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Deregulated expression of the *PER1*, *PER2* and *PER3* genes in breast cancers

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Disruption of circadian rhythm may be a risk factor in the development of breast cancer, but molecular changes in circadian rhythm controlled genes in breast cancer cells are still unexplored. We used immunohistochemical staining, methylation specific PCR and direct sequencing methods to analyze molecular changes in three most important genes, namely *PER1*, *PER2* and *PER3*, in circadian rhythm in 55 cases of breast cancer of Taiwanese women. Our results reveal disturbances in the expression of the three period (*PER*) genes in most (>95%) of the breast cancerous cells in comparison with the nearby non-cancerous cells. The *PER* gene deregulation is not caused by genetic mutations but most probably by methylation of the *PER1* or *PER2* promoter. Methylation of the *PER* gene promoters has a strong correlation with c-erbB2 expression ($P = 0.017$). Since the circadian clock controls expression of cell-cycle related genes, we suggest that disturbances in *PER* gene expression may result in disruption of the control of the normal circadian clock, thus benefiting the survival of cancer cells and promoting carcinogenesis. Differential expression of circadian genes in non-cancerous and cancerous cells may provide a molecular basis for chronotherapy of breast cancer.

Introduction

Breast cancer is a common malignancy affecting adult females worldwide especially in developed countries. Epidemiological studies have suggested multiple risk factors (1–5). The molecular mechanism governing the development of breast cancer is a multiple step process which includes activation of oncogenes, inactivation of tumor suppressor genes, defects in DNA-mismatch repair genes and other tumor-related genes involving apoptosis, proteolysis, adhesion and angiogenesis (6–9). Recent studies have shown that a putative new risk factor, the circadian rhythm, may play an important role in the development of breast cancer (10,11).

Abbreviations: PBS, phosphate buffered saline; *PER*, period genes; Period 1, *PER1*; Period 2, *PER2*; Period 3, *PER3*; SCN, suprachiasmatic nucleus.

In mammals, physiological and hormonal processes as well as behavioral reactions follow circadian rhythms that are driven by an endogenous master clock. The master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and produces self-sustaining circadian rhythms that are synchronized by external cues. Recent studies have shown that the circadian system is a master-and-slave structure: the master pacemaker (SCN) synchronizes slave oscillators (peripheral tissues) of mammals (12–14). Recently, circadian rhythms similar to those operating in the SCN have been found in most mammalian cells and peripheral tissues, and these peripheral circadian rhythms may be driven or synchronized by the central pacemaker in the SCN (14). The human circadian rhythm is controlled by at least nine circadian genes: Period 1 (*PER1*), Period 2 (*PER2*), Period 3 (*PER3*), Cryptochrome 1 (*CRY1*), Cryptochrome 2 (*CRY2*), *CLOCK*, *BMAL1*, Casein kinase 1ε (*CK1ε*) and Timeless (*TIM*) (12–18). The three period (*PER*) genes encode individualized PER–ARNT–SIM (PAS) domain proteins that function in the nucleus but do not directly bind to DNA. The *CLOCK* and *BMAL1* genes encode PAS helix–loop–helix transcription factors. The products of these genes are assembled into a molecular clockwork which is composed of interlocked feedback loops in gene expression. The current model of these oscillators is based on autoregulatory transcription and translation feedback loops of these circadian genes in which the *PER* genes occupy a central position (12–18).

Disruption of circadian rhythm may be a risk factor in the development of breast cancer (10,11,19–22) but molecular changes in these circadian genes in breast cancer cells are still unexplored. In the present study, we used immunohistochemical staining, methylation-specific PCR and direct sequencing methods to explore molecular changes in the three most important circadian genes (*PER1*, *PER2* and *PER3*) in breast cancers.

Materials and methods

Samples

From July 1997 to October 1998, 55 cases of breast cancer patients had been operated on by C.S.T. at Changhua Christian Hospital, and the breast tissue samples of these patients were used for this study. Totally, 55 resected breast cancers and paired non-cancerous tissues were collected. The paired non-cancerous tissue was collected from the normal part of breast tissue without contamination with cancerous cells, which was further confirmed by histopathological analysis. The age of the patients ranged from 27 to 84 years with a mean of 58.9 years. The tissues were frozen or formalin-fixed immediately after surgical resection and stored in liquid nitrogen until DNA extraction as previously described (23). The breast tissue specimens were surgically obtained at the following time points: 19 cases between 10:00 and 12:00, 12 cases between 12:00 and 14:00, 13 cases between 14:00 and 16:00, 8 cases between 16:00 and 18:00, and 3 cases between 18:00 and 20:00. Clinically, 2 cases were stage 0, 15 cases were stage I, 30 cases were stage II, 6 cases were stage III and 2 cases were stage IV. Pathologically, 6 cases were grade I, 36 cases were grade II and 13 cases were grade III breast cancer. Histopathologically, 36 cases were intraductal carcinoma, 7 cases were infiltrating ductal carcinoma, 9 cases were intraductal carcinoma with infiltrating ductal

carcinoma, 2 cases were ductal carcinoma *in situ* and one case was intraductal carcinoma with Paget's disease. This study was approved by the Institute Review Board of the China Medical University Hospital and the Changhua Christian Hospital.

Analysis of expressions of PER1, PER2 and PER3 proteins by immunohistochemistry

Paraffin-embedded tissue sections (4 μm) on poly-1-lysine coated slides were deparaffinized. After treatment with 3% H_2O_2 in methanol, the sections were hydrated with gradient alcohol and phosphate buffered saline (PBS), incubated with 10 mM citrate buffer and, finally, heated at 100°C for 20 min in PBS. After incubation with antibodies for PER1, PER2 and PER3 (Santa Cruz Biotechnology, CA) for 20 min at room temperature, the slides were thoroughly washed three times with PBS before being incubated with an HRP/Fab polymer conjugate for another 30 min. The sites of peroxidase activity were visualized using 3,3′-diamino-benzidine tetrahydrochloride as a substrate. Hematoxylin was used as the counterstain. Appropriate positive and negative controls were also included.

Mutational analysis of the PER1, PER2 and PER3 genes

Amplification of the coding region of the *PER* genes was carried out by PCR. The PCR primers and the primer sequences will be provided on request. PCR was performed in a 50 μl final volume containing 200 nM of each primer, 200 μM of each dNTP, 3.5 mM $MgCl_2$, 2 U *Taq* DNA polymerase (Promega, Madison, WI) and 1× PCR buffer. The amplification procedure was carried out as follows: 35 cycles of PCR reactions including denaturing at 95°C for 1 min, annealing at the temperature depending on the melting temperature (*T*m) of each primer set for 1 min, and an extension cycle at 72°C for 2 min. The PCR products were subject to gel purification and direct sequencing. DNA sequencing was performed by ABI Prism 310 Genetic Analyzer and the BigDye Terminator cycle sequencing kit (Applied Biosystem, USA) according to the manufacturer's protocol.

Methylation-specific PCR analysis of PER1, PER2 and PER3

Genomic DNA was modified with sodium bisulfite and methylation-specific PCRs were performed as described (24) with some modifications. Primer pairs for detection of the methylated and unmethylated sequences in the promoter of *PER1*, *PER2* and *PER3* are shown in Table I. Briefly, ~4 μg of genomic DNA in 40 μl H_2O was denatured by incubation with 10 μl 1 N NaOH at 37°C for 10 min followed by modification with 30 μl of 10 mM hydroquinone and 520 μl 1.5 M sodium bisulfite (pH 5.0) at 50°C for 16 h. The DNA samples were eluted with 100 μl prewarmed H_2O (65–70°C) in a wizard DNA purification kit (Promega). Fifty microlitres of 1 N NaOH was added to the eluent and the mixture was incubated at room temperature for 5 min. After the pellet was precipitated with 150 μl 100% isopropanol and washed with 70% ethanol, it was resuspended in 45 μl H_2O. Modified DNA was amplified in a total volume of 20 μl solution containing 1× PCR buffer, 1.0 mM $MgCl_2$, 100 ng of each primer, 0.2 mM of each dNTP and 2.5 U *Taq* polymerase. The PCR was performed in a thermal cycler for 35 cycles; each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for both methylated and unmethylated primers for 1 min extension at 72°C for 1 min and a final 5 min extension at 72°C. PCR products were then loaded and electrophoresed on 3.5% agarose gels, stained with ethidium bromide and visualized under UV illumination.

CpG methylase (*Sss*I)-treated genomic DNA was used as the positive control for methylation-specific primers. DNA samples extracted from blood samples of healthy individuals tested negative in methylation and positive in unmethylation in the three *PER* genes were used as positive controls for unmethylation. To ensure specificity in methylation in the three *PER* genes, unmodified genomic DNA samples from non-cancerous and cancerous parts of the breast cancer patients were also carried out as negative controls.

Sequencing of methylation-specific PCR products

To determine the number of methylated CpG sites, products of methylation- and unmethylation-specific PCR experiments were subject to direct sequencing analysis. DNA sequencing was performed by ABI Prism 310 Genetic Analyzer and the BigDye Terminator cycle sequencing kit (Applied Biosystem, USA) according to the manufacturer's protocol.

Results

Immunohistochemical analyses of the three PER proteins

We first examined the relative abundance of the PER1, PER2 and PER3 proteins in 55 paired breast cancerous and non-cancerous tissues by immunohistochemical staining. The results revealed three different protein profiles: type I (2 cases) was synchronized expression of all three PER proteins in cancerous and paired non-cancerous cells (Figure 1A); type II (5 cases) was asynchronized expression of all three PER proteins in cancerous and paired non-cancerous cells (Figure 1B); type III (48 cases) showed differential expression of one or two PER proteins in the paired tissue samples (Figure 1C–E). On the other hand, the cancerous tissue of the same patient in the type II and type III PER protein profiles showed different PER protein staining patterns in the cancerous cells: some cells were strong or positively stained, some cells were weak or negatively stained (we have designated this as the heterogeneous staining pattern); such staining patterns were found to occur in one, two or all three PER proteins.

Mutational analysis of the three PER genes

To elucidate the mechanism for the observed expression patterns of the three *PER* genes in breast cancer described above, we analyzed by direct sequencing the cases with abnormal expression patterns for possible mutations in the coding region of the *PER* genes. No function-related changes were found, but 15 polymorphisms of the *PER* genes were detected in cancerous and non-cancerous tissues (Table II). However, 11 of the 15 polymorphic sites were silent changes; the remaining 4 sites resulted in changes in amino acid residues of similar characteristics. We found no correlation between the detected polymorphisms and the *PER* gene expression patterns. These results suggest that *PER* gene mutations do not play a significant role in the perturbed expression patterns of the *PER* genes in breast cancer cells.

Methylation PCR analysis of promoter sequences of the PER genes

To investigate whether aberrant CpG methylation of the promoter region was the cause for differential *PER* gene expression in breast cancer, we analyzed the methylation status of the promoter sequences of the three *PER* genes. For each of the *PER* genes, we designed two primer pairs to discriminate

Table I. Primers used for the detection of CpG methylation and unmethylation in the promoter of the *PER1*, *PER2* and *PER3* genes

Promoter	Location	Methylation (5′ → 3′)	Unmethylation (5′ → 3′)
PER1	M: 298 bp	Up: ATTTAGGTTTACGTGCGTTC	TAGTATTAGTATTTAGGTTTATGTGTGTTT
	U: 318 bp	D: CGACTCAAAAACGAAAATCG	AACAACAATCCAACTCAAAAACAAAAATCA
PER2	M: 140 bp	Up: GCGGTTTCGTTGCGGTTTAC	GTGGTGTGGTGTGGTTTTGTTGTGGTTTAT
	U: 160 bp	D: GCCGACGCCGTTTCAAACCG	ACACCCCCACACCAACACCATTTCAAACCA
PER3	M: 145 bp	Up: CGGTTTTCGTTCGAGTTCGC	GGTTGAGTGTTGGTTTTTGTTTGAGTTTGT
	U: 165 bp	D: ACGATTAATCGTCGAAACCG	CCCAAACCATACAATTAATCATCAAAACA

M, size of methylation specific PCR product; U, size of unmethylation specific PCR product; Up, upstrand primer; D, downstrand primer.

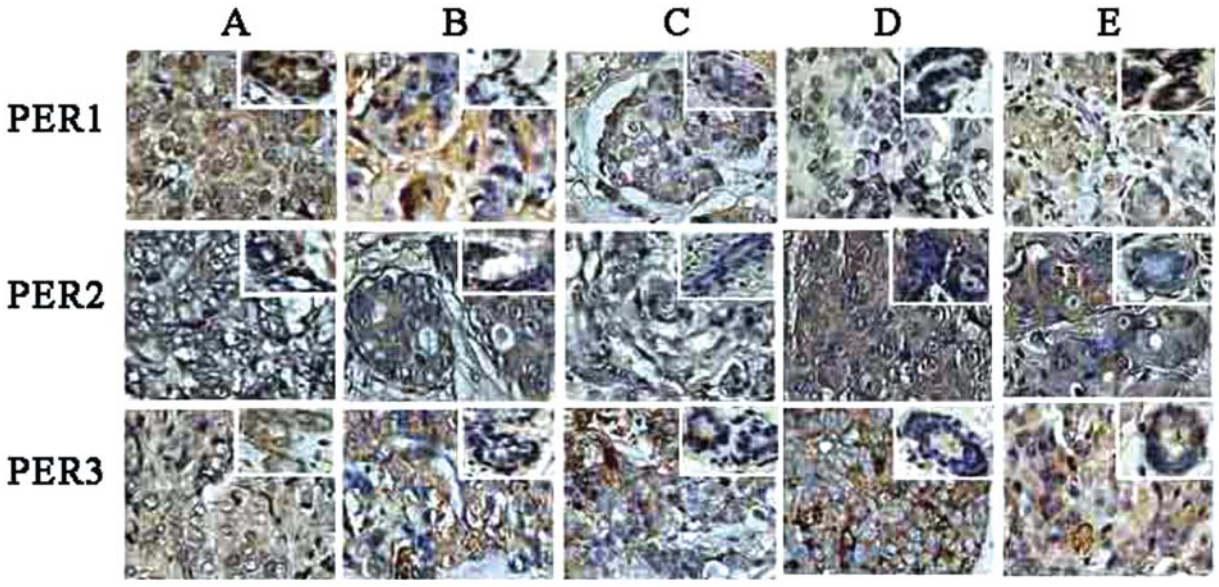

Fig. 1. Immunohistochemical analysis of PER1, PER2 and PER3 for representative cases. (**A**) Synchronized expression of the three PER proteins was found in the breast cancer case in comparison with the paired non-cancerous cells (right upper column). (**B**) Asynchronized expression of the three PER proteins was found in cancerous cells in comparison with paired non-cancerous cells. (**C**) Asynchronized expression of PER3 in cancerous cells was found in comparison with paired non-cancerous cells, and the cancerous cells also showed heterogeneous populations of stained cells. (**D**) Asynchronized expression of PER3 in cancerous cells was found in comparison with paired non-cancerous cells, and the PER staining of the cancer cells showed a picture of homogenous population. (**E**) Asynchronized expression of PER1 and PER2 in cancerous cells was found in comparison with paired non-cancerous cells, and the PER2 staining of cancerous cells also showed a picture of heterogeneity.

Table II. Mutational analysis of the *PER* genes

Gene	Mutation	Frequency
PER1	Codon 213 ACA → ACC (Thr → Thr)	0.31
	Codon 749 GGT → GGC (Gly → Gly)	0.67
	Codon 787 ACG → ACA (Thr → Thr)	0.67
	Codon 962 CCC → GCC (Pro → Ala)	0.47
PER2	Codon 655 GCG → GCA (Ala → Ala)	0.10
	Codon 665 TCG → TCA (Ser → Ser)	0.13
PER3	Codon 445 AGT → AGC (Ser → Ser)	0.63
	Codon 745 CCG → CCA (Pro → Pro)	0.82
	Codon 797 TAC → TAT (Tyr → Tyr)	0.05
	Codon 820 GCA → GCG (Ala → Ala)	0.03
	Codon 827 CTG → CCG (Leu → Pro)	1.00
	Codon 856 CCT → GCT (Pro → Ala)	0.05
	Codon 864 TCG → TCA (Ser → Ser)	0.26
	Codon 969 ACC → ACT (Thr → Thr)	0.45
	Codon 1028 ATG → ACG (Met → Thr)	0.13

between methylated and unmethylated alleles and between bisulfite-modified and unmodified DNA. In Figure 2A, we show the methylation status of the *PER1* promoter region for four representative cases. They represent high *PER1* expression in both cancerous and the paired non-cancerous tissue (case 1), higher expression in non-cancerous tissue than the cancerous tissue (case 2), higher expression in cancerous tissues than the paired-non-cancerous tissues (case 3) and low *PER1* expression in both the cancerous and non-cancerous tissues (case 4), respectively. CpG methylation of the *PER1* promoter was observed in 31/55 (56.3%) cancerous tissues and in 16/55 (29.1%) non-cancerous tissues. Among the 16 non-cancerous but CpG-methylated tissues, only 14 showed methylation in the *PER1* promoter in their paired cancerous tissues. In Figure 2B, we show the *PER2* promoter methylation status for two representative cases. They represent similar expression patterns in both cancerous and non-cancerous tissues (case 5) and elevated *PER2* expression in the non-cancerous tissue (case 6), respectively. There are two cases (2/55) with *PER2* promoter methylation in cancerous tissues, but no methylation is found in the non-cancerous tissues (0/55). In contrast to *PER1* and *PER2*, no methylation of the *PER3* promoter was found in all the 55 breast cancers and their paired non-cancerous tissues analyzed (Figure 2C).

Correlation between the PER1 methylation status and clinical-pathological features in the breast cancer patients

Because the *PER1* promoter was more frequently methylated, *PER1* was the focus of subsequent studies. The results of correlation between methylation status of the *PER* promoters and clinical–pathological features in breast cancer patients are shown in Table III. No correlation between *PER1* methylation and age, estrogen receptor, progesterone receptor, P53, tumor grade, tumor size and clinical stage. There is, however, a strong correlation between promoter methylation and c-erbB2 expression, and c-erbB2 expression in most cases is associated with promoter methylation ($P = 0.017$).

Correlation between the expression of three PER proteins and PER promoter methylation

The correlation between the expression profiles of the three PER proteins and the results of methylation PCR analysis is shown in Table IV. There were two cases in the type I protein profile that showed no methylation in the promoters of all three *PER* genes; five cases belonged to the type II protein profile, two of which had no methylation in the *PER* promoters, whilst two showed promoter methylation in *PER1* gene in tumor tissues. The remaining case was methylated in the *PER2* promoter in the tumor tissue. A total of 48 cases belonged to the type III protein profile, 23 of which had no methylation in

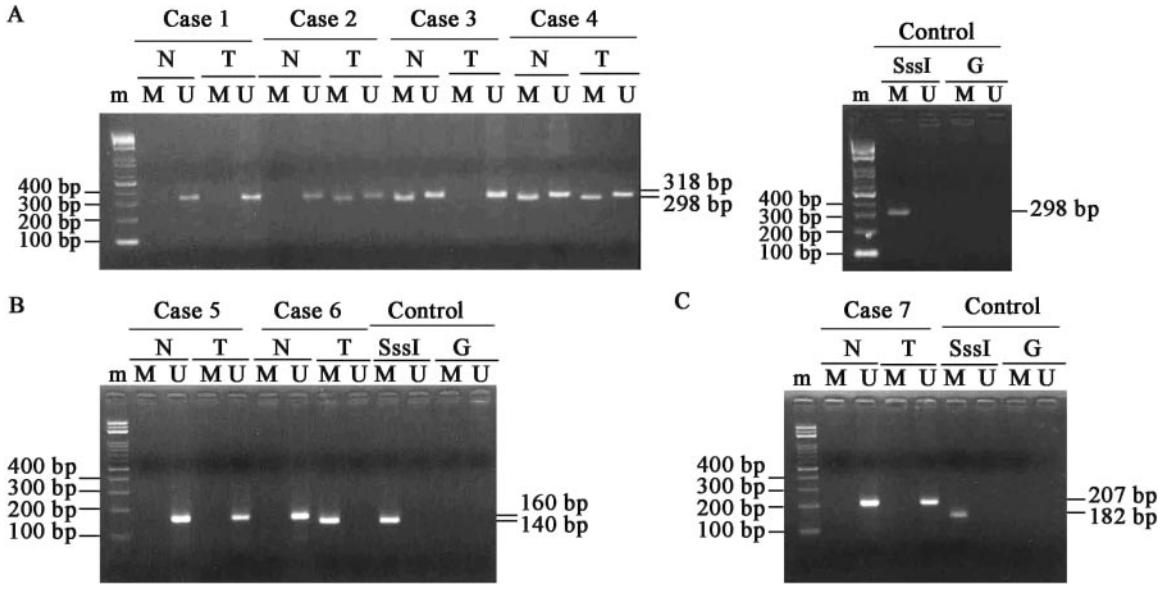

Fig. 2. Methylation-specific PCR analysis of the promoter sequences of the three *PER* genes in breast cancers. (**A**) Analysis of the *PER1* promoter in four breast cancer cases with different PER1 expression. Case 1 had similar abundance of PER1 protein in both cancerous and paired non-cancerous tissues; case 2 had more PER1 protein in non-cancerous tissue; case 3 had more PER1 protein in cancerous tissue than that of non-cancerous tissue; case 4 had similar low expression of PER1 in both cancerous and paired non-cancerous tissue. (**B**) Analysis of the *PER2* promoter in two breast cancer cases with different PER2 expressions. Case 5 had similar abundance of PER2 protein in both cancerous and paired-non-cancerous tissues; case 6 had no expression of PER2 in cancerous tissue but in abundance in the non-cancerous tissue. (**C**) Analysis of the *PER3* promoter in one breast case with abundance expression of PER3 protein in both cancerous and paired non-cancerous tissues. m, 100 bp ladder marker; M and U, mPCR using methylation or unmethylation specific primers; N, non-cancerous tissue; T, cancerous tissue; SssI, *Sss*I methylate treated DNA as positive control; G, unmodified genomic DNA.

Table III. *PER1* methylation status and clinical-pathological features in breast cancer patients

Clinical parameters	Promoter methylation		*P*-value*
	Positive	Negative	
Age			
<50	16	16	0.140
≥50	15	8	
ER			
+	9	12	0.099
−	18	9	
PR			
+	11	11	0.238
−	18	9	
P53			
+	15	7	0.273
−	12	11	
c-erbB2			
+	27	13	0.017
−	1	5	
Grade			
I, II	21	21	0.808
III	6	7	
Tumor size			
<2 cm	2	5	0.418
≥2 cm	25	22	
Stage			
0–II	21	17	0.600
III, IV	3	5	

*, χ^2-test; ER, estrogen receptor; PR, progesterone receptor.

the promoter of all three *PER* genes, 14 were methylated in the *PER1* promoter in non-tumor tissues; 25 showed *PER1* promoter methylation in tumor tissues, and there was only one case with *PER2* promoter methylation in tumor tissue.

Table IV. Relationship between promoter methylation and PER protein patterns in cancerous and non-cancerous tissues of breast cancer

Type of protein profile	No. of cases	No methylation	*mPER1*		*mPER2*		*mPER3*	
			N	T	N	T	N	T
I	2	2	0	0	0	0	0	0
II	5	2	0	2	0	1	0	0
III	48	23	14	25	0	1	0	0

I, Synchronized expression of all three PER proteins between cancerous and non-cancerous tissues; II, asynchronized expression of all three PER proteins between cancerous and non-cancerous tissues; III, differential expression of one or two PER protein between cancerous and their paired non-cancerous cells; *mPER1*, *mPER2* and *mPER3*, methylation of promoter of *PER1*, *PER2* and *PER3*, respectively.

Discussion

The central pacemaker of SCN is greatly influenced by light and other environmental factors. On the other hand, activities of the circadian clock on peripheral tissues are influenced by hormones and neuronal factors and regulated by the central pacemaker (14,25) via various signaling pathways (26–29). *PER1*, *PER2* and *PER3* play important roles in the circadian clock of both central and peripheral tissues (12–17). Several studies have shown that homozygous mutations of the *PER* genes disrupt the circadian clock to display a shorter circadian period with reduced precision and stability (30–32). In this study, we analyzed and compared the expression status of the PER proteins in cancerous and non-cancerous tissues obtained at the same time in each case so that the tissue pairs were synchronized with respect to the same circadian clock, and we found differential expression patterns in the *PER* genes in

cancerous cells in most of the breast cancers cases (53/55) analyzed in comparison with their paired nearby non-cancerous cells. Since expression of the *PER* genes plays a central role in the circadian rhythm, our results suggest that the circadian clock in the cancer cells of most breast cancer cases behaves differently from non-cancerous cells.

Furthermore, we also found different PER expression patterns in different cancer cell populations in the same cancer tissue suggesting that several asynchronized circadian clocks may be in operation in the same cancer tissue. This is indicative of heterogeneity in cancer cell populations in breast cancer. Our results may provide a molecular basis for designing clinical protocols for chronotherapy in breast cancer treatment based on differences in the circadian clock between cancerous and non-cancerous cells and, therefore, differences in drug sensitivities (33–38).

In this study, we found that the PER protein profiles were disturbed in breast cancerous cells. To elucidate a possible mechanism to explain the PER expression patterns, we first sequenced and found no specific genetic alterations in the coding regions of the *PER1*, *PER2* and *PER3* genes in the cases with low or no *PER* gene expression indicating that *PER* gene mutations do not contribute to altered gene expression. It has been shown that CpG methylation of promoter sequences, an epigenetic alteration, can inactivate promoter functions leading to downregulation and inhibition of gene expression (39). We further explored whether CpG methylation had occurred in the *PER* genes. Our results show that ~50% of the differential expression of the PER proteins in the breast cancerous tissues can be explained by promoter methylation of the *PER* genes, and because the cancerous cells were also defective in the cell cycle or signal transduction pathway resulting in disturbance of circadian gene expression. We suggest that the differential expression of the PER proteins between cancerous and non-cancerous tissues in each patient is partly due to promoter methylation of the *PER* gene, or as a result of promoter methylation of other circadian genes resulting in deregulation of the PER proteins, or disruption of the signal transduction pathway or cell cycle influencing the PER protein expression. Our results also show that the methylation status has a strong correlation with the expression of the important *c-erbB2* oncogene. Overexpression of c-erbB2 may result in an aggressive tumor phenotype and reduced survival rates (40). Whether methylation of the *PER* genes influences the c-erbB2 expression, or vice versa, needs to be further investigated. In the methylation study of the *PER1* promoter, we found that four cases of non-cancerous tissues showed methylation in the *PER1* promoter but no methylation was detected in the promoter in their paired cancerous cells. This phenomenon has also been found in other cancer-related genes (24,41) and suggests that a mechanism leading to deregulation in CpG-island methylation may be involved in the early carcinogenesis process (41). Our results may indicate different biological functions for the PER proteins, probably related with the development and progression stage of the cancer *in vivo*, and that methylation of the *PER* promoters may play a role in these biological functions. Fu *et al*. (42) reported that mice deficient in the *mPer2* gene were cancer prone and that these mice showed a markedly increased rate in tumor development and reduced apoptosis in thymocytes after radiation. Based on these results, the authors suggested that *mPer2* was a tumor suppressor gene. In this study, we found only two cases (3.6%) had methylation in the promoter of *PER2* gene, but ~50% cases had *PER1* promoter methylation resulting in disruption of *PER1* expression and disorder in the circadian clock. Taken together, we suggest that *PER1* inactivation may play a more important role in the development of breast cancer. Recently, Matsuo *et al*. (43) have shown that the circadian clock controls the expression of cell cycle related genes. They also noted that the intracellular circadian clockwork is able to control the cell-division cycle directly and in a unidirectional mode in proliferating cells. In *mPer2* mutant mice, the temporal expression of genes involved in cell cycle regulation and tumor suppression was also deregulated (42). Based on these results, we propose that inactivation of the *PER* genes in breast cancer cells may result in deregulation of the cell cycle favoring proliferation of breast cancer cells. In conclusion, deregulated expression of the *PER* genes is common in breast cancer, and may play a role in the development of breast cancer by benefiting the survival and proliferation of cancer cells.

Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org/.

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Conflict of Interest Statement: None declared.

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