

# Abnormal expression of *PER1* circadian-clock gene in oral squamous cell carcinoma

Rui Chen  
Kai Yang  
Ning-Bo Zhao  
Dan Zhao  
Dan Chen  
Chun-Rong Zhao  
Hong Tang

Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing, People's Republic of China

**Background:** The *PER1* circadian-clock gene plays an important role in the regulation of many normal physiological rhythms in vivo. It has been revealed recently that abnormal expression of *PER1* correlates closely with the occurrence and development of many cancers. However, the expression and significance of *PER1* in oral squamous cell carcinoma (OSCC) remains unknown. The purpose of the present study was to investigate the direct links between aberrant *PER1* expression and clinicopathological features of OSCC.

**Methods:** *PER1* expression in cancerous and adjacent noncancerous tissues from 41 patients with OSCC was detected by immunohistochemical staining and real-time reverse transcriptase polymerase chain reaction, and correlations were sought with clinicopathological features in patients.

**Results:** Expression of *PER1* mRNA and protein in OSCC was significantly reduced compared with that in adjacent noncancerous tissue ( $P < 0.05$ ). Expression of *PER1* protein in oral phase III–IV SCC specimens was significantly lower than that in phase I–II specimens ( $P < 0.05$ ), and stage  $T_1$ – $T_2$  patients expressed significantly higher levels of *PER1* protein than  $T_3$ – $T_4$  patients ( $P < 0.05$ ). Expression of *PER1* in patients without lymph node metastasis was significantly higher than that in those with lymph node metastasis ( $P < 0.05$ ). *PER1* protein expression showed no significant correlation with patient gender and age, or with degree of tumor cell differentiation ( $P > 0.05$ ).

**Conclusion:** Changes in *PER1* expression may play an important role in the development, invasion, and metastasis of OSCC, and may also provide novel ideas and methods for investigation of the occurrence, development, and targeted treatment OSCC.

**Keywords:** oral cancer, squamous cell, circadian gene, *PER1*, immunohistochemistry, real-time reverse transcriptase polymerase chain reaction

## Introduction

Many physiological activities in living subjects, such as body temperature, heart rate, hormone secretion, cell proliferation, and metabolism, change rhythmically with the alternation between day and night. Such circadian rhythm is one of the basic characteristics of an organism's life activities and is controlled by the circadian system in vivo, which is composed of a series of circadian-clock genes.<sup>1</sup> To date, there are at least nine demonstrated major circadian-clock genes, ie, *period1* (*Per1*), *period2* (*Per2*), *period3* (*Per3*), *circadian locomotor output cycles kaput* (*Clock*), *cryptochrome1* (*Cry1*), *cryptochrome2* (*Cry2*), *brain and muscle Arnt-like protein 1* (*Bmal 1*), *casein kinase 1ε* (*CK1ε*), and *timeless* (*Tim*).<sup>2</sup> It has been shown recently that the occurrence, development, prognosis, and treatment of cancer are closely related to the abnormal

Correspondence: Kai Yang  
Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuzhong District, Chongqing 400016, People's Republic of China  
Tel +86 23 8901 2569  
Fax +86 23 8901 2569  
Email cqfyk@hotmail.com

expression of certain circadian-clock genes.<sup>3-9</sup> Therefore, further understanding of the behavior of circadian-clock genes may provide novel ideas and methods for investigation of the occurrence, development, and treatment of tumors.

*PER1*, a core circadian-clock gene, is located on the short arm of human chromosome 17 (17p12–p13.1), spanning about 16 kb.<sup>1,3</sup> The *PER1* gene not only plays an important role in the regulation of many normal circadian rhythms in vivo, but also correlates closely with development of cancers.<sup>4-9</sup> Studies have shown that the PER1 protein inhibits growth of prostate and colon cancers,<sup>4,5</sup> and that abnormal expression of the *PER1* gene leads to the occurrence and promotes the development of endometrial cancer, leukemia, and breast cancer.<sup>6-9</sup> Data from published research have also indicated that expression and function of the *PER1* gene varies significantly between different tumors.<sup>4,5,7,10,11</sup> Gery et al have shown that *PER1* plays a proapoptotic role and is downregulated in prostate and colon cancers,<sup>10</sup> while Sato et al have demonstrated that the *PER1* gene is highly expressed in pancreatic and liver cancers and exerts an anti-apoptotic effect.<sup>11</sup> These findings imply that the *PER1* gene may play important but different roles in the occurrence and development of different tumors. However, there is still a lack of studies focusing on the relationship between expression of the *PER1* gene and oral cancers.

The overall incidence of oral cancer is approximately 4.7–32.2/10,000 globally, among which more than 90% are SCC.<sup>12,13</sup> The current overall 5-year survival rate of oral squamous cell carcinoma (OSCC) post treatment is only 50%–60%,<sup>13</sup> so exploring new methods for the treatment of OSCC is of great significance.

The present study detected expression of the *PER1* gene in both cancerous and adjacent noncancerous tissues from 41 patients with OSCC and investigated their clinical significance by analyzing the correlation between *PER1* expression in OSCC and clinicopathological parameters, trying to, from the perspective of changes in *PER1* gene expression, provide new ideas for further investigation of the occurrence, development, and treatment of OSCC.

## Materials and methods

### Reagents and instruments

A streptavidin-peroxidase immunohistochemistry kit and horseradish peroxidase-labeled goat anti-rabbit IgG antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd (Beijing, China). The rabbit anti-human PER1 polyclonal antibody was sourced from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The polymerase

chain reaction (PCR) kit was purchased from Takara Bio (Shiga, Japan). The light microscope was a Leica TS-26 (Solms, Germany); the frozen low-speed centrifuge was a Z233MK-2 (Hermle, Wehingen, Germany); conventional PCR reactions were done using a S1000TM thermal cycler (Bio-Rad, Hercules, CA); and quantitative real-time PCR was performed using a Bio-Rad C-1000TM thermal cycler. The UV-GeneQuant spectrophotometer was sourced from Amersham (Uppsala, Sweden).

### Clinical data

Cancerous and adjacent noncancerous tissues were obtained from 41 patients with OSCC treated in the Department of Oral and Maxillofacial Surgery, First Affiliated Hospital of Chongqing Medical University from January 2011 to March 2012. The specimens were harvested between 10:50 am and 14:30 pm. The inclusion criteria were as follows: pathological diagnosis of OSCC; no preoperative radiotherapy, chemotherapy, or other treatments; concomitant neck dissection during surgery; and pathological confirmation of cervical lymph node metastasis. In total, 41 specimens were obtained from 24 males and 17 females of mean age 51.6 (range 22–81) years. According to the 2010 Union for International Cancer Control staging criteria,<sup>14</sup> six, 12, 16, and seven of the tumor cases were stage T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub>, respectively; 14 cases had cervical lymph node metastasis and 27 were without cervical lymph node metastasis. Clinical phase I, II, III, and IV accounted for six, 16, 13, and six cases, respectively. Pathological classification showed 13, 20, and eight cases to be well, moderately, and poorly differentiated, respectively. Each sample was divided into two parts; one part was fixed with 4% formalin and embedded in paraffin, and the other part was placed rapidly in liquid nitrogen. The study was approved by the human research ethics committee of the First Affiliated Hospital of Chongqing Medical University and written informed consent was obtained from all patients who participated.

### Immunohistochemistry

The paraffin-embedded tissues were cut into sections 4 μm thick. Streptavidin-peroxidase immunohistochemical staining was performed following the kit instructions. Briefly, the sections were routinely dewaxed, rehydrated, and permeabilized; endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub>; antigen retrieval was done under high temperature and pressure to expose the epitopes; and blocking with 5% normal goat serum, incubation with primary and secondary antibodies, and color development with DAB were carried

out sequentially in accordance with the kit instructions. The sections were then stained with hematoxylin, dehydrated, cleared, and observed under a light microscope after mounting with neutral resin. Slides with phosphate-buffered saline added instead of primary antibody were used as the negative control. The positive slide provided in the kit was used as the *PER1* positive control. Positive *PER1* staining appeared as a yellow color in the nucleus or cytoplasm. The results were assessed by semiquantitative scoring.<sup>15</sup> The intensity of positive staining (no staining, 0; light yellow, 1; brown, 2; and dark brown, 3) and the percentage of positive cells (number of positive cells  $\leq$  5%, 0; 6%–25%, 1; 26%–50%, 2; 51%–75%, 3;  $\geq$ 76%, 4) were scored, and the multiplication of both scores was used finally to evaluate *PER1* expression as follows: 0, negative; 1–4, positive;  $>$ 4, strongly positive. All positive and strongly positive sections were expressed as positive.

### Real-time reverse transcriptase PCR

Total RNA was extracted from the frozen cancerous tissue and adjacent noncancerous tissues and used for real-time reverse transcriptase PCR detection. The experiment was repeated three times as follows: total RNA was extracted from the cancerous tissue and adjacent noncancerous tissues using an RNAiso Plus kit, and RNA concentration and purity was determined by measuring absorbance at 260 nm and 280 nm. cDNAs were synthesized using a PrimeScript reverse transcriptase reagent kit according to the manufacturer's manual. Briefly, the reaction (20  $\mu$ L in total volume) was incubated at 37°C for 15 minutes and then at 85°C for 5 seconds. Real-time reverse transcriptase PCR primers for the target *PER1* gene and the internal control gene ( *$\beta$ -actin*) were designed using Oligo7.0 software (Table 1); the reaction system contained 2  $\times$  SYBR Premix Ex Taq™ II 12.5  $\mu$ L, 0.4  $\mu$ M forward and reverse primers (1  $\mu$ L each), a 2  $\mu$ L DNA template (100 ng), molecular grade H<sub>2</sub>O 8.5  $\mu$ L, in

a total volume of 25  $\mu$ L. The reaction was denatured at 95°C for 1.5 minutes, and cycling was carried out at 95°C for 10 seconds and 60°C for 30 seconds, for 40 cycles, the fluorescent signal was collected during a 60°C extension step, and the  $2^{-\Delta\Delta C_t}$  method was used to quantify expression of the *PER1* gene.

### Statistical analysis

All data were analyzed by  $\chi^2$  tests and *t*-tests using the Statistical Package for the Social Sciences version 17.0 statistical software (SPSS Inc, Chicago, IL), and the results are presented as the mean  $\pm$  standard deviation. A *P* value  $<$  0.05 was considered to be statistically significant.

## Results

### *PER1* mRNA and protein expression in OSCC

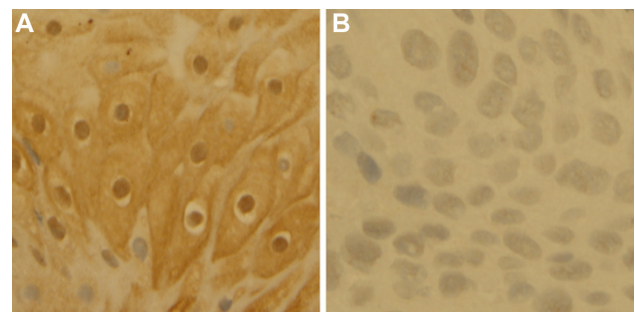
The results of immunohistochemistry show that the rate of positive *PER1* protein expression in OSCC and adjacent noncancerous tissues was 82.93% (34/41) and 100% (41/41) respectively, and the former was significantly lower than the latter ( $\chi^2 = 7.653$ , *P*  $<$  0.05). Expression of *PER1* protein in adjacent noncancerous tissues was mainly in the nucleus and cytoplasm and was strongly positive, whereas expression of *PER1* was weakly or moderately positive in tumor cells (Figure 1). The results of quantitative real-time PCR also confirm that the relative expression level of *PER1* mRNA in OSCC ( $3.71 \pm 0.42$ ) was significantly lower than that in adjacent noncancerous tissues ( $11.95 \pm 0.77$ , *t* =  $-16.171$ , *P*  $<$  0.001).

### *PER1* protein expression and clinicopathological features of OSCC

Expression of *PER1* protein in patients with phase I and II OSCC was significantly higher than that in patients with

**Table 1** Primer sequence and product size for *PER1* and  *$\beta$ -actin* genes

Gene	Primer sequence	Product (bp)
<i>PER1</i>	Forward: 5'-ACCCTGATGACCCA CTCTTCTC-3'	170
	Reverse: 5'-CTCCTCCATAGCCA AGTCCTGA-3'	
<i><math>\beta</math>-actin</i>	Forward: 5'-CTTCTACAATGAGC TGCGTGTG-3'	169
	Reverse: 5'-AGAGGCGTACAGGGA TAGCACAG-3'	



**Figure 1** Immunohistochemical staining of *PER1* protein (SP 400 $\times$ ). Expression of *PER1* protein in adjacent noncancerous tissues is strongly positive (A) and expression of *PER1* protein in oral squamous cell carcinoma is moderately or weakly positive (B).

phase III and IV disease ( $P < 0.05$ ), while stage T<sub>1</sub> and T<sub>2</sub> patients expressed significantly higher levels of PER1 protein compared with T<sub>3</sub> and T<sub>4</sub> patients ( $P < 0.05$ ). Expression of PER1 protein was significantly higher in patients without lymph node metastasis compared with patients having lymph node metastasis ( $P < 0.05$ ), but there was no significant correlation with patient age, gender, or degree of differentiation ( $P > 0.05$ , Table 2).

## Discussion

The results of this study show that expression of the *PER1* gene in OSCC is significantly decreased compared with expression in adjacent noncancerous tissues at both the protein and mRNA levels. Further analysis indicates that expression of the PER1 protein in stage T<sub>3</sub> and T<sub>4</sub> patients, those with lymph node metastasis, and those with clinical phase III and IV disease, is significantly lower than that in stage T<sub>1</sub> and T<sub>2</sub> patients, those without lymph node metastasis, and those with clinical phase I and II disease. These results suggest that expression of PER1 protein in OSCC is gradually decreased with tumor development, implying that PER1 may play a tumor suppression role, and its expression may primarily correlate with invasion and metastasis of

OSCC. Yeh et al have reported the mechanism of decreased PER1 expression in breast cancer and endometrial carcinoma to be methylation of cytosine-phosphate guanosine sites in *PER1* promoter sequences, leading to downregulation and inhibition of PER1 expression.<sup>6,16</sup> However, the mechanism needs further investigation in more detail.

A basic characteristic of malignant tumors is uncontrolled proliferation.<sup>17</sup> *PER1* is one of the important circadian-clock genes controlling the normal physiological rhythm of cell proliferation and metabolism in vivo.<sup>1-3,18,19</sup> Changes in *PER1* expression alter the proliferation rhythm of normal cells, and may easily lead to tumorigenesis. Because a circadian-clock system was not recognized in vivo until recently, the exact mechanism needs to be clarified. In recent reports from Chang et al<sup>20</sup> and Klein et al,<sup>21</sup> *PER1* was demonstrated to regulate the proliferation, invasion, and migration of cancer cells by regulating expression of matrix metalloproteinase-2 and intracellular distribution of laminin receptor 1. Decreased PER1 protein expression upregulates the expression of matrix metalloproteinase-2 and increases the distribution of laminin receptor 1 onto the cell membrane, thereby enhancing tumor invasion, migration, and metastasis. However, due to the lack of sufficient studies, more detailed mechanisms remain unclear.

In summary, circadian-clock genes form a unique system in living subjects. Further research focusing on these genes may, from the perspective of biological rhythms, provide novel ideas and methods for a better understanding of the occurrence and development of tumors, and for individualized treatment of cancer. The results of this study suggest that the *PER1* gene may be used as a marker to determine clinical staging, to evaluate the metastatic risk of OSCC, and as a novel target for the prevention and treatment of OSCC.

## Disclosure

The authors report no conflicts of interest in this work.

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**Table 2** The expression of PER1 protein and its relationship with clinicopathological features of patients with OSCC

Parameters	cases	PER1 expression		$\chi^2$	P
		Negative	Positive		
<b>Tissue type</b>					
Tumor	41	7	34	7.653	0.012
Peritumor	41	0	41		
<b>Age</b>					
≥60	10	2	8	0.131	0.937
45–60	20	3	17		
<45	11	2	9		
<b>Gender</b>					
Female	17	3	14	0.007	1.000
Male	24	4	20		
<b>Tumor differentiation</b>					
Well	13	3	10	0.510	0.775
Moderate	20	3	17		
Poor	8	1	7		
<b>T staging</b>					
T <sub>1</sub> + T <sub>2</sub>	18	0	18	6.606	0.012
T <sub>3</sub> + T <sub>4</sub>	23	7	16		
<b>Lymph node metastasis</b>					
No	27	1	26	9.982	0.004
Yes	14	6	8		
<b>Clinical stage</b>					
I + II	22	1	21	5.262	0.036
III + IV	19	6	13		

**Abbreviation:** OSCC, oral squamous cell carcinoma.

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