Expression of circadian genes correlates with liver metastasis and outcomes in colorectal cancer

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Abstract. Circadian rhythms are daily oscillations in various biological processes, generated by the feedback loops of eight core circadian genes: *Period1 (Per1), Period2 (Per2), Period3 (Per3), Cryptochrome1 (Cry1), Cryptochrome2 (Cry2), Clock, Bmal1* and *Casein Kinase I ε* (*CKIε*). Recent studies have suggested that circadian genes participate in the growth and development of various cancers. This study examined the relations of circadian gene expression to clinicopathological factors and outcomes in patients with colorectal cancer. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with untreated colorectal cancer. The relative expression levels of the circadian genes in the specimens were measured by quantitative real-time, reverse-transcription polymerase chain reaction. Expression of the *Clock* gene and the *CKIε* gene in cancer tissue were significantly higher compared to that in adjacent normal mucosa. Expression of the *Per1* and *Per3* genes in cancer tissue was significantly lower compared to that in adjacent normal mucosa. Analysis of the relations between clinicopathological features and expression of the eight circadian genes in cancer tissue showed that high expression of the *Bmal1* gene and low expression of the *Per1* gene correlated with liver metastasis. On analysis of the relations between outcomes and gene expression, high expression of the *Per2* gene was associated with significantly better outcomes than low expression of the *Per2* gene. Overexpression of the *Bmal1* gene and reduced expression of the *Per1* gene may thus be useful predictors of liver metastasis. Moreover, reduced expression of the *Per2* gene may be a predictor of outcomes in patients with colorectal cancer.

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Introduction

Circadian rhythms are daily oscillations in various biologic processes. In mammals, the master circadian pacemaker is located in the suprachiasmatic nuclei (SCN) (1). The master circadian clock coordinates peripheral circadian clocks within virtually every cell in the body (2). This coordination is accomplished directly through autonomic nervous system innervation and indirectly through daily rhythmic synthesis and release of an array of hypothalamic, pituitary, and dispersed endocrine hormones (3-6).

The molecular mechanism of circadian oscillation in the SCN and peripheral cells is based on the feedback loops of eight core circadian genes (3,7,8). These eight genes are *Period1 (Per1), Period2 (Per2), Period3 (Per3), Cryptochrome1 (Cry1), Cryptochrome2 (Cry2), Clock, Bmal1*, and *Casein Kinase I ε (CKIε)*. The feedback loops of the eight core circadian genes are as follows. The *Clock* gene remains steady throughout the 24-h day. High levels of Bmal1 promote the formation of Bmal1/Clock heterodimers. These heterodimers bind to E-box sequences in the promoters of the *Cry* and *Per* genes to activate transcription. Bmal1/Clock heterodimers can also inhibit Bmal1 transcription. After transcription and translation, the Per proteins accumulate in the cytoplasm and are phosphorylated by CKIε. The phosphorylated forms of Per are unstable and are degraded by ubiquitylation. Cry accumulates in the cytoplasm, promoting the formation of stable Per/Cry/CK1ε complexes, which enter the nucleus. Once in the nucleus, Cry disrupts the Bmal1/Clock-associated transcriptional complex, resulting in the inhibition of Cry and Per transcription and the derepression of Bmal1 transcription (Fig. 1). In the peripheral tissues, the molecular clock coordinates the transcription of the circadian genes. The circadian genes are largely tissue specific and link key tissue functions to the circadian environ-

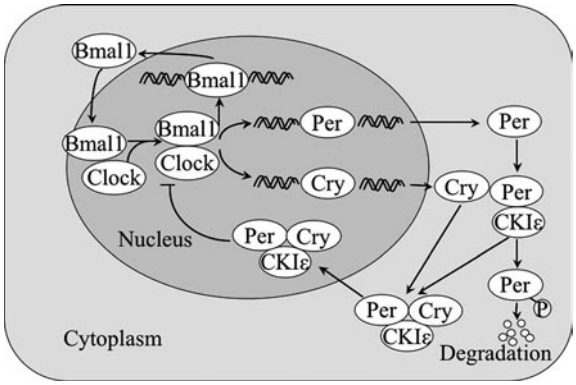

Figure 1. The feedback loops of eight core circadian genes. The molecular mechanism of circadian oscillation in the SCN and peripheral cells is based on the feedback loops of eight core circadian genes. The *Clock* gene remains steady throughout the 24-h day. High levels of Bmal1 promote the formation of Bmal1/Clock heterodimers. These heterodimers bind to E-box sequences in the promoters of the *Cry* and *Per* genes to activate transcription. Bmal1/Clock heterodimers can also inhibit Bmal1 transcription. After transcription and translation, the Per proteins accumulate in the cytoplasm and are phosphorylated by CKIε. The phosphorylated forms of Per are unstable and are degraded by ubiquitylation. Cry accumulates in the cytoplasm, promoting the formation of stable Per/Cry/CK1ε complexes, which enter the nucleus. Once in the nucleus, Cry disrupts the Bmal1/Clock-associated transcriptional complex, resulting in the inhibition of Cry and Per transcription and the derepression of Bmal1 transcription.

ment, making these key functions available at specific times during each day, when they are most needed (9-11).

Disruption of circadian organization has significant effects on human health, causing sleep disorders, gastrointestinal and cardiovascular illnesses, and depression. It is also associated with an increased incidence of several epithelial cancers (12-15). In mouse models, transplanted tumors grow twice as fast in SCN-lesioned mice than in sham-lesioned animals (16). These studies have suggested a close connection between circadian organization and the development of various cancers. Relations between circadian genes and cancer have been demonstrated in recent years. The host circadian clock has been reported to play an important role in the endogenous control of tumor progression (16). As for circadian genes, Bmal1 was shown to be a positive regulator of tumor growth and metastasis in cancer (17). Moreover, overexpression of Per1 in prostate cancer cells causes significant growth inhibition and apoptosis (18). In addition, Per2 plays a key role in tumor suppression, controlled by genes such as *c-myc* and *cyclin D1* through the activity of Bmal1/Clock heterodimers (19), and *Per2* gene overexpression induces cancer cell apoptosis (20). Per2 overexpression has also been found to inhibit the growth of pancreatic cancer cells and to act synergistically with cisplatin (21). However, studies assessing the relations of circadian gene expression to clinicopathological features and outcomes in colorectal cancer have not been reported. We therefore examined whether the expressions of circadian genes were related to clinicopathological characteristics and outcomes in patients with colorectal cancer.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with untreated colorectal cancer. The patients underwent surgery at Yokohama City Medical Center, Gastroenterological Center, and at Kanagawa Cancer Center from January 2002 through January 2005. The duration of observation was longer than 5 years. Informed consent was obtained from each patient, and the ethics committees of Yokohama City Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study.

All tissue samples were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately stored at -80˚C until use. No patient had any other malignancies. The histopathological features of specimens stained with hematoxylin and eosin were examined, and sections that consisted of >80% cancer cells were used to prepare total RNA.

Quantitative real-time, reverse-transcription polymerase chain reaction (PCR). Total RNA isolated from colorectal cancer and adjacent normal mucosa was prepared with the use of TRIzol (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with an iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl containing cDNA derived from 75 ng of mRNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP, and dTTP at concentrations of 400 μM each, and 50 units/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C, followed by 50 cycles of denaturation of the cDNA for 30 sec at 94˚C, annealing for 30 sec at an appropriate temperature (Table I), and a primer extension for 1 min at 72˚C followed by 10 min at 72˚C. The PCR primer sequences of *Per1, Per2, Per3, Cry1, Cry2, Clock, Bmal1*, *CK1ε*, and *β-actin*, used as an internal control, are shown in Table I.

Statistical analysis. Gene expression levels of colorectal cancer were compared with those of adjacent normal mucosa by the Wilcoxon test. Relations between gene expression and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion, and liver metastasis, were evaluated with the χ^2 test. The postoperative survival rate was analyzed by the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test. A Cox proportional hazard regression model was used for multivariate analyses. All statistical analyses were performed using IBM SPSS Statistics 18.0 (SPSS, Inc., Chicago, IL, USA). Two-sided P-values were calculated, and a difference was considered significant if the P-value was <0.05.

Results

Comparison of circadian gene mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *Clock* and *CK1ε* gene expression levels were higher in cancer than in adjacent normal mucosa (P<0.0001, P<0.0001; Fig. 2F and H). *Per1* and *Per3* gene expression levels were higher in adjacent

Table I. PCR primers and conditions.

Gene	Primer	Temperature (˚C)	Product size (bp)
Per1	5'-AGGCAACGGCAAGGACTC-3' 5'-GGCTGTAGGCAATGGAACTG-3'	60.2	101
Per2	5'-CTACAGCAGCACCATCGTC-3' 5'-CCACTCGCAGCATCTTCC-3'	58.9	78
Per3	5'-TGGTGGTGGTGAATGTAAGAC-3' 5'-GGCTGTGCTCATCGTTCC-3'	57.2	104
Cry1	5'-CAACCTCCATTCATCTTTCC-3' 5'-CTCATAGCCGACACCTTC-3'	58.9	151
Cry2	5'-TGGGCTTCTGGGACTGAG-3' 5'-GGTAGGTGTGCTGTCTTAGG-3'	57.2	136
Clock	5'-GCAGCAGCAGCAGCAGAG-3' 5'-CAGCAGAGAGAATGAGTTGAGTTG-3'	61.9	149
Bmal1	5'-TGCCACCAATCCATACACAGAAG-3' 5'-TTCCCTCGGTCACATCCTACG-3'	60.9	123
CKIε	5'-TCAGCGAGAAGAAGATGTC-3' 5'-GAAGAGGTTGCGGAAGAG-3'	58.9	149
β-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

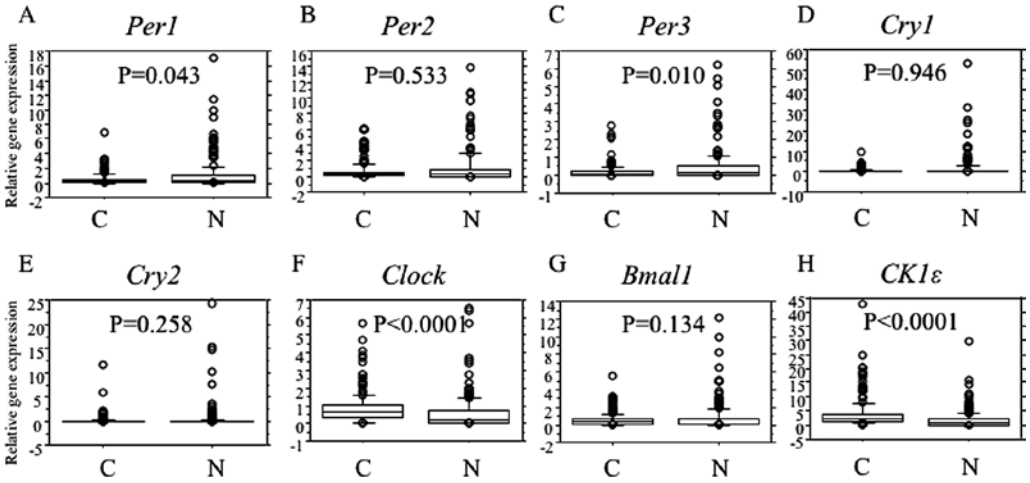

Figure 2. Comparison of circadian gene mRNA expression levels between colorectal cancer tissue (C) and adjacent normal mucosa (N). Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Wilcoxon test. *Clock* and *CK1ε* gene expression levels were higher in cancer than in adjacent normal mucosa (P<0.001). *Per1* and *Per3* gene expression levels were higher in adjacent normal mucosa than in cancer. *Per2*, *Cry1*, *Cry2*, and *Bmal1* gene expression levels were similar in cancer and adjacent normal mucosa.

normal mucosa than in cancer (P=0.043, P=0.010; Fig. 2A and C). *Per2*, *Cry1*, *Cry2*, and *Bmal1* gene expression levels were similar in cancer and adjacent normal mucosa (Fig. 2B-G).

Relations of circadian gene expression levels to clinicopathological features. Expression levels of the circadian genes were categorized as low or high according to their median values. The relations between the expression levels of these genes and clinicopathological features were then examined. Expression levels of the circadian genes were unrelated to age, gender, tumor size, lymph node metastasis, lymphatic invasion, and venous invasion. High expression of the *Bmal1* gene and low expression of the *Per1* gene correlated with liver metastasis (Table II).

Relations of Bmal1 and Per1 gene expression levels to liver metastasis. The highest rate of liver metastasis was associated with high expression of the *Bmal1* gene and low expression of the *Per1* gene (Fig. 3).

Table II. Relations of circadian gene expression levels to clinicopathological features.

A, Relationship between expression of *Per1*, *Per2*, *Per3* or *Cry1* genes and clinicopathological features

	Expression of *Per1*			Expression of *Per2*			Expression of *Per3*			Expression of *Cry1*		
Variables/categories	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value
Age	65.6±11.3	66.0±10.3	0.775	65.6±11.1	66.0±10.5	0.805	65.7±11.2	65.8±10.4	0.917	65.1±11.0	66.5±10.6	0.344
Gender												
Male	49	61	0.090	53	57	0.572	54	56	0.778	51	59	0.258
Female	52	40		48	44		47	45		50	42	
Tumor size (cm)												
<5	57	55	0.777	58	54	0.571	61	51	0.157	59	53	0.396
≥5	44	46		43	47		40	50		42	48	
Histological type												
Well differentiated	24	35	0.193	31	28	0.469	28	31	0.563	27	33	0.428
Moderately differentiated	63	52		59	56		61	54		62	53	
Poorly differentiated	14	14		11	17		12	16		12	16	
Depth of invasion												
T1	8	9	0.319	10	7	0.081	10	7	0.540	9	8	0.659
T2	39	54		53	40		45	48		46	47	
T3	50	30		31	49		42	38		42	3	
T4	4	8		7	5		4	8		4	8	
Lymph node metastasis												
Absent	43	50	0.323	45	48	0.672	48	45	0.672	46	47	0.888
Present	58	51		56	53		53	56		55	54	
Location												
Colon	59	50	0.204	62	47	0.034	59	50	0.204	62	47	0.034
Rectum	42	51		39	54		42	51		39	54	
Lymphatic invasion												
Absent	63	69	0.375	69	63	0.375	67	65	0.767	67	65	0.767
Present	38	32		32	38		34	36		34	36	
Venous invasion												
Absent	32	43	0.109	37	38	0.884	40	35	0.467	38	37	0.884
Present	69	58		64	63		61	66		63	64	
Liver metastasis												
Absent	63	77	0.033	73	67	0.360	71	69	0.760	73	67	0.360
Present	38	24		28	34		30	32		28	34	

Table II. Continued.

B, Relationship between expression of *Cry2*, *Clock*, *Bmal1* or *CK1ε* genes and clinicopathological features

Variables/categories	Expression of *Cry2*			Expression of *Clock*			Expression of *Bmal1*			Expression of *CKIε*		
	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value
Age	66.8±10.6	64.8±10.9	0.187	65.3±11.1	66.3±10.5	0.484	66.4±10.4	65.2±11.2	0.387	66.0±11.1	65.7±10.5	0.837
Gender												
Male	52	58	0.400	54	56	0.778	47	63	0.024	50	60	0.158
Female	49	43		47	45		54	38		51	41	
Tumor size (cm)												
<5	55	57	0.777	53	59	0.396	59	53	0.400	57	55	0.777
≥5	46	44		48	42		42	48		44	46	
Histological type												
Well differentiated	29	30	0.888	26	33	0.432	27	32	0.251	22	37	0.066
Moderately differentiated	59	56		62	53		63	52		63	52	
Poorly differentiated	13	15		13	15		11	17		16	12	
Depth of invasion												
T1	5	12	0.230	7	10	0.570	11	6	0.593	8	9	0.322
T2	48	45		45	48		45	48		41	52	
T3	40	40		41	39		40	40		44	36	
T4	8	4		8	4		5	7		8	4	
Lymph node metastasis												
Absent	47	46	0.888	43	50	0.323	44	49	0.480	46	47	0.888
Present	54	55		58	51		57	52		55	54	
Location												
Colon	55	54	0.888	58	51	0.323	60	49	0.121	61	48	0.066
Rectum	46	47		43	50		41	52		40	53	
Lymphatic invasion												
Absent	60	72	0.843	66	66	1.000	69	63	0.375	64	68	0.554
Present	41	29		35	35		32	38		37	33	
Venous invasion												
Absent	40	35	0.467	37	38	0.884	37	38	0.884	32	43	0.109
Present	61	66		64	63		64	63		69	58	
Liver metastasis												
Absent	72	68	0.542	70	70	1.000	77	63	0.033	66	74	0.223
Present	29	33		31	31		24	38		35	27	

Table III. Univariate analysis of clinicopathological factors and circadian genes expression for outcomes.

Variables/categories	no.	Survival rate (%) 1-year	3-year	5-year	P-value
Age (years)					
<65	92	95.6	87.9	75.3	0.3202
≥65	110	90.9	77.3	71.7	
Gender					
Male	110	91.8	79.9	71	0.4833
Female	92	93.5	83.7	75.1	
Tumor size (cm)					
<5	112	96.4	92.2	81.7	<0.0001
≥5	90	87.8	67.2	60.9	
Histological type					
Wel, mod	174	95.4	85.4	75.1	0.0093
Por	28	74.3	62.4	43.3	
Serosal invasion					
Absent	110	96.3	92.7	91.3	<0.0001
Present	92	87	69.2	57.3	
Lymph node metastasis metastasis					
Absent	93	97.8	94.6	90.5	<0.0001
Present	109	87.1	70.7	58.2	
Location					
Colon	109	92.6	86.1	77.8	0.0941
Rectum	93	92.5	77.9	67.0	
Lymphatic invasion					
Absent	132	98.5	89.9	82.3	<0.0001
Present	70	81	66.8	53.2	
Venous invasion					
Absent	75	96	89.2	72.6	0.1884
Present	127	89.7	77.4	70.8	
Liver metastasis					
Absent	140	97.9	93.9	89.2	<0.0001
Present	62	80.4	53.8	34.2	
Expression of *Per1*					
High	101	90.1	82	74.6	0.7583
Low	101	95	81.2	66.7	
Expression of *Per2*					
High	101	95	91	81.2	0.0048
Low	101	90.1	72.5	63.3	
Expression of *Per3*					
High	101	94.2	87.9	79.8	0.0551
Low	101	91.3	76.5	64.2	
Expression of *Cry1*					
High	101	94.1	86.2	79.9	0.0586
Low	101	91	73.3	66.3	
Expression of *Cry2*					
High	101	90	76.6	69.5	0.0962
Low	101	95	86.7	76	
Expression of *Clock*					
High	101	92.1	83.8	69.8	0.9903
Low	101	93	79.6	75.7	
Expression of *Bmal1*					
High	101	90	75.9	70.7	0.1673
Low	101	95	87.1	74.9	
Expression of *CK1ε*					
High	101	92.1	80.6	73.4	0.7486
Low	101	93	82.7	70.8	

Survival time was determined using the Kaplan-Meier method and compared using the log-rank test. Wel, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma.

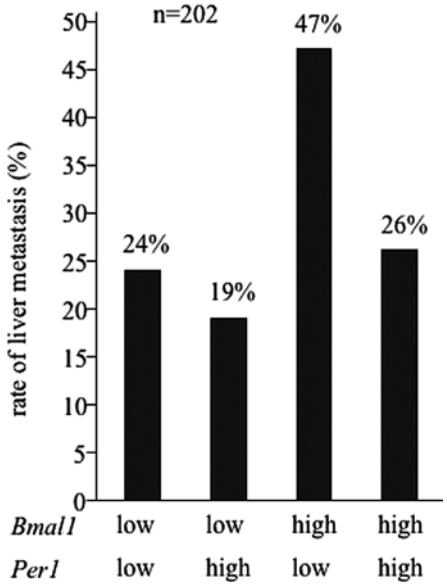

Figure 3. Relations of Bmal1 and Per1 gene expression levels to liver metastasis. The highest rate of liver metastasis was associated with high expression of the *Bmal1* gene and low expression of the *Per1* gene.

Univariate analysis of clinicopathological factors and the expression levels of the circadian genes for outcomes. Univariate analysis revealed that tumor size, serosal invasion, lymph node metastasis, lymphatic invasion, liver metastasis, and the expression of the *Per2* gene positively influenced outcomes (Table III).

Multivariate analysis of clinicopathological factors and the expression levels of the circadian genes for outcomes. On multivariate analysis using Cox proportional hazard regression analysis, the expression of *Per2* gene expression was an independent variable affected outcomes of patients with colorectal cancer (P=0.006) (Table IV).

Table IV. Multivariate analysis using Cox proportional hazard regression model.

Valiables/categories	Hazard ratio	95% CI	P-value
Per2 expression			
High vs. low	0.401	0.208-0.771	0.006
Tumor size			
<5 cm vs. ≥5 cm	0.568	0.289-1.118	0.101
Histological type			
Wel, mod vs. por	0.806	0.388-1.676	0.564
Serosal invasion			
Present vs. absent	1.378	0.616-3.081	0.435
Lymph node metastasis			
Present vs. absent	3.069	1.281-7.351	0.012
Lymphatic invasion			
Present vs. absent	1.357	0.684-2.689	0.382
Liver metastasis			
Present vs. absent	6.169	2.880-13.213	<0.001

CI, confidence interval; wel, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma.

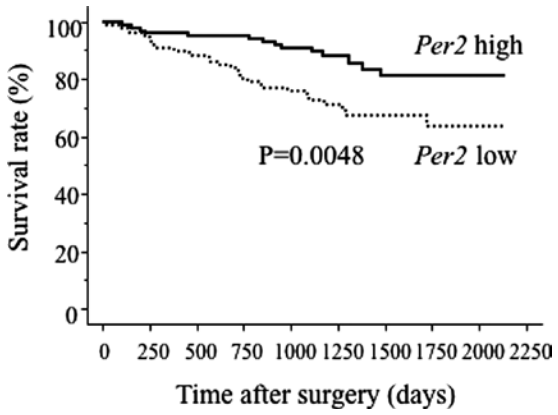

Figure 4. The relations between expressions of the circadian genes and outcomes. High expression of the *Per2* gene was associated with significantly better outcomes than low expression of the *Per2* gene (P=0.0048).

Relations between expressions of the circadian genes and outcomes. High expression of the *Per2* gene was associated with significantly better outcomes than low expression of the *Per2* gene (P=0.0048) (Fig. 4).

Discussion

In this study, we examined the expression levels of circadian genes in colorectal cancer and in adjacent normal mucosa. We also studied the relations of the expression levels of these genes to outcomes and clinicopathological features. Our results suggest that overexpression of the *Bmal1* gene and reduced expression of the *Per1* gene are useful predictors of liver metastasis, whereas reduced expression of the *Per2* gene is linked to poor outcomes in patients with colorectal cancer.

Several previous studies have compared expression levels of circadian regulators between cancer tissue and adjacent normal mucosa. One study found that 95% of breast cancer tissue samples displayed loss or deregulated levels of Per1 and Per3 proteins as compared with adjacent normal tissue (22). Moreover, the expressions of Per1 and Per2 in both sporadic and familial primary tumors are significantly lower than those in normal breast tissues (23). In human endometrial carcinoma, loss of Per1 protein is commonly observed in tumor cells, but not in the adjacent normal cells (24). A meta-analysis of microarray expression studies showed that *Per1* is down-regulated in human prostate cancer as compared with normal prostate tissue (18). *CK1ε* gene expression was found to be overexpressed in six kinds of cancer tissues as compared with adjacent normal tissues (25). In our study, *Per1* and *Per3* gene expression levels were lower in cancer than in adjacent normal mucosa. In contrast, *CK1ε* and *Clock* gene expression levels were higher in cancer than in adjacent normal mucosa. These results seem to be reasonable for the following reasons. Overexpression of *CK1ε* induces the phosphorylation and degradation of the Period. Reduced Period expression in turn decreases the formation of Per/Cry/CK1ε complexes. Because Per/Cry/CK1ε complexes inhibit the activity of Bmal1/Clock heterodimers, reduced levels of the former promote the activity of the latter. Overexpression of Clock also increases Bmal1/Clock heterodimers, which induce *cyclin D1* expression (19). Cyclin D1 promotes the proliferation of cancer cells (26).

We then examined the relations of the expression levels of circadian genes to clinicopathological features. High expression of the *Bmal1* gene and low expression of the *Per1* gene correlated with liver metastasis. We next examined the relations of Bmal1 and Per1 gene expression levels to liver metastasis. Several previous studies have examined Bmal1 and Per1. Bmal1 was suggested to be a positive regulator of tumor growth and metastasis, acting by expressing vascular endothelial growth factor in cancer (27). Bmal1 epigenetic inactivation contributes to the development of hematologic malignancies by disrupting the cellular circadian clock (28). Per1 inactivation is thought to play an important role in carcinogenesis (29). Moreover, overexpression of Per1 in cancer cells leads to significant growth inhibition and apoptosis (24). In our study, high expression of the *Bmal1* gene and low expression of the *Per1* gene correlated with liver metastasis. Overexpression of the *Bmal1* gene and reduced expression of the *Per1* gene might thus promote liver metastasis through the following mechanism. Reduced Per1 expression decreases the formation of Per/Cry/CKIε complexes. Reduced levels of these complexes promote the activity of Bmal1/Clock heterodimers. Overexpression of Bmal1 also increases the activity of Bmal1/Clock heterodimers, which induce cyclin D1 expression (19). High levels of cyclin D1 expression increase cancer cell proliferation (26), thereby, promoting liver metastasis.

Finally, we examined the relations between the expressions of circadian genes and outcomes. In the expressions of circadian genes, only the expression of the *Per2* gene positively influenced outcomes of patients with colorectal cancer in the univariate analysis. Moreover, the expression of

the *Per2* gene was an independent variable affecting outcomes on multivariate analysis using Cox proportional hazard regression analysis. Previous studies examining the relation between Per2 and cancer have reported that mice without functional Per2 are prone to develop cancer and display altered expression of genes involved in cell cycle regulation, tumor suppression, and apoptosis regulation, such as *cyclin D1, cyclin A, p53*, *c-Myc, Mdm2*, and *Bcl-2*. In particular, *c-Myc* is controlled by *Per2* through the activity of Bmal1/Clock heterodimers (19). Overexpression of the *Per2* gene induces cancer cell apoptosis (20), and inhibits the neoplastic growth of cancer cells (30). Moreover, *Per2* gene mutations have been identified in human colorectal and breast cancers (31), and overexpression of Per2 inhibits tumor proliferation in culture as well as in animals (32,33). In our study, high expression of the *Per2* gene was associated with significantly better outcomes than low expression of the *Per2* gene. Reduced expression of the *Per2* gene might thus shorten survival in patients with colorectal cancer. The following mechanism is thought to be involved. Reduced expression of the *Per2* gene decreases the activity of Bmal1/Clock heterodimers, leading to the induction of *c-Myc*. High levels of c-Myc promote cancer cell proliferation, and reduced expression of Per2 decreases p53 and increases Bcl-2. Reduced p53 expression and increased Bcl-2 expression repress apoptosis and promote cancer cell survival. Increased cancer cell proliferation and survival lead to poor outcomes.

In conclusion, our results suggest that overexpression of the *Bmal1* gene and reduced expression of the *Per1* gene are useful predictors of liver metastasis. Moreover, reduced expression of the *Per2* gene may be a predictor of outcomes in patients with colorectal cancer.

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