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Original Article

Period 1 and estrogen receptor-beta are downregulated in Chinese colon cancers

Yupeng Wang[1*], Tonghai Xing[1*], Li Huang[1*], Guohe Song[1], Xing Sun[1], Lin Zhong[1], Junwei Fan[1], Dongwang Yan[1], Chongzhi Zhou[1], Feifei Cui[1], Fudong Yu[1], Jian Chen[1], Yang Yu[1], Chao Li[1], Huamei Tang[2], Zhihai Peng[1], Xiaoliang Wang[1]

[1]Department of General Surgery, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, People's Republic of China; [2]Department of Pathology, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, People's Republic of China. [*]Equal contributors.

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Abstract: To investigate whether Period 1 (PER1) and Estrogen receptor-beta (ER2) are associated with occurrence and development of Chinese colorectal cancers. By using RT-quantitative PCR, tissue microarray (TMA) and immunohistochemistry, we detected mRNA levels and protein levels of PER1 and ER2 in the cancerous tissues and paired normal adjacent tissues in patients with colorectal cancer. Survival analyses were performed by the Kaplan-Meier method utilizing log-rank test and univariate and multivariate Cox proportional modeling to measure 5-year disease-free survival (DFS) and overall survival (OS). Real-time PCR showed that, the delta Ct value (tumor tissue vs. normal mucosa) of PER1 or ER2 is 8.51±2.81 vs. 7.34±2.08 or 12.39±2.43 vs. 9.76±1.75, expression of PER1 and ER2 decreased significantly in tumor tissues compared with noncancerous mucosas of patients with or without metastasis (both of *P* values <0.001). Spearman test revealed that PER1 and ER2 were significantly down-regulated in cancerous tissues (r=0.283; P<0.001) which was also confirmed by immunohistochemistry of specimens from 203 colon cancer patients in a TMA format. The reduction of PER1 was associated with gender and distant metastasis (P=0.037 and P<0.001, respectively) whereas the decline of ER2 was associated with age (P=0.043) by analyzing the clinical data. However, we were not capable of detecting any association between PER1 level or ER2 level and overall survival (OS) or disease free survival (DFS). It is the first observation of correlated reduction of PER1 and ER2 in Chinese colon cancers, and they do play a certain role in colorectal cancer.

Keywords: Period 1, estrogen receptor-beta, colon Cancer, chronotherapy, prognosis

Introduction

In China, with the improvement of living conditions and the changes of eating habits, the incidence of colorectal cancer is gradually increasing [1]. Although surgery is the main treatment, coupled with radiotherapy and chemotherapy which can prolong patients' survival time, its efficacy is not satisfactory, especially in patients with metastasis. Changes of multiple genes and pathways are involved in CRC; studying these changes could contribute to elucidating carcinogenesis and development of CRC and helping improve anti-cancer therapies.

Circadian rhythm is considered to be integrated by the suprachiasmatic nucleus (SCN), which affects the neuroendocrine system, immune system and other aspects of individuals involved in the tumor development and progression, in vitro experiments also confirmed that some of the circadian genes can also affect the invasive ability of tumor cells via transcription and translation process [2, 3]. In peripheral tissues, it's also found that there are molecular clocks independent of the SCN, such as food entrainable oscillator (FEO) which is responsible for the regulation of intestinal physiological oscillation. Recent researches have shown that *Period* gene may play an important role in the regulation of FEO among mice [4].

Period (*PER*) gene, as one of the circadian genes, can regulate cell cycle and promote DNA repair, which plays an important role in proliferation and differentiation, as well as in double-

strand DNA damage response. For example, PER1 can directly act on ATM and CHK2 to control the checkpoint of G1 phase and prevent cells from shifting into S phase [5]. BMAL1-CLOCK/NPAS2 heterodimer binds to E-box in the upstream promoter region of *PER* gene to activate the transcription, and after PER protein hits a certain concentration, the activity of the heterodimer turns to be reduced to form a negative feedback loop [6]. Studies have found that PER1 gene was down-regulated in a variety of tumors such as breast cancer, non-small cell lung cancer and gastric cancer [7-9]. As seen above, PER1 gene plays an important role in cancer development.

Estrogen receptor-beta (ER2), the dominant subtype in gut [10] and through which 17 beta-estradiol exhibits a protective function from colon tumorigenesis [11], may be regulated somehow by the circadian *PER* gene [12]. ER2 -/- mice showed that lacking ER2 will lead to high proliferative activity, differentiation disorders and abnormal apoptosis of the colonic mucosa cells [13]. Data from our previously established long serial gene expression (SAGE) database based on a small size of samples also indicated low ER2 levels in Chinese patients with colon cancer.

Our previous studies had found that PER3 was associated with the development and prognosis of colorectal cancer [14], which prompted us to analyze the expression levels of another member of the circadian gene *PER1* and its possible target gene *ER2* in colorectal cancer. We used Real-time PCR to detect the mRNA levels of PER1 and ER2 in tumor tissues and matched normal tissues, and expanded the sample size to 203 cases of colorectal cancer patients to analyze their expression utilizing immunohistochemistry.

Materials and methods

Patients and specimens

Clinical data of 203 colorectal cancer patients who underwent radical enterectomy in Shanghai Jiaotong University Affiliated First People's Hospital were collected via medical records. No patients received either chemotherapy or radiotherapy before surgery. Informed consent according to a protocol approved by the Institutional Review Board of the Shanghai First People's Hospital was signed by the patients.

Follow-up was carried out in accordance with the National Comprehensive Cancer Network Practice Guidelines in colon cancer. There were 86 male and 117 female patients, with a median age of 68 years (range, 22-95 years) at the time of operation. The median follow-up time was 61 months (range, 9-89 months).

Tumor tissues and normal adjacent tissues (at least 7 cm from the cancer margin) were obtained from colon cancer patients underwent colectomy were snap-frozen in liquid nitrogen and stored at -80°C. The 6th American Joint Committee on Cancer (AJCC) staging system was also applied for tumor staging.

Total RNA extraction and real-time PCR

Total RNA was extracted from 40 colon and 40 rectal cancer cancerous tissues and paired normal tissues using RNeasy Min Kit (Qiagen, Hilden, Germany) and then the mRNA levels of PER1 and ER2 were examined by Real-time PCR with the SYBR Green RNA PCR kit (Fermentas, Shenzhen, China), according to the manufacturer's instructions. The following primers were used: PER1-Forward, 5'-TACCAGCCATTCCGCCTAACC-3'; PER1-Reverse, 5'-GCAGCCCTTTCATCCACATCC-3'; ER2-Forward, 5'-TCTCCTTTAGTGGTCCATCGC-3'; ER2-Reverse, 5'-GAGCATCCCTCTTTGAACCTG-3'; Actin-Forward, 5'-ACGTGGACATCCGCAAAGAC-3'; Actin-Reverse, 5'-CAAGAAAGGGTGTAACGCAACTA-3'. Each reaction was repeated at least three times and actin transcript was used as an internal control. Using $2^{\Delta\Delta Ct}$ method, the mean mRNA level for each tumor specimen was compared with its matched normal tissue, which was calculated by fold change formula as followed: PER1/ER2_ΔCt=(Avg.PER1/ER2_ΔCt-Avg. actin_ΔCt), PER1/ER2_ΔΔCt=(Avg.PER1/ER2_ΔCtNormal-Avg. PER1/ER2_ΔCtTumor).

Tissue microarray construction and immunohistochemistry

A 2.0-mm-diameter punch instrument was applied to collect 2 cores from each formalin-fixed, paraffin-embedded cancer tissue sample and from each normal mucosa sample. After the construction of tissue microarrays in collaboration with Shanghai Biochip (Shanghai,

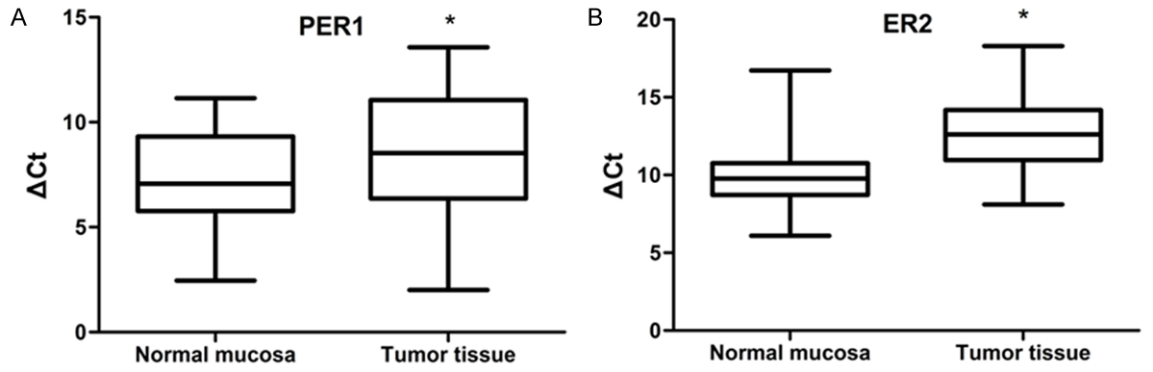

Figure 1. PER1 and ER2 mRNA levels (ΔCt) in cancerous tissue and adjacent normal mucosa by Real-time PCR. *Represented the difference was significant.

China), hematoxylin and eosin (H&E)-stained slides were screened for optimal tumor and related normal mucosa. Immunostaining was performed using anti-PER1 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ER2 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and then incubated with goat anti-mouse Envision System Plus-HRP (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature. Tissue sections were counterstained with Mayer hematoxylin. The negative control was prepared with normal mucosa and without antibody incubation.

To avoid bias, two pathologists who were blind to patient's information independently evaluated immunoreactivity. Staining intensity for PER1 or ER2 was scored as 0 (negative), 1 (weak) and 2 (strong). Judging the percentage of positively-stained cells, staining extent was score as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The specimens were divided into following three groups according to final staining score (sum of intensity score and extent score): negative (0-1), weakly positive (2-4), and strongly positive (5-6). Weakly positive and strongly positive were also deemed to be positive.

Statistical analysis

Statistical analyses were performed by SPSS 19. 0 statistics software (SPSS, Chicago, IL). For continuous variables, data are expressed as mean ± standard deviation and paired t-tests were performed to indicate the global expression (ΔCt) of PER1 or ER2 in normal and cancer tissues from colorectal cancer patients. For categorical variables, data are represented as the numerical count and percentage and compared with Pearson χ^2 test or Fisher's exact test. McNemar's test and Wilcoxon signed rank tests were used to compare PER1 or ER2 staining and PER1 or ER2 expression levels in primary tumor and normal adjacent tissues, respectively. The correlation between PER1 and ER2 was calculated by Spearman's test. Cox proportional hazard models were applied to detect association of PER1 or ER2 expression levels and subjects' characteristics. Significant factors in univariate Cox proportional hazard models were selected for the final multivariate regression model using the forward conditional method. Kaplan-Meier curves using log-rank test were utilized to represent cumulative survival proportion for OS time and DFS time by PER1 or ER2 levels. A P value <0.05 was considered to be statistically significant.

Results

Reduced PER1/ER2 expression in colon cancer compared with adjacent normal tissue

The Real-time PCR results showed that in 80 pairs of randomly selected patients with colorectal cancer (40 cases of colon cancer, 40 cases of colorectal cancer), the ΔCt value of PER1 in matched normal tissues is 7.34±2.08 (range 2.45-11.14), whereas in cancer tissues the ΔCt value of being 8.51±2.81 (range 2.01-13.57) decreased significantly (P<0.001, **Figure 1A**). For ER2 expression, the ΔCt value in related normal mucosa is 9.76 ± 1.75 (range 6.10-16.72), while in cancer tissues, the ΔCt value which is 12.39 ± 2.43 (range 8.11-18.30)

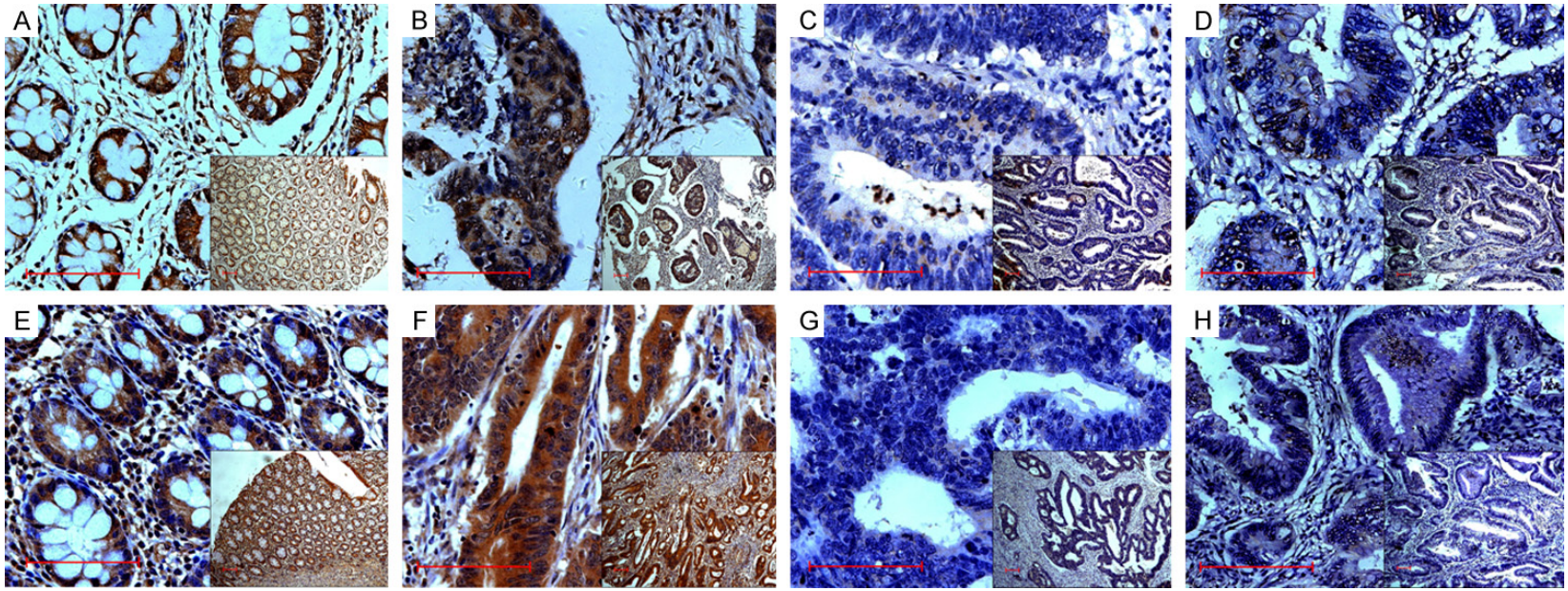

Figure 2. PER1/ER2 expression in normal and tumor tissue by immunochemistry. A and E. Positive staining of PER1 and ER2 in normal tissue, respectively; B and F. Tumor tissue exhibiting positive expressions of PER1 and ER2, respectively; C and G. Tumor tissue exhibiting negative expressions of PER1 and ER2, respectively; D and H. Negative staining of PER1 and ER2 in the same tumor slice, respectively. Bar=100 um; original magnification ×400 (×100 for insets).

Table 1. Expressions of PER1 and ER2 in normal and cancerous tissues

Expression of PER1	Normal tissue	Tumor tissue	P^a	Expression of ER2	Normal tissue	Tumor tissue	P^b
All subjects			<0.001*	All subjects			<0.001*
No. of subjects	203	203		No. of subjects	203	203	
Negative	1 (0.5%)	21 (10.3%)		Negative	22 (10.8%)	86 (42.4%)	
Positive	202 (99.5%)	182 (89.7%)		Positive	181 (89.2%)	117 (57.6%)	
Subjects with metastasis			0.029*	Subjects with metastasis			<0.001*
No. of subjects	95	95		No. of subjects	95	95	
Negative	0 (0%)	6 (6.3%)		Negative	9 (9.5%)	36 (37.9%)	
Positive	95 (100%)	89 (93.7%)		Positive	86 (90.5%)	59 (62.1%)	
Subjects without metastasis			<0.001*	Subjects without metastasis			<0.001*
No. of subjects	108	108		No. of subjects	108	108	
Negative	1 (0.9%)	15 (13.9%)		Negative	13 (12.0%)	50 (46.3%)	
Positive	107 (99.1%)	93 (86.1%)		Positive	95 (88.0%)	58 (53.7%)	
P^c	1.000	0.077		P^d	0.558	0.227	

[a,b]Normal and tumor tissues; *P* values derived from McNemar's test. [c,d]Metastatic vs. nonmetastatic subjects in normal tissue and tumor tissue; *P* value derived from Pearson χ^2 test or Fisher's exact test. *Represented the difference was significant

Table 2. Association between PER1 expression and ER2 expression in colon cancer patients

ER2 expression	PER1 expression			*P*
	Negative	Weakly positive	Strongly positive	
All subjects				<0.001*
Negative	16 (76.2%)	53 (45.7%)	17 (25.8%)	
Weakly positive	4 (19.0%)	57 (49.1%)	42 (63.6%)	
Strongly positive	1 (4.8%)	6 (5.2%)	7 (10.6%)	

P value derived from Spearman's test. *indicated the association between the variables.

similarly diminished significantly (*P*<0.001, **Figure 1B**). A total of 57.5% (n=46) and 78.75% (n=63) of colon cancer patients had significantly reduced expression for PER1 and ER2, respectively.

Immunohistochemistry displayed that PER1 and ER2 were positively expressed in normal tissues (**Figure 2A** and **2E**, respectively). In slices without using antibodies against ER2 and PER1, no expression of ER2 or PER1 was detected. PER1/ER2 was also expressed in cancerous mucosa (**Figure 2B**, **2C**, **2F** and **2G**). As shown in **Table 1**, a larger proportion of cancerous tissues were negative compared with normal tissues (10.3% vs. 0.5%, respectively; *P*<0.001), so is ER2 expression (42.4% vs. 10.8%, respectively; *P*<0.001). Moreover, this phenomenon could be observed in patients with or without lymph node metastasis.

It's worth noting that simultaneously downward expression of PER1 and ER2 was found in the same section of tumor tissue (**Figure 2D**, **2H**). A significant correlated down-regulation of PER1 and ER2 expression in tumor tissues was observed by Spearman's test (*r*=0.283; *P*<0.001, **Table 2**).

Association of reduced PER1/ER2 expression in colon cancer with clinical pathologic parameters

In the 203 enrolled patients, there are 21 patients with PER1-negative tumor and 86 cases of subjects with ER2-negative tumor (**Table 3**). No significant relationship was found between tumor location, T stage, N stage, differentiation, AJCC stage, vascular invasion, recurrence and survival and PER1 or ER2 level. Notably in **Table 3**, PER1 levels were significantly associated with gender (*P*=0.037), and a greater percentage of PER1-negative tumors was observed in females than that in males (71.4% vs. 28.6%, respectively). In addition, age appeared to have a vital influence on the distribution of ER2 expression in colon cancer (*P*=0.043), as in those with ER2-negative cancer the rate of elderly subjects (≥65 y) was higher than the younger ones (<65 y) (52.3% vs. 47.7%, respectively). Moreover, the expression of PER1 was significantly associated with distant metastasis (*P*<0.001).

Association between expression of PER1 or ER2 and colorectal patient survival

We used Kaplan-Meier curves (log-rank test) to explore the possible association between

Table 3. Associations between PER1/ER2 expression in tumor tissues and clinical factors of studied subjects

Variable	PER 1 expression, *n* (%)			*P*	ER2 expression, *n* (%)			*P*
	Negative (*n*=21)	Weakly positive (*n*=116)	Strongly positive (*n*=66)		Negative (*n*=86)	Weakly positive (*n*=103)	Strongly positive (*n*=14)	
Age				0.365				0.043*
<65 y	8 (38.1)	51 (44.0)	22 (33.3)		41 (47.7)	38 (36.9)	2 (14.3)	
≥65 y	13 (61.9)	65 (56.0)	44 (66.7)		45 (52.3)	65 (63.1)	12 (85.7)	
Gender				0.037*				0.075
Male	6 (28.6)	44 (37.9)	36 (54.5)		30 (34.9)	47 (45.6)	9 (64.3)	
Female	15 (71.4)	72 (62.1)	30 (45.5)		56 (65.1)	56 (54.4)	5 (35.7)	
Tumor location				0.064				0.992
Right colon	4 (19.0)	52 (44.8)	28 (42.4)		36 (41.9)	41 (39.8)	7 (50.0)	
Transverse colon	6 (28.6)	7 (6.0)	6 (9.1)		9 (10.5)	9 (8.7)	1 (7.1)	
Left colon	1 (4.8)	12 (10.3)	7 (10.6)		9 (10.5)	10 (9.7)	1 (7.1)	
Sigmoid colon	10 (47.6)	45 (38.8)	25 (37.9)		32 (37.2)	43 (41.7)	5 (35.7)	
T category				0.751				0.659
T1	0 (0.0)	6 (5.2)	2 (3.0)		4 (4.7)	4 (3.9)	0 (0.0)	
T2	3 (14.3)	12 (10.3)	8 (12.1)		10 (11.6)	11 (10.7)	2 (14.3)	
T3	5 (23.8)	45 (38.8)	26 (39.4)		26 (30.2)	44 (42.7)	6 (42.9)	
T4	13 (61.9)	53 (45.7)	30 (45.5)		46 (53.5)	44 (42.7)	6 (42.9)	
N category				0.055				0.290
N0	15 (71.4)	59 (50.9)	34 (51.5)		50 (58.1)	52 (50.5)	6 (42.9)	
N1	3 (14.3)	32 (27.6)	26 (39.4)		20 (23.3)	34 (33.0)	7 (50.0)	
N2	3 (14.3)	25 (21.6)	6 (9.1)		16 (18.6)	17 (16.5)	1 (7.1)	
M category				<0.001*				0.636
M0	17 (81.0)	107 (92.2)	61 (52.6)		77 (89.5)	94 (91.3)	14 (100.0)	
M1	4 (19.0)	9 (7.8)	55 (47.4)		9 (10.5)	9 (8.7)	0 (0.0)	
Vessel invasion				0.245				0.755
No	21 (100.0)	105 (90.5)	63 (95.5)		79 (91.9)	97 (94.2)	13 (92.9)	
Yes	0 (0.0)	11 (9.5)	3 (4.5)		7 (8.1)	6 (5.8)	1 (7.1)	
Differentiation				0.378				0.512
Well	10 (47.6)	55 (47.4)	34 (51.5)		37 (43.0)	55 (53.4)	7 (50.0)	
Moderate	5 (23.8)	46 (39.7)	23 (34.8)		34 (39.5)	36 (35.0)	4 (28.6)	
Poor	6 (28.6)	15 (12.9)	9 (13.6)		15 (17.4)	12 (11.7)	3 (21.4)	
AJCC stage				0.207				0.659
I	3 (14.3)	13 (11.2)	8 (12.1)		10 (11.6)	12 (11.7)	2 (14.3)	
II	11 (52.4)	45 (38.8)	25 (37.9)		38 (44.2)	39 (37.9)	4 (28.6)	
III	3 (14.3)	49 (42.2)	28 (42.4)		29 (33.7)	43 (41.7)	8 (57.1)	
IV	4 (19.0)	9 (7.8)	5 (7.6)		9 (10.5)	9 (8.7)	0 (0.0)	
Recurrence and metastasis				0.504				0.613
No	15 (71.4)	72 (62.1)	38 (57.6)		56 (65.1)	60 (58.3)	9 (64.3)	
Yes	6 (28.6)	44 (37.9)	28 (42.4)		30 (34.9)	43 (41.7)	5 (35.7)	
Survival				0.943				0.971
No	8 (38.1)	43 (37.1)	23 (34.8)		31 (35.2)	38 (36.9)	5 (35.7)	
Yes	13 (61.9)	73 (62.9)	43 (65.2)		57 (64.8)	65 (63.1)	9 (64.3)	

P derived from Pearson χ^2 test or Fisher's exact test. *Represented a significant association among the variables.

expression of PER1/ER2 and patient survival (**Figure 3**). The OS of 1-year, 3-year and 5-year in patients with PER1- positive cancer was 98%, 86% and 55%, respectively, while in sub-

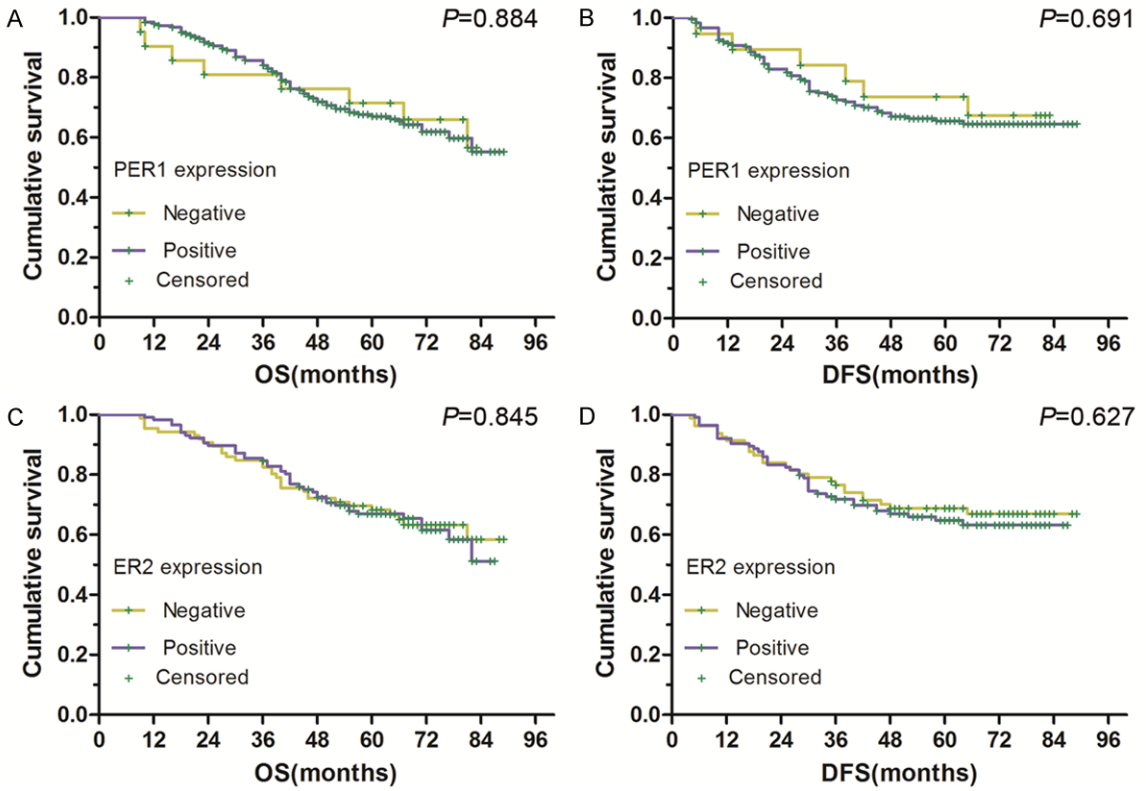

Figure 3. Association of PER1/ER2 expression with survival time of patients with colorectal cancer. A and B. OS and DFS for PER1, respectively; C and D. OS and DFS for ER2, respectively. Data are shown using Kaplan-Meier curves by log-rank test.

jects with PER1-negative cancer the 1-year, 3-year and 5-year OS was 90%, 81% and 67%, respectively. The 1-year, 3-year and 5-year OS of ER2-positive cancer patients was 99%, 85% and 55%, respectively, whereas in patients with ER2-negative tumors, the 1-year, 3-year and 5-year OS was 95%, 85% and 59%, respectively. No significant difference for estimated mean OS and DFS were found between patients with PER1-negative tumors and PER1-positive tumors (65.5±5.9 months vs. 69.5±2.0 months for OS, P=0.884; 66.7±6.0 months vs. 66.8±2.4 months for DFS, P=0.691), so were the calculated mean OS and DFS between patients with ER2-negative tumors and ER2-positive tumors (69.5±3.0 months vs. 68.3±2.4 months for OS, P=0.845; 68.3±3.4 months vs. 65.0±2.9 months for DFS, P=0.627). Similarly, no significant differences were observed in OS and DFS when the expressions of PER1 and ER2 were grouped as: negative, only one negative and both positive (data not shown).

Cox models exhibited factors that influenced patients' survival (**Tables 4** and **5**). By univariate analysis, OS and DFS were significantly related with N category, M category, vascular invasion, differentiation and AJCC stage (all P<0.05), nevertheless, no significant association was found between PER1/ER2 expression and patients' survival time. Moreover, in multivariate analysis, only differentiation and AJCC stage were found significantly associated with OS (P=0.002 and P=0.006, respectively), while DFS was found significantly related with N category, M category and differentiation (P<0.001, P=0.022 and P=0.005, respectively).

Discussion

In different races of colorectal cancer patients, changes at the genetic levels may differ; this study focused on the association of PER1/ER2 expression with colorectal cancer in Chinese population, assessing their values for diagnosis and prognosis as biomarkers, and may to a certain extent help better anti-cancer treatment.

As is prompted by our Long SAGE analysis, we evaluated PER1/ER2 expression in an ampli-

Table 4. Association between clinical characteristics and OS by Cox regression model analysis

Variable	OS			
	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age				
≥65 y vs. <65 y	0.963 (0.606-1.533)	0.875		
Gender				
Female vs. male	1.343 (0.836-2.158)	0.223		
Tumor location				
Transverse vs. right	0.799 (0.331-1.929)	0.618		
Left vs. right	0.959 (0.420-2.189)	0.920		
Sigmoid vs. right	1.064 (0.643-1.761)	0.808		
T category				
T2 vs. T1	0.303 (0.043-2.150)	0.232		
T3 vs. T1	0.949 (0.220-4.090)	0.944		
T4 vs. T1	2.812 (0.684-11.551)	0.152		
N category				
N1 vs. N0	4.021 (2.177-7.427)	<0.001*	1.631 (0.361-7.372)	0.525
N2 vs. N0	14.070 (7.537-26.266)	<0.001*	3.748 (0.844-16.643)	0.082
M category				
M1 vs. M0	14.741 (8.148-26.668)	<0.001*		
Vessel invasion				
Yes vs. no	4.677 (2.545-8.595)	<0.001*		
Differentiation				
Moderate vs. well	2.368 (1.342-4.178)	0.003*	1.747 (0.970-3.148)	0.063
Poor vs. well	7.499 (4.112-13.678)	<0.001*	2.972 (1.470-6.009)	0.002*
AJCC stage				
II vs. I	2.076 (0.468-9.025)	0.336	1.911 (0.430-8.488)	0.395
III vs. I	9.512 (2.292-39.469)	0.002*	3.905 (0.521-29.628)	0.185
IV vs. I	72.117 (16.172-321.605)	<0.001*	19.641 (2.347-164.401)	0.006*
PER1 expression				
Weak vs. negative	1.093 (0.511-2.337)	0.819		
Strong vs. negative	0.995 (0.443-2.233)	0.990		
ER2 expression				
Weak vs. negative	1.065 (0.663-1.713)	0.794		
Strong vs. negative	0.925 (0.359-2.384)	0.872		

HR: hazard ratio; CI: confidence interval. *meant the 95% CI of HR was not including 1.

fied sample size of 203 cases of Chinese colon cancers at both mRNA level and protein level, and determine clinical values of the disturbance of the two genes in colon cancer in this research.

In consistency with discoveries of Krugluger et al. [15], we found the decrease of PER1 in cancerous tissues was associated with gender. In chronotherapy which is playing an increasingly important role in treatment for colon cancer [16-18], gender is a quite reliable predictor for therapeutic effects [19]. Recently, a mathematical model using the expression of Reverba and Bmal-members of circadian genes-in livers as input data to determine optimal delivery time of irinotecan had been established by Li et al. [20], so it is reasonable this study may to a degree consummate chronotherapy. Additionally, our data exhibited the abated expression of ER2 in tumor tissues was associated with age, which can be used to determine curative effects of certain forms of anti-cancer therapy, such as postmastectomy radiation therapy

Table 5. Association between clinical characteristics and DFS by Cox regression model analysis

Variable	DFS			
	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age				
≥65 y vs. <65 y	1.063 (0.647-1.749)	0.808		
Gender				
Female vs. male	1.221 (0.745-2.002)	0.427		
Tumor location				
Transverse vs. right	0.859 (0.328-2.252)	0.758		
Left vs. right	1.135 (0.489-2.634)	0.768		
Sigmoid vs. right	1.251 (0.731-2.140)	0.413		
T category				
T2 vs. T1	0.326 (0.046-2.318)	0.263		
T3 vs. T1	0.933 (0.215-4.040)	0.926		
T4 vs. T1	2.610 (0.633-10.764)	0.184		
N category				
N1 vs. N0	3.433 (1.809-6.515)	<0.001*	3.172 (1.661-6.058)	<0.001*
N2 vs. N0	14.180 (7.477-26.892)	<0.001*	9.177 (4.584-18.374)	<0.001*
M category				
M1 vs. M0	9.028 (4.322-18.855)	<0.001*	2.563 (1.144-5.744)	0.022*
Vessel invasion				
Yes vs. no	5.162 (2.735-9.742)	<0.001*		
Differentiation				
Moderate vs. well	2.340 (1.306-4.193)	0.004*	1.707 (0.937-3.112)	0.081
Poor vs. well	6.363 (3.350-12.087)	<0.001*	2.863 (1.369-5.984)	0.005*
AJCC stage				
II vs. I	2.002 (0.452-8.872)	0.361		
III vs. I	9.052 (2.185-37.496)	0.002*		
IV vs. I	42.100 (8.886-199.459)	<0.001*		
PER1 expression				
Weak vs. negative	1.182 (0.500-2.799)	0.703		
Strong vs. negative	1.187 (0.481-2.929)	0.711		
ER2 expression				
Weak vs. negative	1.140 (0.686-1.895)	0.612		
Strong vs. negative	1.058 (0.406-2.757)	0.908		

HR: hazard ratio; CI: confidence interval. *meant the 95% CI of HR was not including 1.

seems to be beneficial to breast cancer patients younger than 40 years old in spite of ER status [21]. Judging from these, our discovery could be helpful to identify patients who may or may not benefit from close monitoring after surgery and develop optimal individualized treatment.

It's the first time in Chinese population, we convince, that significantly relevant reduction of PER1 expression and ER2 level was disclosed in colorectal cancerous tissues. This may provide indirect evidence the hypothesis that *ER2* gene might be downstream regulated by *PER1* gene.

However, different from results of the study conducted in HeLa cells of Cai et al. [12], we uncovered that the down-regulated expressions of PER1 and ER2 in CRC were correlated, as they considered that being a negative regulator, PER1 can inhibit the ER2 transcription through CLOCK-BMAL1 complex. This may be explained by diversely investigated cell lines or tissues-HeLa cells deprive from reproductive

system whose circadian oscillation is mainly generated by hypothalamus-pituitary-ovarian axis of SCN were used by Cai and his co-workers, while alimentary colon tissues in which there exists FEO independent of SCN to regulate circadian oscillation, were adopted for our study. Owing to the mechanism remains to be unclear, more studies are needed to elucidate how FEO controls physiological circadian rhythm of gastrointestinal tract.

Another possible explanation of joint decrease of PER1 and ER2 in colorectal cancer was similar epigenetic mechanism [22]. Highly conserved E-box motifs, serving as binding site of CLOCK-BMAL1 heterodimer which regulates *Period* gene transcription, exists in proximal promoter of both *PER1* and *ER2* [12]. DNA methylation is common in early tumorigenesis, Li L et al. had observed the low methylation of *PER1* promoter in the ER (+)/PR (+) breast cancers, which is significantly different from ER (-)/PR (-) breast cancers [23]. In prostate cancers, one of the reasons for *ER2* gene inactivation may be the CpG island methylation of promoter region [24]. Similar study on the relationship of PER1 and ER2 was also implemented on thirty Caucasian colon cancer patients by Mostafaie N et al. [22], whereas he failed to validate their findings at protein level and explore the association between expressions of the two genes and patients' survival.

Our data showed the reduction of PER1 in cancer tissues was significantly associated with M category, suggesting PER1 may get involved in metastatic process of colon cancer. Discrepancy between Mazzoccoli G et al. [25] and our study is that his team discovered that down-regulation of PER1 was significantly related to shorter survival time of colon cancer patients, yet no significant association was disclosed between downward level of PER1 or ER2 and the subjects' survival (OS and DFS) in this investigation. This maybe interpreted by that patients in our study were from Han Chinese population, which was different from the previous study using Caucasian population, and distinct ethnic genetic background probably leads to the contradictory result. Therefore, A larger sample size will be needed and further research on the down-regulation mechanism of PER1 and ER2 in the CRC of Han population is supposed to be performed.

In summary, concomitant decrease of PER1 and ER2 in Chinese colorectal cancer patients was observed for the first time, and although PER1 or ER2 seems valueless for diagnosis and prognosis, our investigation do contribute to be helpful in optimizing and individualizing anti-cancer therapy as well as designing further studies to figure out the mechanism of down-regulation of PER1 and ER2 in colon cancer patients of Han Chinese population.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiaoliang Wang and Zhihai Peng, Department of General Surgery, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200080, People's Republic of China. Tel: (+86)21-63240090-3131; Fax: +86-2163241377; E-mail: xiaoliangwang1975@hotmail.com(XLW); E-mail: zhihai_peng58@163.com(ZHP)

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