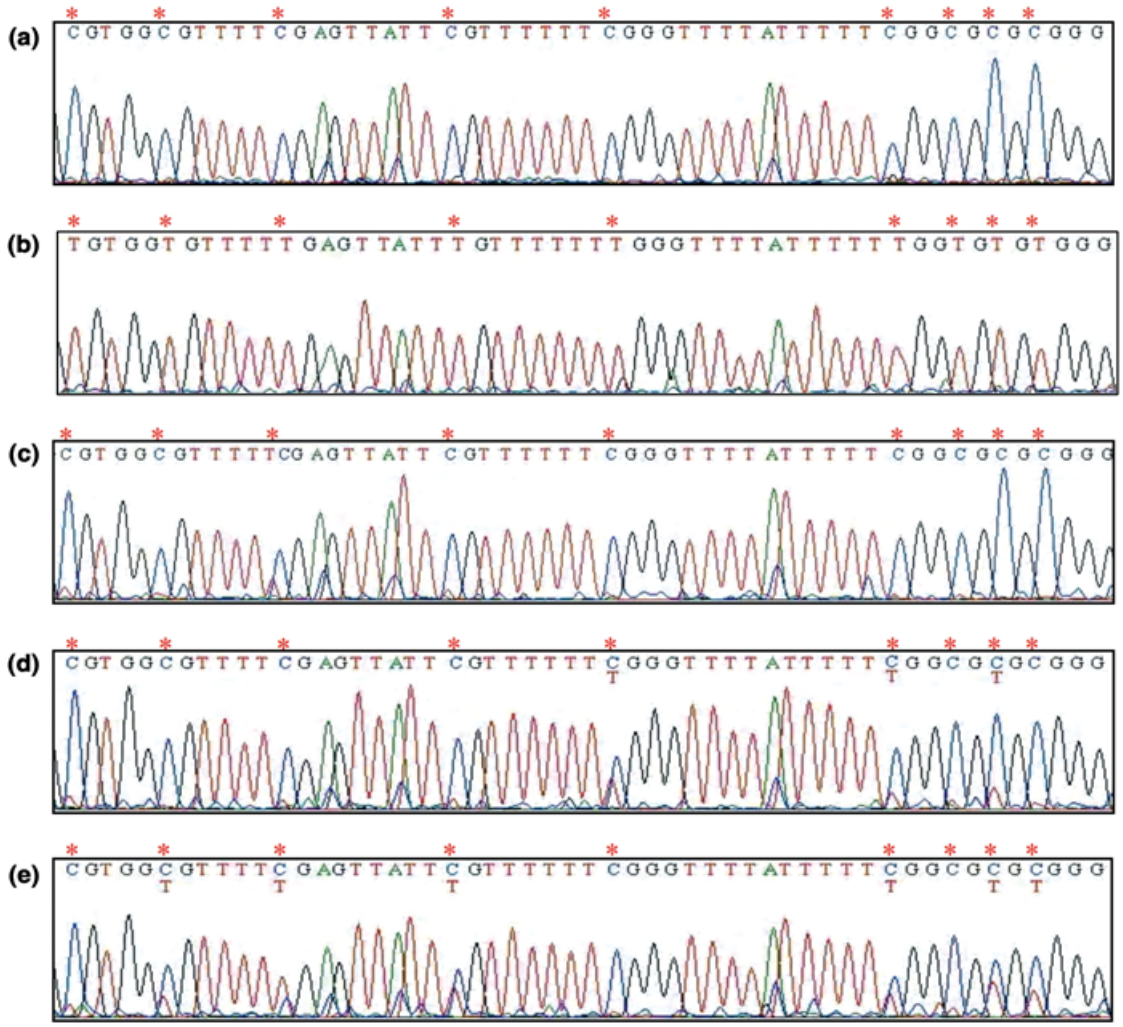


Fig. 3. Examples of methylation analysis of CpG islands at the promoter area of *hPER3* gene. The sequence shown corresponds to the -336~-389 to the transcriptional start site of *hPER3* gene. (a) The cytosines remains intact in the *Sss I*-treated, sodium bisulphate-modified DNA. (b) All the cytosines convert to thymines in a PER3-U positive case. (c) A CML patient showed significantly reduced expression of *hPER3* with all the Cs in CpG dinucleotides remains as C in the sodium bisulphate-modified DNA. (d) and (e) CML patients showed different levels of reduced *hPER3* expression, and their modified DNA showed some methylated CpG sites C and some partially methylated C/T.

As the hallmark of epigenetic transcriptional suppression is reversibility, we attempted to reverse the hypermethylated status of the CML cells K562 by treatment with 5-aza-2′-deoxycytidine and/or Trichostatin A. In all of the three different treatments, increased *hPER3* gene expression was observed after 2 days and carried on for at least 5 days. Decreased intensities of PER3-M PCR products and slightly increased intensities of PER3-U PCR products were also observed. Results from our demethylation studies of K562 cells have demonstrated that the loss of *hPER3* gene expression is caused mainly by CpG-site methylation at the promoter area. In a previous study, it was demonstrated that transcription from densely methylated promoters can only be partially restored by 5-aza-2′-deoxycytidine treatment but can be enhanced by cotreatment with an inhibitor of histone deacetylase;[34] the same phenomenon was also observed in the present study. Our results also provided evidence for the theory that another mechanism, such as deacetylation change, also plays some role in the inactivation of CpG islands at the promoter area.

The *PER1*, *PER2* and *PER3* genes belong to the same *Period* gene family. *PER1* and *PER2* are important in regulating the circadian clock,[3,17,25] but the exact role of *PER3* has not been well described. It has been suggested that the three *mPer* genes have distinct functions in the SCN circadian clock,[35,36] and *mPer1* and *mPer2*, but not *mPer3*, play critical roles within the core circadian feedback loop. However, a recent study has shown that the *hPER1*, *hPER2*, *hPER3* and *hDec1* genes are expressed in a similar circadian manner in human PB mononuclear cells, with the peak level occurring during the habitual time of activity,[37] suggesting that the oscillation of *hPER3* may also be an essential factor in maintaining circadian rhythm. Besides, downregulation of the *hPER1*, *hPER2* and *hPER3* genes has also been observed in breast cancer in a recent study.[38] The fact that human CML patients exhibit decreased *hPER3* levels by methylation of the CpG sites at the promoter, and the methylation frequencies are correlated with patients' clinical phases, demonstrates the possibility that deregulation of multiple molecular pathways plays a role in the development of CML, caused by tissue-specific *hPER3* inactivation. In addition, our other studies showed that *hPER3* expression declined both in acute myeloid leukemia and acute lymphoid leukemia patients (data not shown), suggesting that *hPER3* is important in regulating and maintaining the circadian clock. It is also reasonable to hypothesize that deregulation of *hPER3* is crucial

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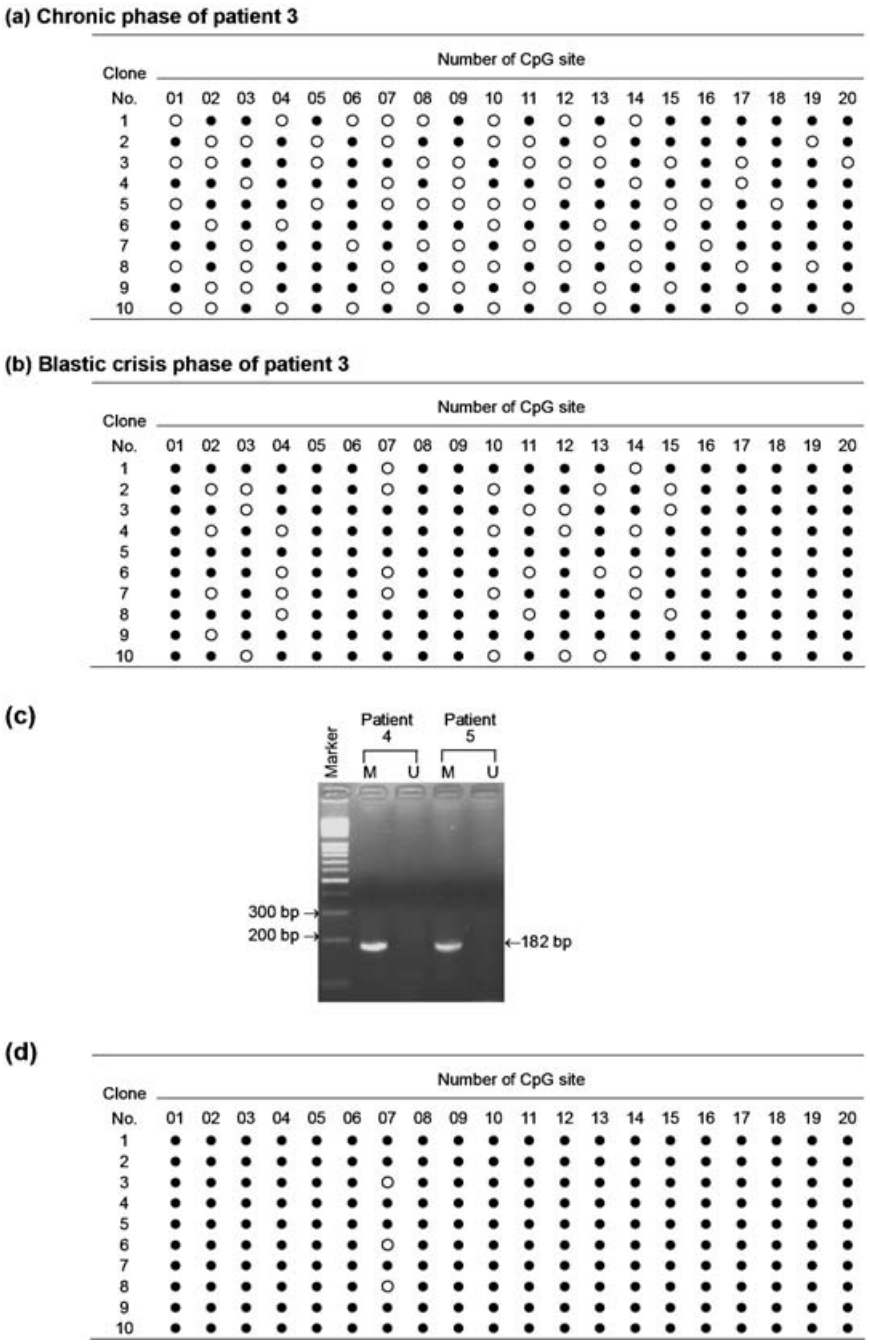

Fig. 4. Allelic patterns of CpG sites methylation at the promoter area of *hPER3* gene. Methylation of the *hPER3* CpG island located between positions -269 and -465 corresponding to the transcriptional starting site was examined by genomic bisulfite sequence analysis. Ten clones were randomly selected and sequenced. Methylated CpG sites are marked as filled circles (●) and unmethylated sites as open circles (○). (a) The allelic patterns of chronic phase of patient 3 in Fig. 3. (b) The allelic patterns of blastic crisis of patient 3 in Fig. 3. (c) Methylation-specific PCR of two CML cases (patient 4 and patient 5) with nearly undetectable *hPER3* expression. They are positive for PER3-M but are negative for PER3-U primer sets. M and U indicate methylation-specific PCR using PER3-M and PER3-U primer sets, respectively. (d) The allelic patterns of CpG sites methylation of *hPER3* gene of patient 5.

in the development of leukemia, but its defined significance remains to be clarified.

It is believed that the t(9,22) chromosomal aberration is the initiating event in human CML. The t(9,22) translocation gives rise to fusion of the *ABL* and *BCR* genes and synthesis of a chimeric BCR–ABL p210 protein with deregulated tyrosine kinase activity. We assumed that the function of the *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2* and *BMAL1* genes is located upstream of the activity of BCR–ABL. Therefore, when *PER3* in chronic phase is further downregulated, the inhibition of *BCR–ABL* is weakened and thus allows the disease to further progress to blastic crisis. Genetic alteration (point mutations and chromosomal deletions) and epigentic alteration (aberrant methylation of the CpG islands in the promoter region) are considered to be the two major types of events involved in systemic gene inactivation. A recent study showed that structural variation of *PER3* exon 18 is associated with increased risk of breast cancer among premenopausal women,[39] suggesting genetic alteration as a possible reason for the altered *PER3* function. However, hypermethylation of the gene promoter leading to abnormal silencing of transcription is also seen commonly in cancers.[25] Evidence that the methylation frequency of CpG sites in the promoter region of *PER3* is significantly higher in patients at blastic crisis compared with those in chronic phase supports the possible role of methylation in the downregulation of *PER3* expression. Recently, Cardone *et al*. showed that the circadian clock may be controlled by sumoylation of *BMAL1*.[40] Whether the sumoylation of *BMAL1*, or another circadian gene, plays a role in the disturbance of CML circadian rhythm needs to be studied further.

Table 5. ***hPER3*** **gene expression levels and methylated CpG frequencies at the promoter area in healthy individuals and CML patients.** ***hPER3*** **gene expression was determined by real-time quantitative RT-PCR. The amount of** ***hPER3*** **was normalized to the endogenous reference GAPDH to obtain the ΔC_T value for each sample. The normalized** ***hPER3*** **expression (ΔC_T) of CML cases was first related to the ΔC_T of healthy individuals to obtain the relative threshold cycle ($\Delta\Delta C_T$) and then the relative expression levels ($2^{-\Delta\Delta CT}$) were calculated. In methylation study, all the normal cases are positive for the unmethylation-specific PCR and all the CML cases are positive for methylation-specific PCR. The number of CpG and T/Cp G were calculated from direct sequencing results of PER3-M or PER3-U PCR products of bisulfite-modified DNA**

	n	Real-time quantitative RT-PCR			Methylation study		
		ΔC_T(PER3-GAPDH)	$\Delta\Delta C_T$(CML-Normal)	Relative Expression	Methylation rate	No. of CpG	No. of T/Cp G
Healthy individuals	53	7.16 ± 0.27*		1	0/53 (0%)	0	0
CML							
Blastic crisis	19	19.60 ± 1.26†	12.44	1/5544.7	19/19 (100%)	8.24 ± 0.73‡	10.47 ± 0.67‡
Chronic phase	16	12.34 ± 087†	5.18	1/36.20	16/16 (100%)	4.48 ± 0.48	14.67 ± 0.46

*: Results are the mean ± SE.
†: $P < 0.001$ when compared with healthy individuals.
‡: $P < 0.001$ when compared with chronic phase.

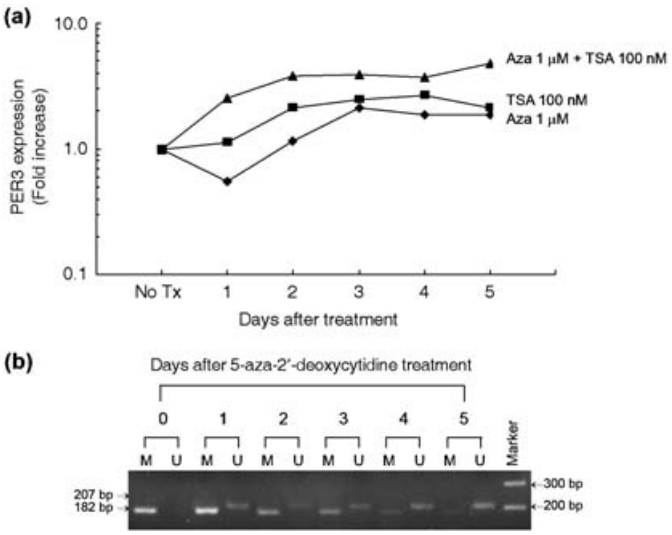

Fig. 5. Demethylation studies using K562 cells treated with 5-aza-2′-deoxycytidine and/or Trichostatin A. 10^6 cells were treated with 1 μM 5-aza-2′-deoxycytidine or/and 100 nM Trichostatin A for 1, 2, 3, 4, and 5 days respectively then harvested for *hPER3* expression and methylation-specific PCR analysis. (a) *hPER3* gene expression levels in K562 cells were increased after 5-aza-2′-deoxycytidine, Trichostatin A, and combined treatments for two days and persisted for 5 days as determined by real-time quantitative RT-PCR. Data are presented as folds increased compared to untreated K562 cells. Each data point was calculated from 3 duplicate studies. (b) Methylation-specific PCR of PER3-M and PER3-U of K562 cells after 5-aza-2′-deoxycytidine treatment. PER3-M PCR products showed a time-dependent decrease of intensities and PER3-U PCR products showed a slight increase of intensities. Marker represents the 100-bp ladder DNA marker.

In conclusion, we have examined the expression of nine circadian genes in CML patients. Our data indicated that the expression of *hPER1*, *hPER2*, *hPER3*, *hCRY1*, *hCRY2* and *hBMAL1* was downregulated in CML, which correlated with the patients' clinical phases. The downregulated expression of *hPER1*, *hCRY1*, *hCRY2* and *hBMAL1* was not owing to CpG-site methylation at the promoter area. Further investigations will be made to clarify the mechanism responsible for the downregulated expression of these four genes. However, the downregulation was due predominantly to methylation of the *hPER3* promoter and less significantly to other factors, such as deacetylation. The methylation frequencies were also correlated with the patients' clinical phases. Therefore, we suggest that the downregulation of *hPER3* may disrupt the circadian rhythm of leukemic cells and result in different circadian clock regulation compared with normal cells. Recently, it was shown that the circadian clock controls the expression of cell cycle-related genes.[41] The temporal expression of genes involved in cell cycle regulation and tumor suppression was also shown to be deregulated in *mPer2* mutant mice.[17] According to these results, we also suggest that downregulation of the circadian genes in CML may result in deregulation of the cell cycle, favoring proliferation of CML cells, and down-regulation of the circadian genes may play a role in the development of CML by benefiting the survival and proliferation of tumor cells.

Acknowledgments

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