Int J Clin Exp Pathol 2017;10(2):1008-1018
www.ijcep.com /ISSN:1936-2625/IJCEP0030362

Original Article

Daily rhythmic variations of VEGF in esophageal squamous cell carcinoma patients: a correlation study of clock gene PER1, PER2 and VEGF expression

Xia Deng[1], Guangming Li[2], Xin Hu[2]

[1]*The First People's Hospital of Yibin, Yibin 644000, Sichuan, China;* [2]*Nanchong Central Hospital, Nanchong 637000, Sichuan, China*

Received April 12, 2016; Accepted November 18, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Background: Esophageal Squamous Cell Carcinoma (ESCC) has a poor prognosis. The application of targeted drugs in ESCC is limited. Aim: To investigate the correlation between vascular endothelial growth factor (VEGF) and clock genes PER1, PER2, determine the daily rhythm of VEGF in ESCC patients. Methods: PER1, PER2 and VEGF were evaluated by Quantitative RT-PCR, western blot and Immunohistochemistry (IHC). The concentration of VEGF was evaluated by enzyme linked immunosorbent assay (ELISA). 36 blood samples and 36 urine samples were collected from patients with ESCC, and 36 blood samples were collected from normal people at four time points in a day. Results: Decreased level PER1 and PER2 and increased level of VEGF were observed in ESCC cancer tissues. The levels of PER1 and PER2 were negatively, while the level of VEGF was positively associated with factors of lymph node metastasis and clinical staging ($P < 0.001$). A significant negative correlation was found between PER1, PER2 expression and VEGF expression in cancer specimen and the blood of ESCC patients. The concentrations of VEGF differed in the blood and urine of ESCC patients and in normal person blood at different times in a day (03:00, 09:00, 15:00, 21:00). In particularly, the expression level of VEGF exhibited daily rhythmic variations, reaching the highest at 15:00 and lowest at 21:00. Conclusion: Down-regulation of PER1, PER2 and increased expression of VEGF might promote tumor progression in ESCC. The optimized time selection for VEGF administration for ESCC patients is at 15:00, when VEGF expression reaches peak.

Keywords: Esophageal squamous cell carcinoma, vascular endothelial growth factor, daily rhythm, clock gene

Introduction

Esophageal cancer (EC) is the disease that malignant cancer cells form in the esophagus. Esophageal Squamous Cell Carcinoma (ESCC) is the most frequent subtype of EC, approximately 70% of the worldwide cases of ESCC occur in China [1]. However, ESCC has a poor prognosis that the 5-year survival rate ranges from only 10% to 25% [2]. Therefore, research on the treatment of ESCC has always been a key topic.

For the treatment of ESCC, reports have indicated some valuable targets, i.e., vascular endothelial growth factor receptor (VEGR) and c-MET [3]. VEGF is a potent angiogenic factor and an important growth factor for vascular endothelial cells [4]. Previous studies demonstrated that in ESCC patients, the expression level of VEGF in tumor tissues is increased than in normal tissues, the up-regulation of VEGF was positively associated with lymphatic metastasis and clinical stages of ESCC, but was negatively associated with the prognosis of ESCC patients [5]. As VEGF is up-regulated in many types of tumors, anti-VEGF strategies aim to inhibit neovascularization by targeting the pro-angiogenic activity and the anti-apoptotic functions of VEGF.

Many aspects of human function display circadian rhythms driven by clock genes [6]. PER1 and PER2 play an important role in the development of various tumors, including gastric, pancreatic, endometrial, and breast cancers [7]. The rhythm variations of clock genes in human were different from those in animals [8]. Based

on the present outcomes of clinical research, the effectiveness of targeted therapies applied in ESCC is limited. Therefore, conducting further clinical studies to determine the effect and to explore the possible causes are necessary.

The present manuscript aims to explore the correlation between VEGF and clock genes (PER1, PER2), and determine the daily rhythm of VEGF in ESCC patients, thereby delivering theoretical basis to indicate the best time for targeted drugs administration.

Materials and methods

Clinical materials in this research was approved by local trials committee of the Nanchong ethical association and written informed consent was gained from all patients according to the guidelines of The Declaration of Helsinki 2000.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cancer specimens and tissues adjacent to cancer, non-tumorous fresh tissues were obtained from three patients with ESCC and all patients did not accept any other treatments. The normal tissue: the incisal edge more than 5 cm far away from cancer lesion edge; adjacent cancer: the tissue 2 cm away from cancer lesion edge to the outside; cancer tissue: the tissue from cancer lesion edge to the inside. Total RNA was extracted from cancer specimens and adjacentatypical hyperplasia, non-tumorous tissues. NANO DROP2000 was used to test total RNA concentration and optical density (OD). 1 μg RNA was reverse-transcripted by retrovirus kit (K1622, Thermo Fisher Scientific). The obtained cDNA was used as template, under SYBR Green Master Mix (Roche, Switzerland) and the CFX 96 Real-Time PCR System (Bio-Rad, USA). The ΔCt method was used to calculate the expression levels of the samples, and normalized against β-actin. Each PCR amplification was performed under the following conditions: 95°C for 10 min, then 95°C for 30 s, and 60°C for 30 s, 72°C for 30 s through 35 cycles, and all measurements were executed in triplicate. Amplification specificity was confirmed by melting curve analysis. Primer sequences used were as follows: PER1, F: 5'-AAGTTCGTCTTCTGCCGTATC-3', R: 5'-AGGCGGAATGGCTGGTA-3'; PER2, F: 5'-TCGTAAAACATCTTATGCCTGACT-3', R: 5'-GGCATAGGCAGCTGATGTTA-3'; VEGF, F: 5'-CAACAAATGTGAATGCAGACC-3', R: 5'-CTCCAGGGCATTAGACAGC-3'; β-actin, F: 5'-GGACTTCGAGCAAGAGATGG-3', R: 5'-AGCACTGTGTTGGCGTACAG-3'.

Western blot analysis

Whole-tissue protein extracts were prepared by dissolving cells in RIPA buffer containing protease inhibitors (cOmplete, Mini, EDTA-free; Roche, Welwyn Garden City, United Kingdom). Protein output was quantified using the BCA Protein Quantification Kit (Thermo scientific, USA). SDS-PAGE was used to separate the equal amounts of protein before wet transferring onto PVDF membrane (Millipore, USA). After membrane transferring, the PVDF membrane was immersed in TBST for 10 s and then in the Ponceau S staining solution for 5 min in the tunnel and the protein can be observed on the membrane. Non-specific binding sites were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween (2.4 g Tris (pH 7.6), 8 g NaCl, 0.1% Tween 20) for 2 h. PER1, PER2 and VEGF primary antibodies were purchased from Abcam (Cambridge, United Kingdom): anti-PER1 (catalog number ab3443, diluted 1:1000), anti-PER2 (catalog number ab179813, diluted 1:1000), and anti-VEGF (catalog number ab46154, diluted 1:1000). Anti-β-actin primary antibodies (catalog number TDY051F, diluted 1:10,000) were purchased from Beijing TDY Biotech CO., Ltd. Protein complexes were visualized with chemiluminescence (Alphalmager 2200).

Immunohistochemistry (IHC)

144 patients diagnosed with ESCC who were treated at Nanchong Central Hospital between July 2013 and August 2015. All of them did not accept other treatments before surgery. Tumor Node Metastasis staging (TNM-staging) was collected from pathological reports verified by pathologists. The tissues were surgically obtained at the following time points: 86 cases were between 11:00 and 13:00, 35 cases were between 13:00 and 15:00, and 23 cases were between 15:00 and 17:00.

Cancer specimens and tissues adjacent to cancer, non-tumorous fresh tissues were gained from patients, including 144 cases with esophageal squamous cell carcinoma, 50 cases with

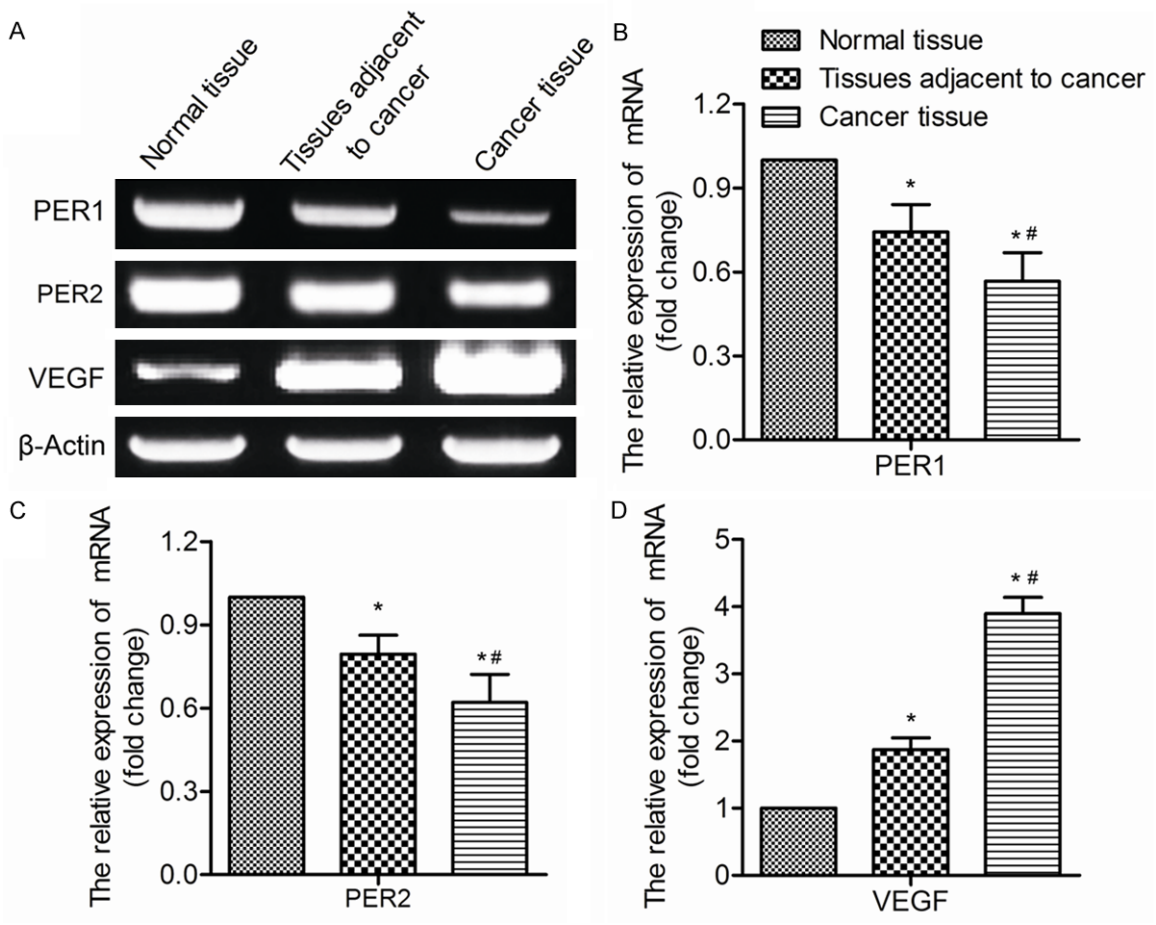

Figure 1. The mRNA level of PER1, PER2 and VEGF in cancer tissues, tissues adjacent to cancer and normal tissues. (A) The levels of PER1, PER2 and VEGF were determined by qRT-PCR; relative mRNA expressions of PER1 (B), PER2 (C) and VEGF (D) were quantified using Image-Pro Plus 6.0 software and normalized to β-actin. $^{*}P < 0.05$ versus Normal tissue, $^{\#}P < 0.05$ versus tissues adjacent to cancer.

tissues adjacent to cancer, and 144 cases with normal esophagealmucosa. We used usually IHC standard methods. Cancer specimens and adjacentatypical hyperplasia, non-tumorous fresh tissues were obtained from patients. All tissues were processed with formaldehyde for 24 hours, and pathological section was conducted by running dehydration of tissue (LEICA PELORIS II), embedding (LEICA EG1150H). The sections were subsequently immersed in citrate buffer (pH 6.0), repaired for 20 minutes using hot steam and cooled to room temperature. This conduction was followed by incubation overnight with a primary antibody anti-PER1 (catalog number ab3443, diluted 1:1000) and anti-PER2 (catalog number ab179813, diluted 1:1000) in a humidified container at 4°C and incubated with the corresponding secondary antibodies after being washed three times with PBS. Then the sections were stained with DAB and finally counterstained with hematoxylin.

The IHC consequences were evaluated by two independent viewers who were blinded to the patient information and specialized in esophageal squamous cell carcinoma. The overall PER1 and PER2 immunostaining scores were calculated using the percentage of positively stained cancer cells and the staining intensity. For each sample, one score was obtained according to the percentage of positive cells as this: ≤ 25% = 1, > 25% to ≤ 50% = 2, > 50% = 3. And another score was get according to the intensity of staining as follows: negative = 0, weak = 1, moderate = 2, and strong = 3. The total scores were get using the formula [HScore = % positive × (intensity + 1)], scores were assigned as follows: negative = 0, 1 = weak, 2 = medium, and 3 = strong.

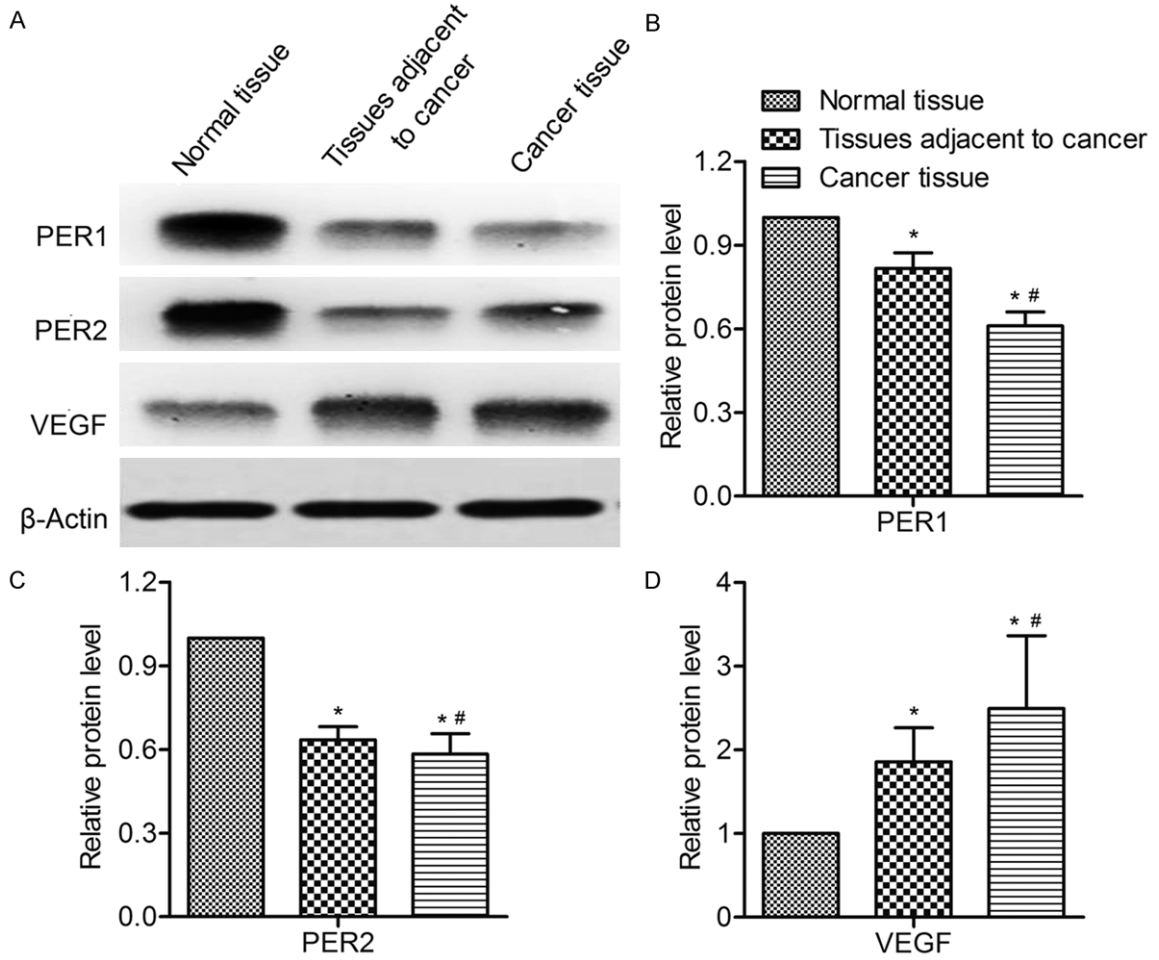

Figure 2. Protein levels of PER1, PER2 and VEGF. (A) The expressions of PER1, PER2 and VEGF were determined by western blot; relative mRNA expressions of PER1 (B), PER2 (C) and VEGF (D) were quantified using Image-Pro Plus 6.0 software and normalized to β-actin. $^{*}P < 0.05$ versus Normal tissue, $^{\#}P < 0.05$ versus tissues adjacent to cancer.

Enzyme linked immunosorbent assay (ELISA)

144 blood samples were collected respectively from patients with ESCC who also contributed tissues before surgery, and especially, 9 patients agreed with us to collect their blood and urine samples at four time points in a day (03:00, 09:00, 15:00, 21:00). At the same time, 36 blood samples from 9 normal persons were collected at four time points in a day (03:00, 09:00, 15:00, 21:00). ELISA kit (Cat# DVE00, R&D Systems, MN, USA) was used to measure the concentration of VEGF. This VEGF enzyme linked immunosorbent assay employed the quantitative sandwich enzyme immunoassay technique. The microplate provided in this kit has been pre-coated with a monoclonal antibody specific for VEGF. Standards or samples are then pipetted into the microplate wells, and VEGF present in the samples or standards binds to antibodies adsorbed to the microplate wells. In order to quantitatively determine the amount of VEGF present in the samples, the horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for VEGF were added to each well. The microplate was incubated for 1 h, and then the wells were thoroughly washed to remove any unbound components. The substrate solution A and B was respectively added to each well. After letting the enzyme (HRP) and substrate react for a short period, the reaction was stopped by adding 50 ul stop solution to each well. The optical density of each well was measured within 30 minutes, using a microplate reader set to the wavelength of 450 nm.

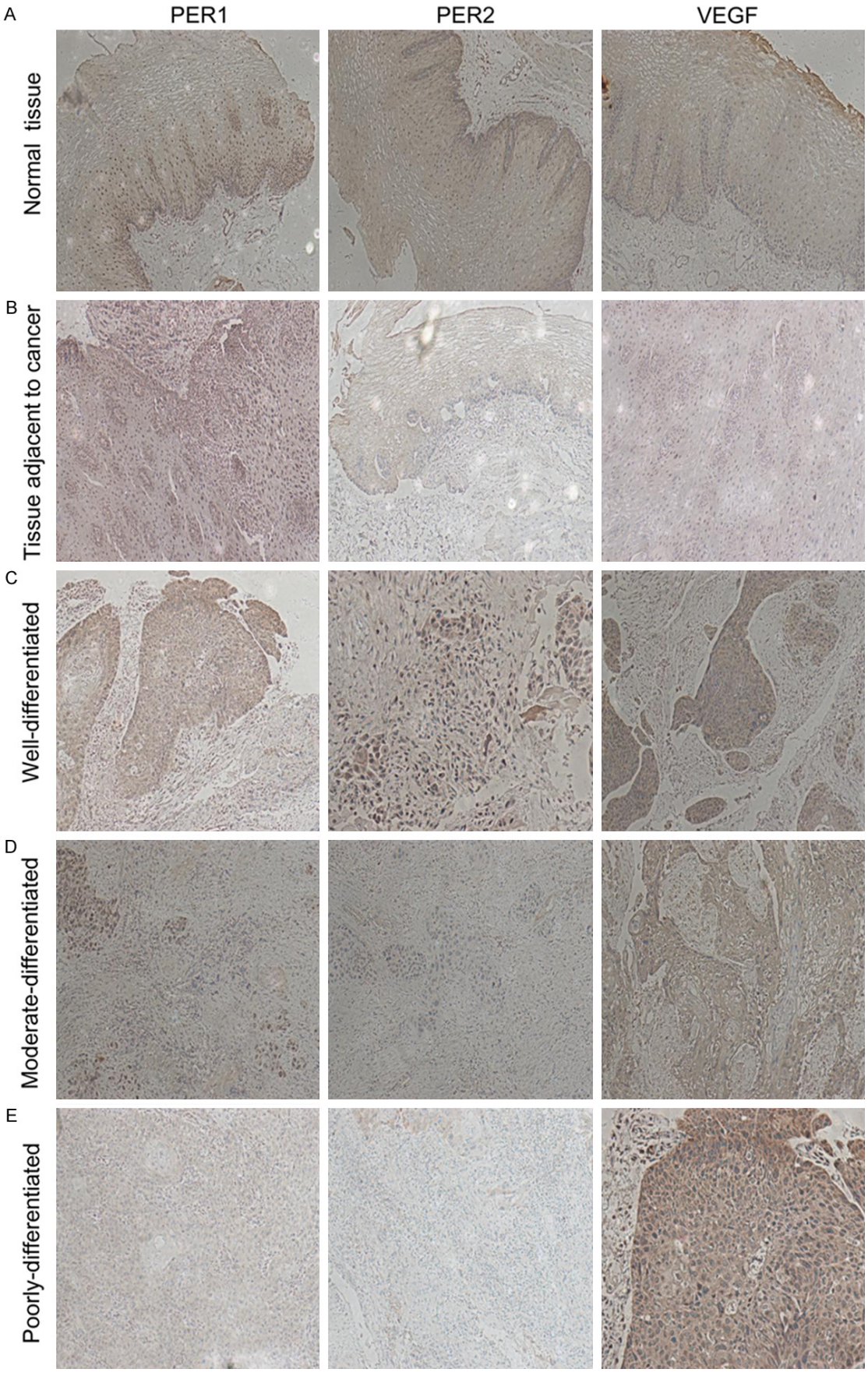
A
PER1
PER2
VEGF
Normal tissue
B
Tissue adjacent to cancer
C
Well-differentiated
D
Moderate-differentiated
E
Poorly-differentiated

Figure 3. The expressions of PER1, PER2 and VEGF in esophageal squamous cell carcinoma with different differentiation. A. Represent normal tissues, B. Represent tissues adjacent to cancer, C-E. Represent esophageal squamous cell carcinoma (C present well-differentiated, D present moderate-differentiated, E present poorly-differentiated) (100×).

Statistical analysis

SPSS 13.0 was used for all analyses (SPSS, Chicago, IL, USA). ANOVA and Kruskal-Wallis H test were used to determine the differences in the mRNA and protein expression of PER1, PER2 and VEGF in cancer tissues, tissues adjacent to cancer and normal tissue. The correlation between protein (PER1, PER2, VEGF) expressions in tissues and clinicopathologic features were measured with Pearson's correlation coefficient. The relationship of VEGF expression in blood, PER1, PER2, VEGF expressions in tissues and clinicopathologic characteristics were measured with Spearman's rank correlation coefficient. Results were presented as mean and standard deviation. $P < 0.05$ was considered to be statistically significant.

Results

The expression of clock gene PER1, PER2 and VEGF was observed in tumor tissues

As shown in **Figure 1**, PER1 and PER2 expressions were decreased in tumor tissues compared with that in non-tumorous tissues ($P < 0.05$), especially, PER1 and PER2 levels were decreased in adjacent compared with that in non-tumorous tissues but were enhanced compared with that in cancer specimens ($P < 0.05$). The same results were obtained from western blot analysis (**Figure 2**). The statistical anallysis on the expression of PER1, PER2 and VEGF were summerised in Tables S1, S2, S3.

The level of PER1, PER2 and VEGF was measured in tumors with different differentiation and with different TNM stage

The clinical characteristics of 144 ESCC patients were presented in Table S4. As shown in **Figure 3**, the results demonstrated that compared to normal tissue, PER1 and PER2 were reduced significantly during the tumor progression, especially in cancer tissues that poorly-differentiated. On the contrary, level of VEGF was strongly increased, and this effect was clearly observed even in tissues that adjacent to cancer. The same results were detected in tumors with different TNM stage (**Figure 4**). As showned in **Table 1**, significant negative correlation was found between PER1, PER2 expression and VEGF expression in cancer specimen of ESCC patients ($P < 0.001$).

The correlation between PER1, PER2 and VEGF expression in cancer tissues and clinicopathological characteristics of ESCC patients

As presented in Table S5 and **Figure 3**, a significant negative correlation was observed between PER1, PER2 expression in cancer specimen of ESCC patients and clinical stage, depth of invasion, lymph node metastasis and distant metastasis ($P < 0.001$). However, VEGF expression in cancer tissues of ESCC patients was significantly positively correlated with tumor size, clinical stage ($P < 0.001$), depth of invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$) and distant metastasis ($P < 0.05$), but was negatively correlated with differentiation level ($P < 0.001$) (Table S6; **Figures 3**, **4**).

The association between VEGF expression in ESCC patients' blood and clinic-pathologic characteristics

As presented in **Figure 5**, the concentration of VEGF in the blood of ESCC patients ranging from 481.37 pg/ml to 2,238.48 pg/ml (mean ± SD, 1170.42 ± 501.39 pg/ml). Spearman's rank correlation analysis showed that VEGF expression in blood was positively correlated with clinical stage, invasion of depth, nymph node metastasis, distant metastasis and tumor size, but was negatively correlated with tumor differentiation level. *P* values and correlation coefficients were listed in Table S7.

The correlation between VEGF expression in blood of ESCC patients and PER1, PER2, VEGF expression in cancer specimen

Ranks correlation analysis found that VEGF expression in blood was positively correlated withVEGF expression in cancer tissues ($r = 0.7$, $P < 0.001$), but was negatively correlated with PER1 expression ($r = -0.4$, $P < 0.001$) and PER2 expression ($r = -0.6$, $P < 0.001$) in tumor tissues.

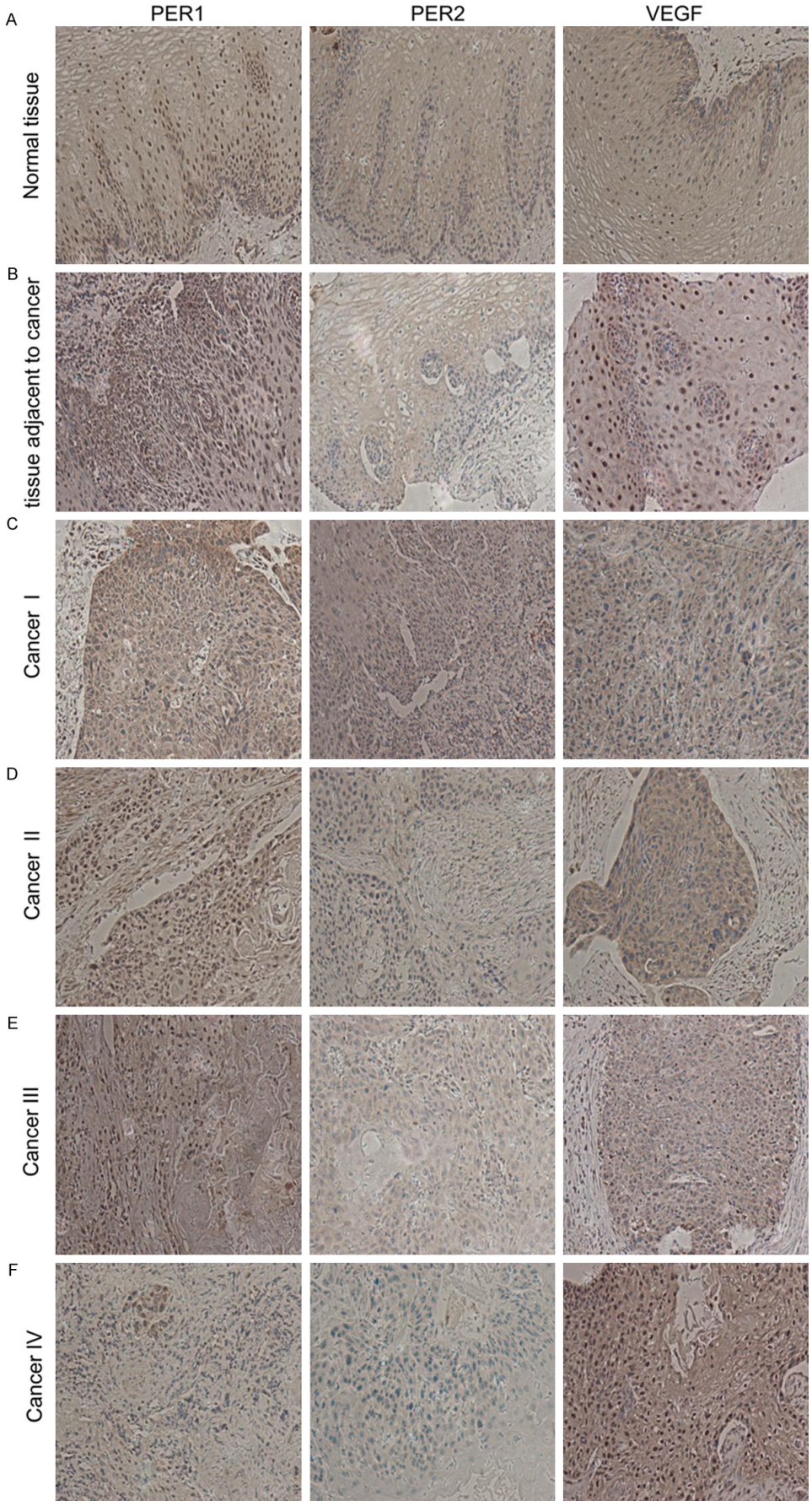
PER1
PER2
VEGF
A
Normal tissue
B
tissue adjacent to cancer
C
Cancer I
D
Cancer II
E
Cancer III
F
Cancer IV

Figure 4. The expressions of PER1, PER2 and VEGF in different tissues. A. Represent normal tissues, B. Represent tissues adjacent to cancer, C-F. Represent esophageal squamous cell carcinoma (C represent TNM stage I, D represent TNM stage II, E represent TNM stage III, F represent TNM stage IV) (200×).

Table 1. Correlation between PER1, PER2 protein expression and VEGF expression in cancer specimen of ESCC patients. 144 patients diagnosed with ESCC who were treated at Nanchong Central Hospital between July 2013 and August 2015 took part in the study, and all of them did not accept other treatments before surgery

Circadian gene	VEGF (Number of cases)			*P* value and correlation coefficient (r)
	Weak	Medium	Strong	P < 0.001
PER1 (Number of cases)				R = -0.6
Weak	0	11	34	
Medium	11	49	17	
Strong	9	13	0	
PER2 (Number of cases)				P < 0.001
Weak	0	9	41	R = -0.8
Medium	9	42	10	
Strong	20	22	0	

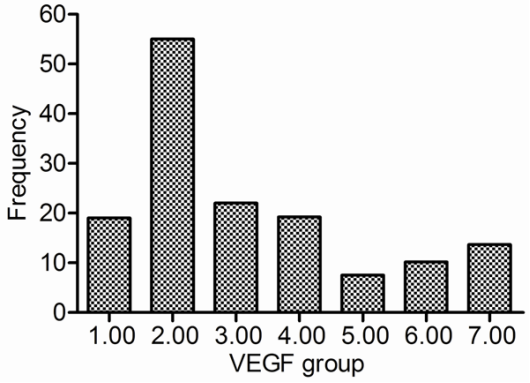

Figure 5. The concentration of VEGF in the blood of ESCC patients.

Fluctuation of VEGF expression in the blood, urine of ESCC patients and normal person at different times

VEGF concentration in the blood and urine of ESCC patients reached its maximum value at 15:00 and minimum value at 21:00 (**Figure 6A**, **6B**). VEGF protein concentration in urine of ESCC patients was higher than that in the blood of ESCC patients (**Figure 6D**). In the control group, VEGF expression at 09:00 was slightly different from that at 21:00, but was enormously different from that at 03:00 and 15:00, reaching the peak at 15:00 and hitting bottom at 09:00 and 21:00 (**Figure 6C**). The concentrations of VEGF differed in the blood and urine of ESCC patients and in normal person blood at different times in a day. (03:00, 09:00, 15:00, 21:00) (P < 0.05) (Table S8; **Figure 6D**).

Discussion

Circadian rhythms in mammals are 24-hour oscillations responsible for regulating a large variety of behavior and physiology rhythms [9]. In the circadian system, molecular oscillations of "clock genes" were generated at the cellular level [10]. In this study, we explored the expression pattern of the circadian clock gene PER1 and PER2, and elucidated the correlation of clock genes and VEGF.

Previous studies have demonstrated that PER1 is a tumor suppressor gene [11]. The down regulation of clock genes PER1 and PER2 exerted carcinogenesis in the development and progression of ESCC. In our research, the expressions of clock gene PER1 and PER2 were decreased significantly in ESCC tumors with different differentiation and different TNM stage. Besides, the expression levels of PER1 and PER2 were negatively associated with factors of lymph node metastasis, distant metastasis and clinical staging, therefore it can be concluded that PER1 and PER2 exhibited suppressive effect in the progression and migration of ESCC.

Angiogenesis is an essential physiological process to allow small, established tumors grow beyond a critical size of a few millimeters [12]. Expression level of VEGF is a predictor of differentiation, TNM stage, distant metastasis, OS, and DMFS in resectable ESCC patients [13]. In this study, the level of VEGF was increased significantly in ESCC and the elevated level of VEGF was detected in tumor tissues with different differentiation and TNM stage,

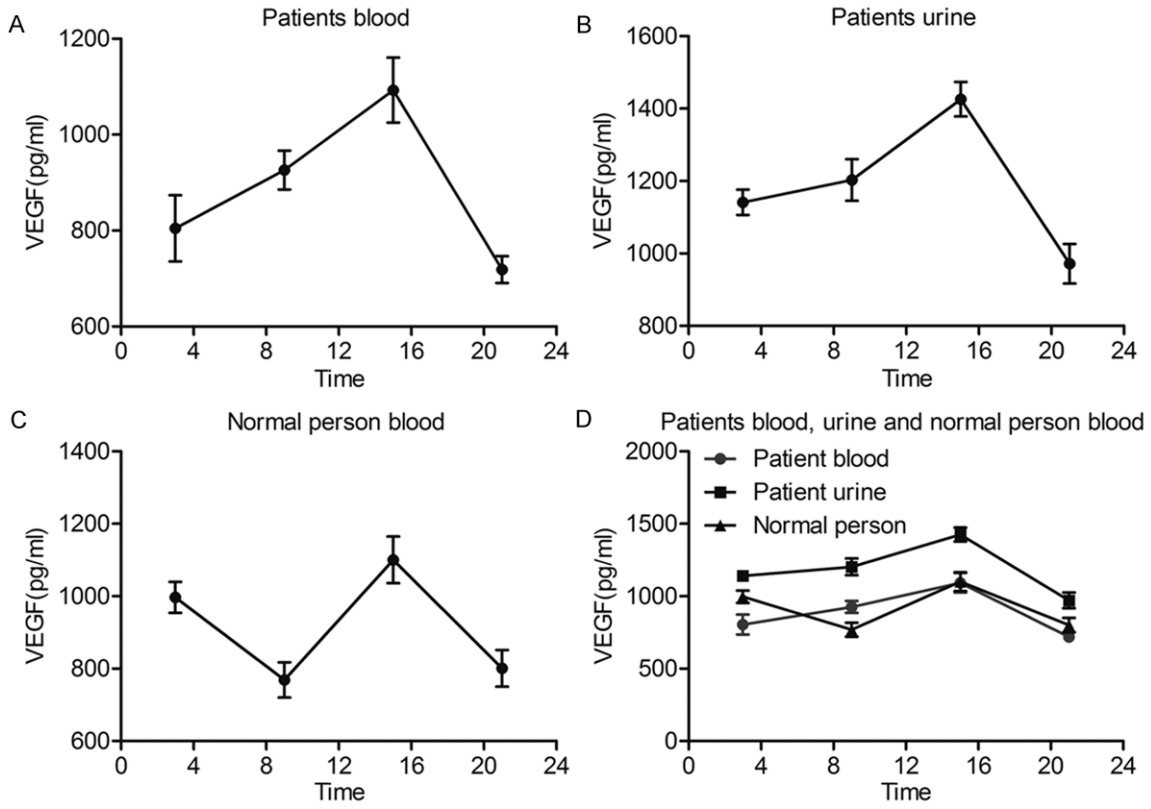

Figure 6. The expression levels of VEGF in ESCC patients and normal person. The expression levels of VEGF in the blood (A) and urine (B) of ESCC patients and in the blood of normal person (C) at different times in a day (03:00, 09:00, 15:00, 21:00). (D) The expression levels of VEGF in patients and normal person at different times in a day were summarized. $^{*}P < 0.05$ versus Normal person.

suggesting that VEGF plays a remarkable role in tumor-inducing progress. Notably, a significant negative correlation was found between PER1, PER2 expression and VEGF expression in cancer specimen of ESCC patients, hinting the decreased level of PER1, PER2 may be related to elevated level of VEGF.

Circadian rhythms are discovered in almost all organisms and affect our physiological activities [14]. Variations of clock genes have been proved to associate with diseases and metabolic disorder [15]. Currently, anti-cancer drugs are administered with optimal timing according to circadian rhythms of anti-cancer action, as well as adverse effects on normal cells [16]. Numerous studies have proved that gene expression of many transporters and protein production varied within a 24-hour circadian rhythm [17]. Iwata K. et al. showed that myelo suppression was 10% mitigated in treatment group of 09:00 than in treatment group of 15:00 [18]. Satoru Koyanagi et al. found that VEGF expression in tumor cells implanted in mice was up-regulated enormously in response to hypoxia, and the expression levels fluctuated rhythmically in a circadian manner [19]. Consistently, this study found that in ESCC patients and in normal person, VEGF expression exhibited daily rhythmic variations, reaching the highest at 15:00 and lowest at 21:00. These results suggested that exploring the circadian rhythm of VEGF in ESCC patients may be beneficial for administration of chemotherapeutic drugs.

In conclusion, down-regulation of PER1, PER2 and the increased expression of VEGF might promote tumor progression in ESCC. The treatment time was at 15:00, when VEGF expression reached maximum and the expression of clock genes PER1, PER2 were the lowest. However, the human body is a unified and complex system, in which the effect of chrono-modulated therapy on normal tissue must be taken into consideration. The treatment time selection to achieve the most favorable clinical outcome remains the topic of our future studies.

Acknowledgements

This work was supported by Research and Development funds of Nanchong NO. 11A0145. The authors thank Nanchong Central Hospital for for providing helpful discussions concerning the present study.

Disclosure of conflict of interest

None.

Address correspondence to: Xia Deng, Department of Oncology, The First People's Hospital of Yibin, 65 Wenxing Street, Yibin 644000, China. E-mail: friendly944@163.com

References

[1] Wei WQ, Chen ZF, He YT, Feng H, Hou J, Lin DM, Li XQ, Guo CL, Li SS, Wang GQ, Dong ZW, Abnet CC and Qiao YL. Long-term follow-up of a community assignment, one-time endoscopic screening study of esophageal cancer in China. J Clin Oncol 2015; 33: 1951-1957.

[2] Zhang L, Zhou Y, Cheng C, Cui H, Cheng L, Kong P, Wang J, Li Y, Chen W, Song B, Wang F, Jia Z, Li L, Li Y, Yang B, Liu J, Shi R, Bi Y, Zhang Y, Wang J, Zhao Z, Hu X, Yang J, Li H, Gao Z, Chen G, Huang X, Yang X, Wan S, Chen C, Li B, Tan Y, Chen L, He M, Xie S, Li X, Zhuang X, Wang M, Xia Z, Luo L, Ma J, Dong B, Zhao J, Song Y, Ou Y, Li E, Xu L, Wang J, Xi Y, Li G, Xu E, Liang J, Yang X, Guo J, Chen X, Zhang Y, Li Q, Liu L, Li Y, Zhang X, Yang H, Lin D, Cheng X, Guo Y, Wang J, Zhan Q and Cui Y. Genomic analyses reveal mutational signatures and frequently altered genes in esophageal squamous cell carcinoma. Am J Hum Genet 2015; 96: 597-611.

[3] Zhang L, Ma J, Han Y, Liu J, Zhou W, Hong L and Fan D. Targeted therapy in esophageal cancer. Expert Rev Gastroenterol Hepatol 2016; 2: 1-10.

[4] Eng L, Azad AK, Qiu X, Kong QQ, Cheng D, Ying N, Tse A, Kuang Q, Dodbiba L, Renouf DJ, Marsh S, Savas S, Mackay HJ, Knox JJ, Darling GE, Wong RK, Xu W, Liu G and Faluyi OO. Discovery and validation of vascular endothelial growth factor (VEGF) pathway polymorphisms in esophageal adenocarcinoma outcome. Carcinogenesis 2015; 36: 956-962.

[5] Mobius C, Freire J, Becker I, Feith M, Brucher BL, Hennig M, Siewert JR and Stein HJ. VEGF-C expression in squamous cell carcinoma and adenocarcinoma of the esophagus. World J Surg 2007; 31: 1768-1772; discussion 1773-1764.

[6] Innominato PF, Lim AS, Palesh O, Clemons M, Trudeau M, Eisen A, Wang C, Kiss A, Pritchard KI and Bjarnason GA. The effect of melatonin on sleep and quality of life in patients with advanced breast cancer. Support Care Cancer 2015; 24: 1097-1105.

[7] Sancar A, Lindsey-Boltz LA, Gaddameedhi S, Selby CP, Ye R, Chiou YY, Kemp MG, Hu J, Lee JH and Ozturk N. Circadian clock, cancer, and chemotherapy. Biochemistry 2015; 54: 110-123.

[8] Mazzoccoli G, Rubino R, Tiberio C, Giuliani F, Vinciguerra M, Oben J, De Cata A, Tarquini R, De Cosmo S, Liu S and Cai Y. Clock gene expression in human and mouse hepatic models shows similar periodicity but different dynamics of variation. Chronobiol Int 2016; 33: 181-190.

[9] Katamune C, Koyanagi S, Shiromizu S, Matsunaga N, Shimba S, Shibata S and Ohdo S. Different roles of negative and positive components of the circadian clock in oncogene-induced neoplastic transformation. J Biol Chem 2016; 5: 112-113.

[10] Boucher H, Vanneaux V, Domet T, Parouchev A and Larghero J. Circadian clock genes modulate human bone marrow mesenchymal stem cell differentiation, migration and cell cycle. PLoS One 2016; 11: 110-118.

[11] Kudo T, Block GD and Colwell CS. The circadian clock gene period1 connects the molecular clock to neural activity in the suprachiasmatic nucleus. ASN Neuro 2015; 7.

[12] Zhu H, Yang X, Ding Y, Liu J, Lu J, Zhan L, Qin Q, Zhang H, Chen X, Yang Y, Yang Y, Liu Z, Yang M, Zhou X, Cheng H and Sun X. Recombinant human endostatin enhances the radioresponse in esophageal squamous cell carcinoma by normalizing tumor vasculature and reducing hypoxia. Sci Rep 2015; 5: 14503.

[13] Hou X, Wei JC, Fu JH, Wang X, Luo RZ, He JH, Zhang LJ, Lin P and Yang HX. Vascular endothelial growth factor is a useful predictor of postoperative distant metastasis and survival prognosis in esophageal squamous cell carcinoma. Ann Surg Oncol 2015; 22: 3666-3673.

[14] Mitchell MI and Engelbrecht AM. Circadian rhythms and breast cancer: the role of per2 in doxorubicin-induced cell death. J Toxicol 2015; 2015: 392360.

[15] Csernus VJ, Nagy AD and Faluhelyi N. Development of the rhythmic melatonin secretion in the embryonic chicken pineal gland. Gen Comp Endocrinol 2007; 152: 148-153.

[16] Wei B, Han Q, Xu L, Zhang X, Zhu J, Wan L, Jin Y, Qian Z, Wu J, Gao Y, Zhou J and Chen X. Effects of JWA, XRCC1 and BRCA1 mRNA expression on molecular staging for personalized therapy in patients with advanced esophageal

squamous cell carcinoma. BMC Cancer 2015; 15: 331.

[17] Zarogoulidis P, Darwiche K, Huang H, Spyratos D, Yarmus L, Li Q, Kakolyris S, Syrigos K and Zarogoulidis K. Time recall; future concept of chronomodulating chemotherapy for cancer. Curr Pharm Biotechnol 2013; 14: 632-642.

[18] Iwata K, Aizawa K, Sakai S, Jingami S, Fukunaga E, Yoshida M, Hamada A, Saito H. The relationship between treatment time of gemcitabine and development of hematologic toxicity in cancer patients. Biol Pharm Bull 2011; 34: 1765-1768.

[19] Koyanagi S, Kuramoto Y, Nakagawa H, Aramaki H, Ohdo S, Soeda S and Shimeno H. A molecular mechanism regulating circadian expression of vascular endothelial growth factor in tumor cells. Cancer Res 2003; 63: 7277-7283.

Table S1. Expression of PER1 in cancer tissues, tissues adjacent to cancer and normal tissues

PER1 expression	Number of cases			Statistic values of H and *P*-value
	Normal tissues	Tissues adjacent to cancer	Cancer tissues	
Weak	4	2	45	H = 10.281
Medium	17	18	77	P < 0.001
Strong	29	23	22	

Table S2. Expression of PER2 in cancer tissues, tissues adjacent to cancer and normal tissues

PER2 expression	Number of cases			Statistic values of H and *P*-value
	Normal tissues	Tissues adjacent to cancer	Cancer tissues	
Weak	3	1	50	H = 13.2
Medium	23	17	52	P < 0.001
Strong	24	25	42	

Table S3. Expression of VEGF in cancer tissues, tissues adjacent to cancer and normal tissues

VEGF expression	Number of cases			Statistic values of H and *P*-value
	Normal tissues	Tissues adjacent to cancer	Cancer tissues	
Weak	23	25	20	H = 11.5
Medium	16	11	73	P < 0.001
Strong	11	7	51	

Table S4. Clinical characteristics of the 144 patients. Collection of patient information Experiment samples included 144 cancer tissues, 50 non-tumorous tissues and 43 tissues adjacent to cancer. The average (mean ± SD) age of patients in the study was 61.9 ± 5.8 years (age range, 45 to 77 years)

	Number of cases (%)
Gender	
Male	101 (70.0)
Female	43 (30.0)
Age (years)	
≤ 60	75 (52.1)
> 60	69 (47.0)
standard of culture	
Primary school or below	111 (77.1)
Junior high school	29 (20.1)
Senior high school or above	4 (2.8)
Location	
Cervical	5 (3.5)
Upper	36 (25.0)
Middle	81 (56.3)
Lower	22 (15.3)
Size (cm)	
≤ 5	118 (81.9)
> 5	26 (18.1)
Clinical stage	
I	12(8.3)
II	68 (47.2)
III	60 (41.7)
IV	4 (2.8)
Depth of invasion	
T1	12 (8.3)
T2	43 (30.0)
T3	64 (44.4)
T4	25 (17.4)
Lymph node metastasis	
N0	89 (30.8)
N1	55 (69.2)
Distant metastasis	
M0	141 (97.9)
M1	3 (2.1)
Histological types	
Well/Moderate differentiation	102 (70.8)
Poor differentiation	42 (29.2)
PER1 expression	
Weak	45 (31.3)
Medium	77 (53.5)
Strong	22 (15.3)
PER2 expression	
Weak	50 (34.7)
Medium	52 (36.1)
Strong	42 (29.2)
VEGF expression in tissues	
Weak	20 (13.9)
Medium	73 (50.7)
Strong	51 (35.4)
VEGF expression in blood (pg/ml)	
≥ 400, < 700	19 (13.2)
≥ 700, < 1000	55 (38.2)
≥ 1000, < 1300	21 (14.6)
≥ 1300, < 1600	19 (13.2)
≥ 1600, < 1900	8 (5.6)
≥ 1900, < 2200	10 (6.9)
≥ 2200, < 2500	12 (8.3)

Table S5. The correlation between PER1, PER2 expression in cancer tissues and clinicopathological characteristics of ESCC patients. 144 patients diagnosed with ESCC who were treated at Nanchong Central Hospital between July 2013 and August 2015 took part in the study, and all of them did not accept other treatments before surgery

Clinicopathological characteristics	PER1			*P* value & correlation coefficient	PER2			*P* value & correlation coefficient
	Weak	Medium	Strong		Weak	Medium	Strong	
Gender				P = 0.8				P = 0.9
Male	34	46	21	r = -0.03	35	35	31	r = 0.01
Female	11	31	1		15	17	11	
Age (years)				P = 0.7				P = 0.2
≤ 60	45	30	0	r = -0.03	39	25	11	r = -0.01
> 60	0	47	22		11	27	31	
Standard of culture				P = 0.8				P = 0.2
Primary school or below	32	69	10	r = -0.02	46	23	42	r = -0.1
Junior high school	9	8	12		0	29	0	
Senior high school or above	4	0	0		4	0	0	
Location				P < 0.001				P < 0.001
Cervical	0	5	0	R = -0.4	0	5	0	r = -0.4
Upper	0	27	9		8	9	19	
Middle	32	36	13		24	34	23	
Lower	13	9	0		18	4	0	
Size (cm)				P = 0.8				P = 0.4
≤ 5	28	68	22	R = -0.1	25	51	42	r = -0.4
> 5	17	0	3		16	2	0	
Clinical stage[1]				P < 0.001				P < 0.05
I	0	0	12	r = -0.5	0	12	0	R = -0.2
II	10	57	1		18	18	22	
III	31	20	9		29	11	20	
IV	4	0	0		3	1	0	
Depth of invasion				P < 0.001				P < 0.001
T1	0	0	12	r = -0.5	0	12	0	r = -0.3
T2	1	41	1		9	22	12	
T3	28	27	9		17	17	30	
T4	16	9	0		24	1	0	
Lymph node metastasis				P < 0.001				P < 0.001
N0	10	57	22	r = -0.6	18	40	31	r = -0.3
N1	35	20	0		32	12	11	
Distant metastasis				P < 0.05				P < 0.05
M0	42	77	22	r = -0.2	47	52	42	r = -0.2
M1	3	0	0		3	0	0	
Histological types[2]				P < 0.001				P < 0.001
Well/Moderate differentiation	3	77	22	r = 0.8	17	43	42	r = 0.6
Poor differentiation	42	0	0		33	9	0	

[1]Tumor Node Metastasis staging (TNM-staging) was collected from pathological reports verified by pathologists. The tissues were surgically obtained at the following time points: 86 cases were between 11:00 and 13:00, 35 cases were between 13:00 and 15:00, and 23 cases were between 15:00 and 17:00. [2]For each sample, one score was obtained according to the percentage of positive cells as this: ≤ 25% = 1, > 25% to ≤ 50% = 2, > 50% = 3. And another score was get according to the intensity of staining as follows: negative = 0, weak = 1, moderate = 2, and strong = 3. The total scores were get using the formula [HScore = % positive × intensity, scores were assigned as follows: 0, 1 = week; 2, 3, 4 = medium; 6, 9 = strong].

Table S6. VEGF expression in cancer tissues of ESCC patients and clinic-pathologic features in IHC

Clinicopathological characteristics	VEGF			*P* value & correlation coefficient (r)
	Weak	Medium	Strong	
Gender				P = 0.3
Male	16	47	38	r = 0.2
Female	4	26	13	
Age (years)				P = 0.08
≤ 60	2	33	40	r = -0.1
> 60	18	40	11	
Standard of culture				P = 0.2
Primary school or below	20	53	38	r = -0.2
Junior high school	0	20	9	
Senior high school or above	0	0	4	
Location				P < 0.001
Cervical	0	5	0	r = 0.4
Upper	9	19	8	
Middle	11	49	21	
Lower	0	0	22	
Size (cm)				P < 0.001
≤ 5	20	72	26	r = 0.4
> 5	0	1	16	
Clinical stage				P < 0.001
I	0	12	0	r = 0.4
II	11	48	9	
III	9	13	38	
IV	0	0	4	
Depth of invasion				P < 0.001
T1	0	12	0	r = 0.4
T2	12	23	9	
T3	9	38	17	
T4	0	0	25	
Lymph node metastasis				P < 0.001
N0	20	60	9	r = 0.6
N1	0	13	42	
Distant metastasis				P < 0.05
M0	20	73	48	r = 0.2
M1	0	0	3	
Histological types				P < 0.001
Well/Moderate differentiation	20	64	18	
Poor differentiation	0	9	33	r = -0.5

Table S7. The correlation of VEGF expression in blood and clinico-pathological characteristics

	Correlation coefficient (r)	*P*-value
bVEGF and differentiation	-0.4	< 0.001
bVEGF and clinical stage	0.3	< 0.001
bVEGF and depth of invasion	0.3	< 0.001
bVEGF and lymph node metastasis	0.6	< 0.001
bVEGF and distant metastasis	0.2	0.01
bVEGF and tumor size	0.5	< 0.001

Table S8. VEGF concentration in the blood and urine of ESCC patients and in normal person at different times in a day. To analyze the aily rhythmic variations of VEGF, the clinical data of 9 ESCC patients (6 males, 3 females, 45-70 years old) and 9 normal person (5 males, 4 females, 28-70 years old) were collected

Sample	VEGF (pg/ml)			
Patient's blood	0:3	0:9	15	21
1	590.624	1014.299	1033.497	791.129
2	824.808	740.451	832.677	596.604
3	1050.236	991.135	1279.997	679.374
4	594.415	899.527	1341.312	766.543
5	550.784	976.876	1003.786	750.691
6	856.765	776.987	854.897	640.786
7	1087.567	1000.412	1301.459	689.876
8	698.624	1105.299	1256.447	871.378
9	989.808	830.551	932.777	681.908
Mean	804.8479	926.1708	1092.983	718.6988
Patients urine				
1	1057.361	966.137	1198.119	899.722
2	1112.503	1087.636	1288.141	1200.952
3	1073.877	1330.23	1606.531	1092.346
4	1028.812	1224.578	1438.569	721.483
5	1127.894	1357.565	1540.041	864.805
6	1093.887	1235.23	1506.541	1086.684
7	1298.849	1129.578	1398.789	821.353
8	1147.257	1006.117	1288.239	917.642
9	1329.518	1487.961	1568.24	1139.876
Mean	1141.106	1202.781	1425.912	971.6514
Normal person's blood				
1	1119.525	819.725	1204.414	963.934
2	1126.98	861.636	1226.762	876.43
3	887.542	588.505	898.05	620.272
4	901.563	725.893	1198.432	899.698
5	1067.98	789.998	1198.891	776.893
6	916.672	679.67	772.146	737.561
7	1089.237	724.45	1190.745	1006.983
8	1086.123	1083.636	1326.962	776.53
9	777.342	348.87	889.04	549.67
Mean	996.996	735.8203	1100.605	800.8857