

# Aberrant TIMELESS expression is associated with poor clinical survival and lymph node metastasis in early-stage cervical carcinoma

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Received September 5, 2016; Accepted November 22, 2016

DOI: 10.3892/ijo.2016.3784

**Abstract.** TIMELESS is a highly conserved protein required for the maintenance of normal mammalian circadian oscillations and for controlling cellular metabolism and proliferation. Recently, TIMELESS was implicated in the tumorigenesis of certain cancers. However, little is known on TIMELESS protein expression and its potential as a prognostic factor in cervical cancer. Here, we investigate TIMELESS expression pattern and its clinicopathological significance in early-stage cervical carcinoma. TIMELESS mRNA and protein expression was evaluated by real-time PCR and western blot analysis in cervical cancer cell lines, a normal cervical cell line, as well as in six pairs of surgically removed cervical cancer and adjacent normal cervical tissues. A total of 189 paraffin-embedded cervical carcinoma specimens were detected and diagnosed by immunohistochemistry (IHC), and the clinical significance of TIMELESS expression was further analyzed. Aberrant TIMELESS mRNA and protein expression were demonstrated in cervical cancer cell lines compared with the normal cervical cell line. TIMELESS mRNA and protein expression were significantly increased in cervical cancer specimens compared with adjacent non-cancerous cervical

specimens. TIMELESS protein expression was significantly associated with the age ( $P=0.011$ ), clinical stage ( $P<0.001$ ), pelvic lymph node metastasis ( $P<0.001$ ), squamous cell carcinoma antigen ( $P=0.003$ ), tumor recurrence ( $P=0.015$ ), vital status ( $P<0.001$ ), tumor differentiation grade ( $P<0.001$ ), property of the surgical margin ( $P=0.036$ ) and lymphovascular space involvement ( $P=0.001$ ). Patients with increased TIMELESS protein expression showed strong tendencies to receive postoperative radiotherapy ( $P=0.002$ ). Upregulation of TIMELESS correlated with poorer overall survival (OS) and disease-free survival (DFS). Univariate and multivariate Cox-regression analyses showed that TIMELESS can be regarded as an independent predictive biomarker for poor clinical outcome for early-stage cervical carcinoma. Our results show that TIMELESS overexpression correlates with pelvic lymph node metastasis, lymphovascular space involvement, as well as unfavorable OS and DFS in human cervical cancer. Therefore, TIMELESS expression may be a potential prognostic biomarker for cervical cancer patients.

## Introduction

Cancer of the uterine cervix is one of the most prevalent malignant neoplasms diagnosed, and remains the fourth main cause of cancer-related mortality among women worldwide (1). Globally, ~529,800 new cases of cervical carcinoma are diagnosed each year, resulting in ~275,100 deaths annually, especially in developing countries (2). Infection with high-risk human papillomavirus (HPV) types is the common cause of cervical cancer, which leads to activation of oncogenes and deactivation of tumor suppressor genes (3). Smoking, multiple sexual partners, and poor sanitation are additional risk factors closely associated with cervical cancer development (4). Although advances in vaccinations, Pap smear screening programs, and therapeutic methods (including surgery techniques, chemotherapy and radiotherapy) have decreased the morbidity and mortality of cervical carcinoma (5,6), these prevention, diagnostic tools and treatments have their

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**Key words:** TIMELESS, pelvic lymph node metastasis, lymphovascular space involvement, cervical cancer, prognosis, biomarker

limitations. Moreover, patients with lymph node metastasis, lymphovascular space involvement, and higher differentiation grade or FIGO stage show poorer clinical outcomes (7-9). While these prognostic indicators have been correlated with biomarkers (such as C14ORF166, B3GNT3, URG4 and C1SD2) (10-14), we still require novel prevention and treatment strategies to improve the outcomes of patients with cervical cancer.

One potential therapeutic or prognostic target is TIMELESS, a 138,658-Da protein that was first characterized in *Drosophila melanogaster* as a core component of the circadian clock (15). Since then, TIMELESS has been shown to be a highly conserved protein that is also involved in the cell cycle checkpoint system, cell survival after damage or DNA replication stress, increasing DNA polymerase epsilon activity, maintaining telomere length, epithelial cell morphogenesis and in the formation of branching tubules (16,17). Recently, circadian rhythm disruption was hypothesized to explain an increased susceptibility to certain malignancies (18). Indeed, previous studies report a relationship between TIMELESS and human cancers of many different organs, including lung, breast, liver, prostate, colon, kidney, bladder, pancreas and blood (19-31). However, the characteristics of TIMELESS expression in human cervical cancer have not been investigated and its clinical significance remains largely unknown.

In the present study, we sought to investigate the expression of TIMELESS in cervical cancer and analyze its clinicopathologic and prognostic value in 189 archival early-stage cervical carcinoma specimens.

## Materials and methods

**Cell lines.** Eight cervical cancer cell lines (HeLa229, MS751, ME180, SiHa, HeLa, CasKi, C33A and HCC94) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in the Department of Experimental Research, Sun Yat-sen University Cancer Center. They were grown in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

**Samples and patient cohorts.** Written informed consent was obtained from each of the cervical cancer patients and the study was approved by the research ethics committee of the Cancer Center of Sun Yat-sen University for the use of these clinical materials for study purposes. The Institutional Review Board (IRB) at the Sun Yat-sen University approved all experimental methods. Included in the present study were tumor specimens from 189 consecutive patients undergoing surgical treatment for cervical cancer at the Department of Sun Yat-sen University Cancer Center from 2006 to 2010. The tumor staging and clinicopathological classification of the tumors were accessed in accordance with the International Federation of Gynecologists and Obstetricians (FIGO, 2009) criteria. The classification of the histological types was based on the World Health Organization (WHO) recommendations.

The 189 patients were in Ib1-IIa2 stage cervical cancer and all received a radical hysterectomy and lymphadenectomy. Patients had not undergone anticancer treatment,

such as immunotherapy, chemotherapy, hormone therapy or radiotherapy, before specimen collection. Patients who received postoperative chemotherapy and/or radiotherapy were those with high-risk factors, including: pelvic lymph node metastasis (PLNM), high differentiation grade, positive parametrial involvement, positive lymphovascular space involvement, positive surgical margin, deep stromal invasion and large tumor size (>4 cm). Patients with only deep stromal invasion or positive surgical margins received postoperative radiotherapy, while patients with only lymphovascular space involvement, high differentiation grade, or large tumor size (>4 cm) received postoperative chemotherapy. No patients received immunotherapy after surgery.

In total, 189 early-stage cervical cancer patients were included (aged 23-68 years, with a median age of 46 years). The media age is used to determine the age gap of patients. Thus, we separated the groups by a 46-year age gap in the present study. Demographic and clinicopathological information were collected from inpatient medical records (Table I). In addition, six pairs of cervical cancer tissues and the matched tumor-adjacent morphologically normal tissues, which were collected from early-stage cervical cancer patients who underwent surgery in 2015, were frozen in liquid nitrogen at -80°C until further use.

**Quantitative and real-time RT-PCR analysis.** To explore the role of TIMELESS in the development of cervical cancer, we evaluated its mRNA expression pattern in cervical cancer cell lines and tissues. RNA extraction from the cultured cell lines and surgically dissected tissue samples was carried out using the TRIzol reagent (Invitrogen) following the manufacturer's guidelines. Agilent Bioanalyzer 2100 was used to evaluate the RNA quality (RIN, 2.4-8.8; median 5.9). For PCR-mediated synthesis and amplification of TIMELESS cDNA, an initial amplification reaction using TIMELESS-specific primers was performed with a denaturation step at 95°C for 10 min, followed by 30 denaturation cycles at 95°C for 60 sec, primer annealing at 55°C for 30 sec, and primer extension at 72°C for 30 sec. A final extension at 72°C for 5 min was carried out before the reaction was stopped and stored at 4°C on completion of the cycling steps. TIMELESS-specific primers were designed using the Primer Express v.2.0 software (Applied Biosystems) as follows: forward 5'-CCATACATCAGTGGACCAACC-3' and reverse 5'-CTCCTCCGGGCTTCTGA-3'. To control the variability in expression levels, expression data of TIMELESS were normalized to the expression of the housekeeping gene, GAPDH. Primers for GAPDH were: forward 5'-TTGAGGTCAATGAAGGGGTC-3' and reverse 5'-GAAGGTGAAGGTCCGAGTCA-3'. Quantitative RT-PCR (qRT-PCR) was performed in a total volume of 10 µl using the LightCycler 480 instrument (Roche Diagnostics, Penzberg, Germany) with the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 15 sec, and a final extension step of 72°C for 5 min. The relative quantitative value was expressed by the  $2^{-\Delta\Delta C_t}$  method, where  $C_t$  represents the threshold cycle for each transcript. All experiments were repeated three times.

**Western blot analysis.** We also evaluated TIMELESS protein expression in cervical cancer cell lines and tissues. The

Table I. Clinicopathological characteristics and tumor expression of TIMELESS in patients with early-stage cervical cancer.

Characteristics	No. of cases, (%)
Age (years)	
≤46	98 (51.9)
>46	91 (48.1)
FIGO stage	
Ib1	83 (43.9)
Ib2	28 (14.8)
IIa1	55 (29.1)
IIa2	23 (12.2)
Histological type	
Squamous carcinoma	179 (94.7)
Adenocarcinoma	10 (5.3)
Tumor size, cm	
<4	142 (75.1)
≥4	47 (24.9)
Squamous cell carcinoma antigen, ng/ml	
≤1.5	91 (48.1)
>1.5	98 (51.9)
HPV infection	
No	37 (19.6)
Yes	152 (80.4)
Pelvic lymph node metastasis	
No	134 (70.9)
Yes	55 (29.1)
Tumor recurrence	
No	160 (84.7)
Yes	29 (15.3)
Vital status (at last follow-up)	
Alive	152 (80.4)
Dead	37 (19.6)
Differentiation grade	
G1	62 (32.8)
G2	111 (58.7)
G3	16 (8.5)
Myometrium invasion	
<1/2	69 (36.5)
≥1/2	120 (63.5)
Property of surgical margin	
No	177 (93.7)
Yes	12 (6.3)
Infiltration of parauterine organ	
No	179 (94.7)
Yes	10 (5.3)
Lymphovascular space involvement	
No	163 (86.2)
Yes	26 (13.8)
Chemotherapy	
No	84 (44.4)
Yes	105 (55.6)

Table I. Continued.

Characteristics	No. of cases, (%)
Radiation therapy	
No	168 (88.9)
Yes	21 (11.1)
Concurrent chemotherapy and radiotherapy	
No	173 (91.5)
Yes	16 (8.5)
TP chemotherapy	
No	27 (14.3)
Yes	93 (49.2)
Radical hysterectomy	
No	9 (4.8)
Yes	180 (95.2)
Expression of TIMELESS protein	
Low or none	65 (34.4)
High	124 (65.6)

cervical tissues and cell lines were prepared and lysed using the cell total protein extraction kits, on ice, according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Western blots were performed according to standard methods in our previous publication (32). Briefly, we separated equal amounts of protein samples (30 µg) on 9% SDS polyacrylamide gels and transferred them to PVDF membranes (Immobilon P; Millipore, Bedford, MA, USA). Anti-TIMELESS rabbit monoclonal (1:1,000; Abcam, Cambridge, MA, USA) and anti-rabbit (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used to detect TIMELESS protein. After detection, the blotted membranes were stripped, and anti-GADPH was detected using a mouse monoclonal antibody (1:2,000; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody (anti-mouse antibody; Santa Cruz Biotechnology) was diluted 1:2,000 in both samples. TIMELESS signals were examined on X-ray film using an ECL prime Western blotting detection reagent (Amersham Biosciences).

*Immunohistochemical staining and assessment.* Paraffin-embedded specimens were obtained from 189 early-stage cervical cancer patients for immunohistochemical analysis. The procedures of immunostaining were carried out with standard protocols. In detail, paraffin tissue slides (4 µm) were dried at 60°C for 1 h, deparaffinized for 10 min in xylene (twice), and rehydrated through a series of incubations in graded ethanol solutions (100, 100, 95, 90 and 80%) for 5 min, respectively. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min at room temperature to quench endogenous peroxidase activity. Following this, all deparaffinized sections were immersed and boiled in buffered ethylenediaminetetraacetic acid (pH 8.0) for 2 min in a pressure cooker to retrieve antigen, and then cooled to room temperature. Then, sections were immersed with 1% bovine serum albumin (BSA) to avoid non-specific binding. Subsequently, the slides were immunostained with a primary

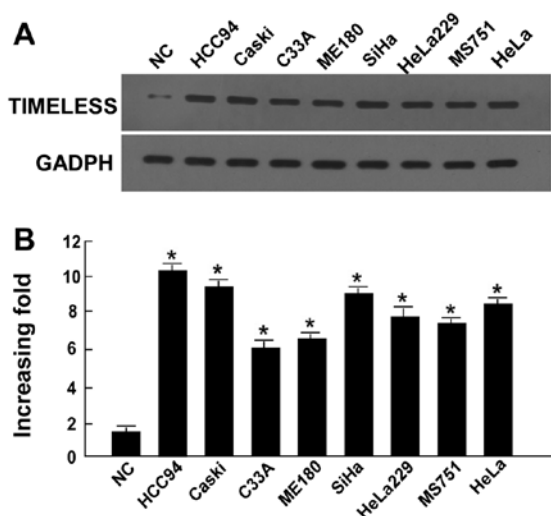


Figure 1. Upregulation of TIMELESS mRNA and protein in cervical cancer cell lines. (A and B) Expression of TIMELESS mRNA and protein in cervical cancer cell lines (HeLa229, MS751, ME180, SiHa, HeLa, CasKi, C33A and HCC94) and normal cervical tissue were examined by (A) western blotting and (B) qPCR. Expression levels were normalized against GAPDH, respectively. Error bars represent the standard deviation of the mean (SD) calculated from three parallel experiments. \* $P < 0.05$ .

antibody against TIMELESS (Abcam) diluted at 1:250 at 4°C overnight in a moist chamber. Phosphate-buffered saline (PBS) was used as a negative control. After washing with PBS buffer, the slides were incubated with prediluted anti-rabbit secondary antibody (Abcam) and then incubated with streptavidin-horseradish-peroxidase complex (Abcam). The tissue sections were immersed in 3-amino-9-ethylcarbazole, counterstained with 10% Mayer's haematoxylin, dehydrated and mounted in Crystal Mount. For visualization, tissue slides were stained with DAB (3,3-diaminobenzidine) for 1 min at room temperature. Finally, the tissue specimens were counterstained with hematoxylin, dehydrated and mounted using neutral balsam (Sigma-Aldrich).

All immunostained tissue slides were assessed independently by two observers without prior knowledge of clinical data. To evaluate IHC expression of TIMELESS, we applied a scoring system based on multiplying the proportion of positively stained tumor cells and the intensity of staining. The staining intensity (SI) was graded according to the following criteria: 0, no staining; 1, weak staining; 2, modest staining; and 3, strong staining. The proportion of positive tumor cells was scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10-50% positive tumor cells); 3 (51-80% positive tumor cells), and 4 (>80% positive tumor cells). The slides were rescored if the difference between the two pathologists was >3. The optimal cut-off values were distinguished and categorized by the median of the immunoreactive score (IRS) results: an SI score of 6 was used to identify tumors with high TIMELESS expression and an SI score of 4 was used for low TIMELESS expression.

**Statistical analysis.** All calculations were carried out using the SPSS version 18.0 software package (SPSS, Inc., Chicago, IL, USA). The relationship of TIMELESS protein expression levels with clinical and histomorphological characteristics

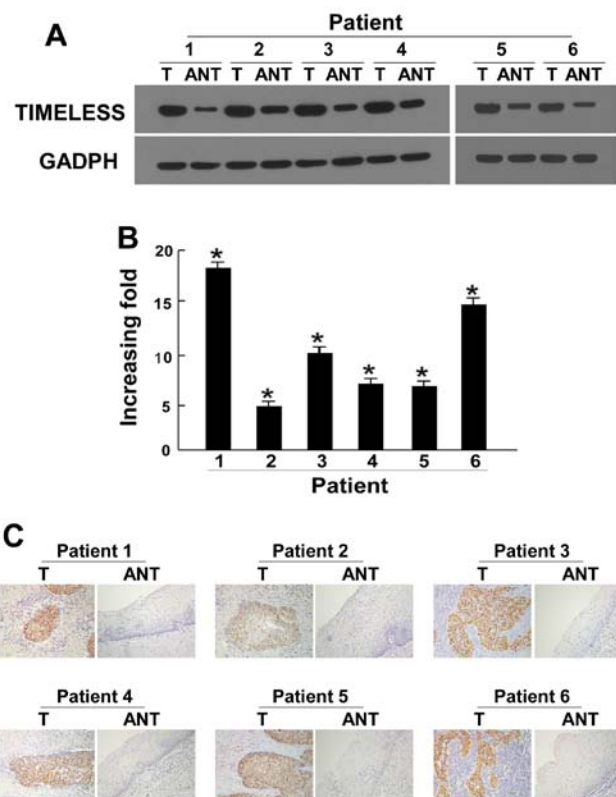


Figure 2. Aberrant expression of TIMELESS mRNA and protein in early-stage cervical cancer tissues. (A) Representative images of western blot analyses of TIMELESS protein expression in six matched pairs of cervical cancer tissue samples (T) and adjacent non-cancerous tissues (ANT). GAPDH was used as the loading control. (B) The average T/ANT ratios of TIMELESS mRNA expression in the paired cervical cancer (T) and adjacent non-cancerous tissue sections (ANT) were quantified using qPCR and normalised against GAPDH. The error bars represent the standard deviation of the mean (SD), which was calculated from three parallel experiments. (C) Immunohistochemical staining of TIMELESS protein in six pairs of matched early-stage cervical cancer tissues. \* $P < 0.05$ .

were determined using a Chi-squared test. The overall survival (OS) and disease-free survival (DFS) curves in association with TIMELESS expression were plotted using the Kaplan-Meier method and the difference between the groups was tested by a log-rank test. Multivariate analysis was conducted independently for each biomarker, including only significant clinical/histomorphological factors from the univariate analysis. Univariate and multivariate survival analyses were carried out using the Cox regression model. A statistically significant difference was determined by a two-sided  $P$ -value of <0.05.

## Results

**Expression of TIMELESS is upregulated in human cervical cancer.** Significantly higher TIMELESS protein expression and mRNA levels were observed in all eight cervical cancer cell lines compared to the normal cervical tissue sample ( $P < 0.05$ ; Fig. 1). TIMELESS protein expression was also significantly upregulated in the six cervical cancer cases compared with matched adjacent non-cancerous cervical tissues ( $P < 0.05$ ; Fig. 2A). Similarly, qRT-PCR data also indicated that TIMELESS mRNA was significantly upregulated in

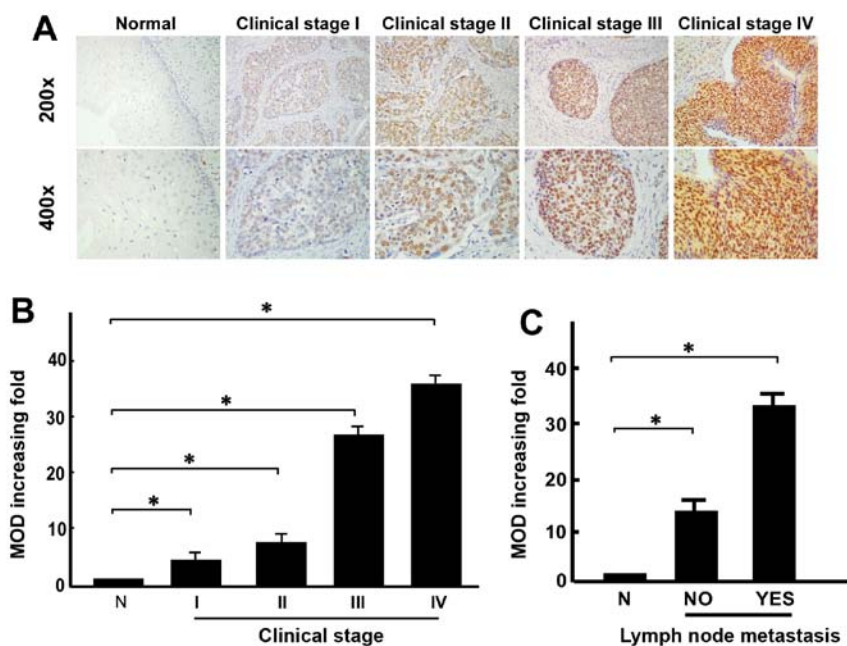


Figure 3. The expression of TIMELESS in cervical cancer tissues from patients at different clinical stages. (A) Representative images from immunohistochemical analyses of TIMELESS protein expression in normal cervical tissues and cervical cancer tissues at different clinical stages. (B) Statistical analyses of the average MOD of TIMELESS staining in normal cervical tissue and cervical cancer specimens at different clinical stages. Values represent the mean  $\pm$  SD from three independent experiments. Results are shown as the fold increase in MOD of TIMELESS staining relative to that of normal cervical tissue. (C) The statistical analyses of the average MOD of TIMELESS staining in the lymph node metastasis group and the lymph node metastasis-free group. \* $P < 0.05$ .

cervical cancer specimens compared to their matched adjacent non-cancerous regions ( $P < 0.05$ ; Fig. 2B). By real-time RT-PCR evaluation, the tumor/adjacent non-cancerous (T/N) ratio of TIMELESS mRNA expression was  $>5$ -fold in all cases, with the highest ratio being  $\sim 18$ -fold (Fig. 2B). In agreement with the results of the western blot analysis and real-time RT-PCR assay, IHC showed high expression of TIMELESS protein in cervical cancer lesions (Fig. 2C). Together, our results showed that TIMELESS expression was elevated at both the transcriptional level and translational levels in the cervical cancer cell lines and tissues.

**TIMELESS overexpression is correlated with clinicopathological variables of early-stage cervical cancer.** We collected 189 paraffin-embedded early-stage cervical cancer tissue samples for IHC, including 83 cases of stage IB1, 28 cases of stage IB2, 55 cases of stage IIA1 and 23 cases of stage IIA2 tumors. Following IHC staining, TIMELESS expression was assessed as positive in 124/189 (65.6%) cases and weakly positive or negative in 65 cases (34.4%). TIMELESS expression was mainly observed in the nucleus and rarely in the cytoplasm of epithelial cells (Fig. 3A). Notably, TIMELESS expression was found in most cancerous lesions in the primary cervical tumors, but was reduced in the adjacent normal cervical regions. TIMELESS protein expression was generally negative in normal cervical tissues, weak in stage IB1 and IB2 cervical cancer tissues, and strong in stage IIA1 and IIA2 cervical cancer tissues; TIMELESS expression obviously increased with the progression of tumor grades ( $P < 0.05$ ; Fig. 3A). Mean optical density (MOD) represents the average response strength of all positive cells in the field of vision. Quantitative IHC data revealed that the MOD values of TIMELESS were significantly associated with the cervical cancer tumor grades

(Fig. 3B), and were higher in cervical cancer tissues with PLNM than in those without PLNM (Fig. 3C).

Next, we examined the relationship between TIMELESS expression and established clinicopathological and investigative parameters using the Chi-squared test and Fisher's exact test (Table II). In patients with early-stage cervical cancer, high TIMELESS expression was markedly associated with age ( $P = 0.011$ ), clinical stage ( $P < 0.001$ ), PLNM ( $P < 0.001$ ), squamous cell carcinoma (SCC) antigen ( $P = 0.003$ ), tumor recurrence ( $P = 0.015$ ), vital status ( $P < 0.001$ ), differentiation grade ( $P < 0.001$ ), property of surgical margin ( $P = 0.036$ ) and lymphovascular space involvement ( $P = 0.001$ ; Table III). Moreover, patients with increased TIMELESS protein expression showed strong tendencies to receive postoperative radiotherapy ( $P = 0.002$ ). However, no significant correlations between TIMELESS expression and the histological type, HPV infection, tumor size, myometrium invasion, or infiltration of the parametrium organ were observed ( $P > 0.05$ ; Table III). Patients with aberrant TIMELESS protein expression also showed no obvious tendency to receive chemotherapy, concurrent chemotherapy and radiotherapy, chemotherapy with TP regimen (paclitaxel plus cisplatin), or radical hysterectomy ( $P > 0.05$ ; Table III). TP chemotherapy is the main chemotherapy of cervical cancer patients which combines paclitaxel with cisplatin.

Association coefficient analyses (Spearman's correlation) further confirmed the above-mentioned relationships between the TIMELESS protein expression levels and the clinicopathological features (Table III).

**High TIMELESS expression is associated with poor prognosis of patients with early-stage cervical cancer.** We examined whether patients with TIMELESS-positive samples had a

Table II. Correlation between TIMELESS protein expression and the clinicopathological features of early-stage cervical cancer.

Characteristics	Total	No or weak TIMELESS expression	Moderate or strong TIMELESS expression	Chi-squared test (P-value)	Fisher's exact test (P-value)
Age (years)					
≤46	98	42 (22.2)	56 (29.6)	0.011	0.014
>46	91	23 (12.2)	68 (36.0)		
Histological type					
Adenocarcinoma	10	2 (1.1)	8 (4.2)	0.325	0.498
Squamous cell carcinoma	179	63 (33.3)	116 (61.4)		
HPV infection					
No	40	14 (7.4)	23 (12.2)	0.623	0.700
Yes	153	51 (27.0)	101 (53.4)		
FIGO stage					
Ib1	83	41 (21.7)	42 (22.2)	<0.001	-
Ib2	28	13 (6.9)	15 (7.9)		
Ila1	55	7 (3.7)	48 (25.4)		
Ila2	23	4 (2.1)	19 (10.1)		
Pelvic lymph node metastasis					
Absent	134	60 (31.7)	74 (39.2)	<0.001	<0.001
Present	55	5 (2.6)	50 (26.5)		
Squamous cell carcinoma antigen, ng/ml					
≤1.5	91	41 (21.7)	50 (26.5)	0.003	0.004
>1.5	98	24 (12.7)	74 (39.1)		
Tumor size (cm)					
<4	142	50 (26.5)	92 (48.7)	0.680	0.726
≥4	47	15 (7.9)	32 (16.9)		
Tumor recurrence					
No	160	63 (33.3)	106 (56.1)	0.015	0.014
Yes	29	2 (1.1)	18 (9.5)		
Vital status (at last follow-up)					
Alive	158	63 (33.3)	89 (47.1)	<0.001	<0.001
Dead	35	2 (1.1)	35 (18.5)		
Differentiation grade					
G1	62	38 (20.1)	24 (12.7)	<0.001	-
G2	111	26 (13.8)	85 (45.0)		
G3	16	1 (0.5)	15 (7.9)		
Chemotherapy					
No	84	32 (16.9)	52 (27.5)	0.338	0.358
Yes	105	33 (17.5)	72 (38.1)		
Radiation therapy					
No	168	64 (33.9)	104 (55.0)	0.002	0.001
Yes	21	1 (0.5)	20 (10.6)		
Concurrent chemotherapy and radiotherapy					
No	177	62 (32.8)	111 (58.7)	0.169	0.270
Yes	16	3 (1.6)	13 (6.9)		
TP chemotherapy					
No	27	11 (9.2)	16 (13.3)	0.167	0.232
Yes	93	25 (20.8)	68 (56.7)		

Table II. Continued.

Characteristics	Total	No or weak TIMELESS expression	Moderate or strong TIMELESS expression	Chi-squared test (P-value)	Fisher's exact test (P-value)
Radical hysterectomy					
No	9	1 (0.5)	8 (4.2)	0.132	0.168
Yes	180	64 (33.9)	116 (61.4)		
Myometrium invasion					
<1/2	69	30 (15.9)	39 (20.6)	0.046	0.057
≥1/2	120	35 (18.5)	85 (45.0)		
Property of surgical margin					
No	177	64 (33.9)	113 (59.8)	0.036	0.037
Yes	12	1 (0.5)	11 (5.8)		
Infiltration of parauterine organ					
No	179	61 (32.3)	118 (62.4)	0.701	0.739
Yes	10	4 (2.1)	6 (3.2)		
Lymphovascular space involvement					
No	163	63 (33.3)	100 (52.9)	0.001	0.001
Yes	26	2 (1.1)	24 (12.7)		

Table III. Spearman correlation analysis of TIMELESS protein expression vs. clinicopathological factors.

Variables	TIMELESS protein expression	
	Spearman's correlation coefficient	P-value
Age	0.185	0.011
Histological type	0.072	0.327
HPV infection	0.036	0.625
Clinical staging	0.326	<0.001
Pelvic lymph node metastasis	0.341	<0.001
Squamous cell carcinoma antigen, ng/ml	0.216	0.003
Tumor size	0.030	0.682
Recurrence	0.177	0.015
Vital status	0.301	<0.001
Differentiation grade	0.404	<0.001
Survival time	-0.526	<0.001
Chemotherapy	0.070	0.340
Radiation therapy	0.221	0.002
Concurrent chemotherapy and radiotherapy	0.100	0.170
TP chemotherapy	0.126	0.169
Radical hysterectomy	-0.110	0.133
Myometrium invasion	0.145	0.046
Property of surgical margin	0.153	0.036
Infiltration of parauterine organ	-0.028	0.703
Lymphovascular space involvement	0.232	0.001

poorer survival rate than those with TIMELESS-negative samples. Survival time was markedly different between patients with TIMELESS-positive and TIMELESS-negative samples based on the log-rank test. Patients with higher TIMELESS expression had shorter OS (log-rank,  $P<0.001$ ; Fig. 4A) and DFS (log-rank,  $P=0.001$ ; Fig. 4B) than those with lower TIMELESS expression. When examining the whole study cohort, patients with high TIMELESS expression had a significantly lower cumulative 5-year survival rate than those with low TIMELESS protein expression (73.1 vs. 96.8%, respectively;  $P<0.05$ ).

We also explored the prognostic value of TIMELESS protein expression in different subgroups of patients with early-stage cervical cancer. Subgroups of patients were stratified according to age, HPV infection, FIGO stage, PLNM, SCC antigen, tumor size, differentiation grade, chemotherapy, radiotherapy, concurrent chemotherapy and radiotherapy, TP chemotherapy, radical hysterectomy, myometrium invasion, properties of the surgical margin, infiltration of parauterine organ and lymphovascular space involvement. TIMELESS protein expression was strongly associated with the OS duration of patients aged  $>46$  years (log-rank test;  $P=0.008$ ), aged  $\leq 46$  years (log-rank test;  $P<0.001$ ), with HPV infection (log-rank test;  $P<0.001$ ), without HPV infection (log-rank test;  $P=0.030$ ), without radiotherapy (log-rank test;  $P<0.001$ ), with SCC antigen  $\leq 1.5$  ng/ml (log-rank test;  $P=0.004$ ), with clinical stage IB1-IB2 (log-rank test;  $P<0.001$ ), with TP chemotherapy (log-rank test;  $P=0.005$ ), with histological differentiation grade (grades 1 and 2, log-rank test,  $P<0.001$ ; grades 2 and 3, log-rank test;  $P=0.011$ ), with tumor size (tumor size  $<4$  cm, log-rank test;  $P<0.001$ ; tumor size  $\geq 4$  cm, log-rank test;  $P=0.033$ ), with radical hysterectomy (log-rank test;  $P<0.001$ ), without infiltration of parauterine organ (log-rank test;  $P<0.001$ ), without PLNM (log-rank test;  $P<0.001$ ), with



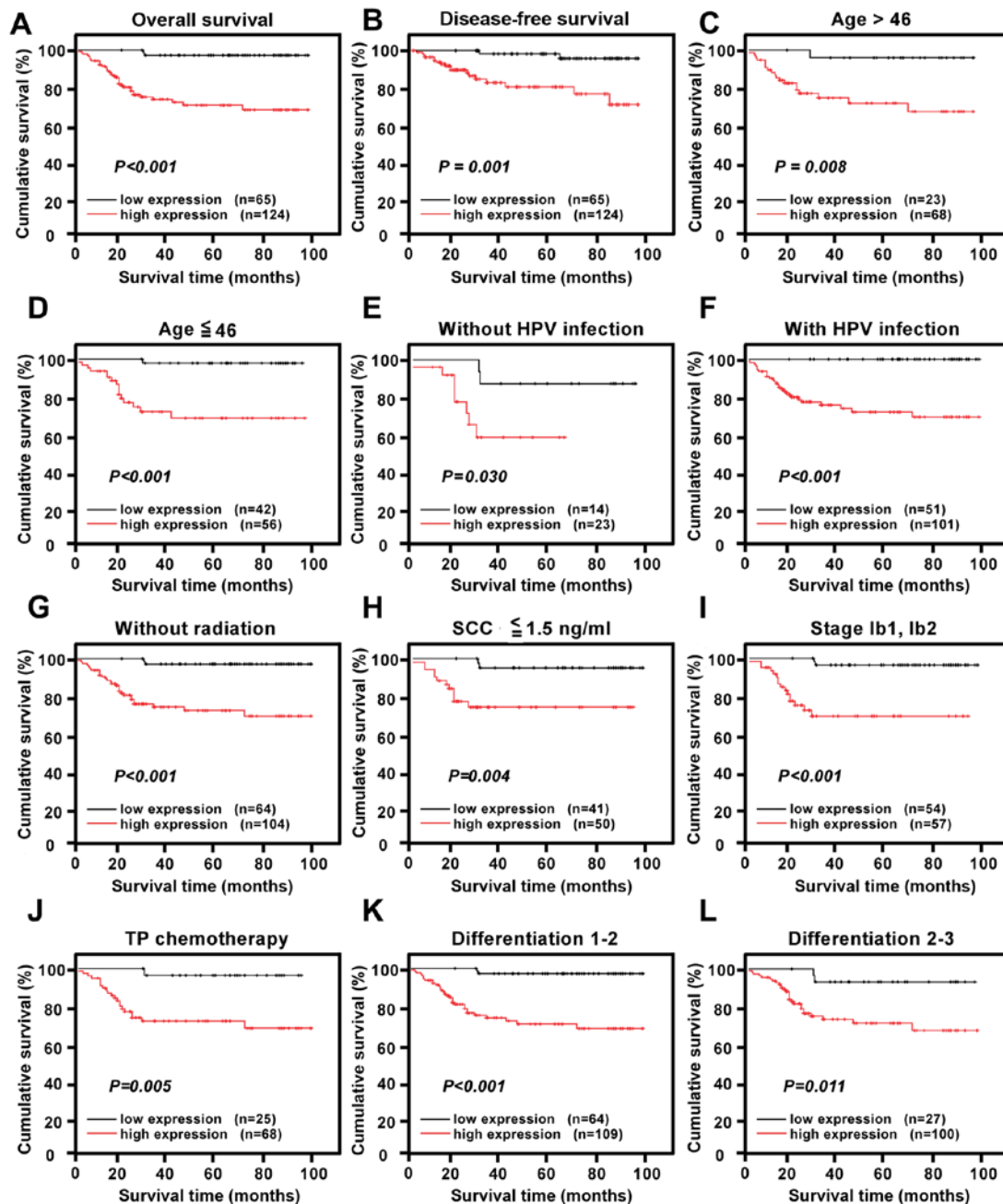


Figure 4. Kaplan-Meier curves of univariate analysis data (log-rank test). The overall survival (OS) and disease-free survival (DFS) for the patients with low TIMELESS expression vs. high TIMELESS expression (A and B). Survival curves for the patients (C) aged >46 years, (D) aged ≤46 years, (E) without HPV infection, (F) with HPV infection, (G) without radiation, (H) with SCC ≤1.5 ng/ml, (I) at stages Ib1-Ib2, (J) with TP chemotherapy, (K) at differentiation grades 1 and 2, (L) at differentiation grades 2 and 3. ( $P<0.05$ , respectively).

SCC (log-rank test;  $P<0.001$ ), without lymphovascular space involvement (log-rank test;  $P<0.001$ ), without properties of the surgical margin (log-rank test;  $P<0.001$ ), without myometrium invasion (log-rank test;  $P=0.001$ ), with myometrium invasion (log-rank test;  $P=0.001$ ) and with chemotherapy (log-rank test,  $P<0.001$ ; Figs. 4 and 5).

Univariate analysis based on the Cox regression model demonstrated that upregulation of TIMELESS protein expression was a significant prognostic biomarker of poor OS ( $P<0.001$ ; Table IV). In addition, early-stage cervical carcinoma patients with PLNM, with lymphovascular space involvement, and those who had undergone radiation therapy, showed poorer prognosis (Table IV). In our multivariate

analysis, only TIMELESS protein expression and tumor recurrence remained significant (Table IV), and served as independent prognostic factors for poor OS in cervical cancer patients ( $P=0.002$ , 95% CI, 2.285-42.639; and  $P<0.001$ , 95% CI, 2.430-10.673, respectively).

## Discussion

The present study demonstrated that TIMELESS is abundantly expressed at both transcriptional and translational levels in human cervical cancer compared with normal cervical tissues. This elevation in TIMELESS expression was strongly associated with age, clinical stage, PLNM,



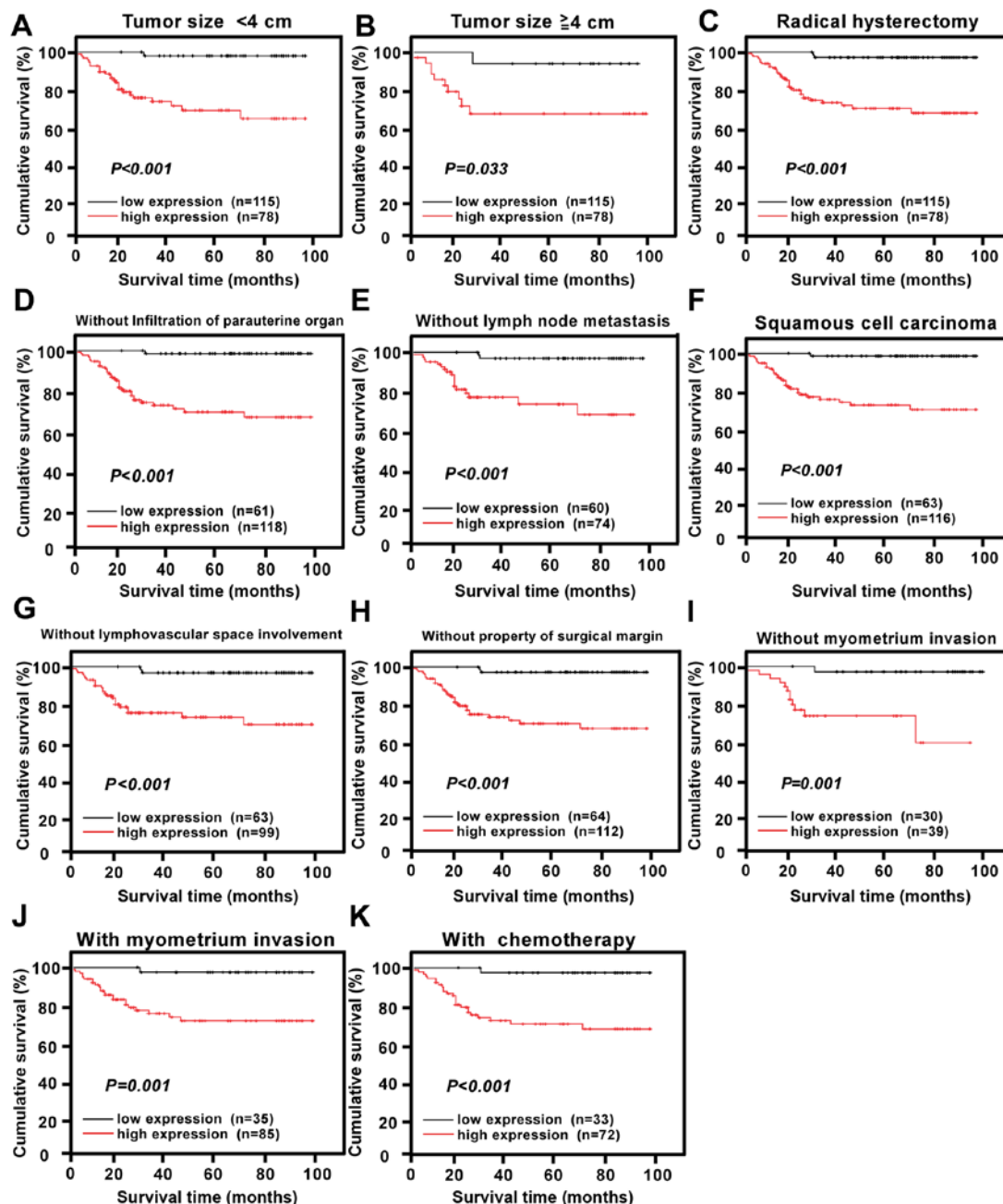


Figure 5. Survival curves for the patients in select patient subgroups (log-rank test). OS rates for patients (A) with tumor size <4 cm, (B) with tumor size  $\geq 4$  cm, (C) with radical hysterectomy, (D) without infiltration of parauterine organ, (E) without lymph node metastasis, (F) with pathologic type of squamous cell carcinoma, (G) without lymphovascular space involvement, (H) without property of surgical margin, (I) without myometrium invasion, (J) with myometrium invasion, (K) with chemotherapy. ( $P<0.05$ , respectively).

SCC antigen, tumor recurrence, vital status, differentiation grade, property of surgical margin and lymphovascular space involvement. Patients with upregulated TIMELESS protein expression were also more likely to receive postoperative radiotherapy. Furthermore, our survival analysis revealed that high TIMELESS expression was an independent biomarker associated with poorer clinical outcomes in cervical cancer patients.

Similar to our findings, recent reports have shown the importance of TIMELESS in many different types of human cancers (19-31). TIMELESS was overexpressed in a panel of human lung cancer cell lines and clinical lung cancer specimens compared to normal lung controls (19). Associations

between TIMELESS and breast cancer risk have also been demonstrated, with TIMELESS being reported as promising marker of tamoxifen resistance in women with estrogen receptor  $\alpha$ -positive breast tumors (20-22). In colorectal cancer, high TIMELESS expression was significantly associated with the clinical and pathological features (25). TIMELESS expression is also closely related to the clinicopathological factors in bladder cancer (29). On the other hand, TIMELESS is downregulated in kidney cancer cells (28), and TIMELESS transcription was significantly lower in pancreatic ductal adenocarcinoma compared to adjacent tissue and benign tissue (30). As we know, it is quite common that some gene may act as an oncogenic role in several types of cancers while

Table IV. Univariate and multivariate analyses of prognostic factors in early-stage cervical cancer using a Cox-regression model.

	Univariate analysis			Multivariate analysis		
	No. of patients	P-value	Regression coefficient (SE)	P-value	Relative risk	95% CI
TIMELESS		<0.001	13.712 (0.729)	0.002	9.871	2.285-42.639
Low expression	65					
High expression	124					
Pelvic lymph node metastasis		0.015	2.237 (0.330)	0.607	1.203	0.595-2.434
Absent	134					
Present	55					
Radiation therapy		0.041	2.372 (0.422)	0.537	1.330	0.537-3.291
No	168					
Yes	21					
Lymphovascular space involvement		0.005	2.748 (0.360)	0.994	0.997	0.450-2.209
No	163					
Yes	26					
Recurrence		<0.001	7.148 (0.348)	<0.001	5.092	2.430-10.673
No	160					
Yes	29					

it plays an anti-oncogenic role in other types of cancers. For example, our previous finding showed that B3GNT3 was upregulated in cervical cancer and is correlated with the progression of cervical cancer (11). Moreover, several reports suggested that B3GNT3 plays an oncogenic role in certain types of cancer such as colon and pancreatic cancer (33,34). However, it is found that B3GNT3 might play a critical role in suppressing the malignant properties of neuroblastoma (35).

With regards to its mechanism of action, TIMELESS appears to regulate the many signaling pathways involved in tumor proliferation, apoptosis, metastasis and the cell cycle. Indeed, TIMELESS has been implicated in cell proliferation in leukemia stem cells (31). It has also been shown to activate the eeF1a2/PI3K/aKt/mtoR axis to contribute to the migratory phenotype in hepatocellular carcinoma, and may also activate the protumorigenic eeF1a2/aKt/mDm4 axis (23). Moreover, TIMELESS was previously shown to be essential for Rad3-related-checkpoint kinase 1 (ATR-Chk1)-mediated and ataxia telangiectasia mutated-checkpoint kinase 2 (ATM-Chk2)-mediated signaling and S-phase arrest (25). While our results also indicate that TIMELESS is important in the development, progression and metastasis of cervical cancer, further studies are warranted to identify its specific role in the related signaling pathways.

Our results also indicate that overexpression of TIMELESS might be an effective biomarker for identifying patients with more aggressive risk factors, such as those with higher clinical stage, with PLNM, with tumor recurrence, higher differentiation grade, with property of surgical margin and with lymphovascular space involvement. In our experience, several clinical characteristics play important roles in the prognosis of cervical cancer and can affect patients' optimal treatment selections. For example, PLNM has been previously shown to

be an important biomarker for the clinical outcome of cervical cancer patients (36), as well as well as for determining the optimal treatment. In the clinic, patients found to have PLNM before surgery will receive chemoradiotherapy instead of undergoing an operation. While many tests (e.g., physical examination, magnetic resonance imaging, biomarkers, among others) are currently available to assess PLNM, they are still prone to errors. Thus, more precise molecular marker are required to avoid misdiagnosis and improper treatment. PLNM also determines the optimal postoperative therapy. For example, after radical hysterectomy plus lymphadenectomy, patients with PLNM will receive postoperative chemotherapy and radiation.

In the present study, we found that TIMELESS expression is clearly associated with PLNM. Thus, TIMELESS may be an effective biomarker for PLNM and for determining the optimal treatment in cervical cancer patients. In previous reports, elevated TIMELESS levels were suggested to play an oncogenic role in hepatocellular carcinoma and promote tumor cell migration (23), and were also associated with tumor metastasis in prostate cancer (24). While our present results are in agreement with these previous studies, we failed to show that TIMELESS is an independent prognostic marker of PLNM, most likely due to the small sample size. In our next study, we aim to detect the utility of TIMELESS as an independent prognostic biomarker of PLNM by examining a larger sample size of early-stage cervical cancer patients. Furthermore, we found a strong correlation between poorer OS and aberrant TIMELESS protein expression in patients without lymph node metastasis. Thus, TIMELESS may be an outstanding marker of PLNM and a good prognostic marker for cervical cancer patients without PLNM, although further studies are required.

Lymphovascular space involvement has also been identified as an important high-risk factor that affects prognosis and determines postoperative treatment (37). Patients with lymphovascular space involvement may face poorer clinical outcomes and should receive more aggressive treatment, such as postoperative chemotherapy (38). Our data illustrated that TIMELESS protein overexpression is markedly correlated with lymphovascular space involvement and unfavorable survival rates. This indicates that patients with lymphovascular space involvement should undergo more aggressive therapy to reduce the mortality rates.

Past evidence has demonstrated that high differentiation grade is an essential indicator for aggressiveness and worse outcomes in cervical cancer (38). Cervical cancer cells of high differentiation grade show strong proliferative and migratory ability. Furthermore, differentiation grade is critical for the selection of therapy in cervical cancer. According to our treatment guidelines, after resection of the tumor of uterine cervix, patients of high differentiation grade cervical cancer should receive postoperative chemotherapy. Here we showed that the expression of TIMELESS is associated with the differentiation grade of cervical cancer and thus, may be used to evaluate prognosis and guide treatments.

The standard treatments for early-stage cervical cancer patients are currently radical hysterectomy plus lymphadenectomy or chemoradiotherapy. Patients with high-risk factors, such as myometrium invasion, will receive postoperative radiation therapy. On the other hand, patients with high-risk factors, such as lymphovascular space involvement, high grade of tumor differentiation, or a tumor size >4 cm will receive postoperative chemotherapy. In addition, patients with PLNM, property of surgical margin, infiltration of the parauterine organ, or lymphovascular space involvement should receive postoperative chemoradiotherapy. Previous reports have proved that postoperative radiation and chemotherapy contribute to the prognosis of patients with high risk factors (39). In the present study, we found that patients with high TIMELESS protein expression showed an increased tendency to receive postoperative radiation therapy. Interestingly, we observed that TIMELESS protein overexpression was significantly correlated with OS in patients with radical hysterectomy, with chemotherapy, with TP chemotherapy, and without postoperative radiation therapy. Thus, TIMELESS protein expression may be useful in identifying patients potentially with shorter OS in these subgroups, who may wish to undertake more aggressive treatments.

While tumor recurrence remains a major issue for cervical cancer mortality (40), it remains difficult to diagnose as the lesions are too small to be seen using available imaging methods. Moreover, no effective techniques or biomarkers are available for predicting tumor recurrence. Our findings demonstrated that TIMELESS protein overexpression is strongly associated with tumor recurrence and is closely correlated with poorer DFS. Thus, TIMELESS protein expression is a useful prognostic biomarker of tumor recurrence in early-stage cervical cancer. Despite our positive results, whether TIMELESS protein or mRNA overexpression could be detected in blood serum and was strongly correlated with tumor recurrence requires further investigation.

Together our results indicate that TIMELESS may be a new therapeutic target for the treatment of cervical cancer. For example, TIMELESS depletion was previously shown to increase sensitivity to doxorubicin, an anticancer drug (26,27). Thus, TIMELESS may represent a novel therapeutic target. However, we must first unravel its exact mechanism of action in cervical cancer to confirm our hypothesis.

In conclusion, our present data demonstrated that TIMELESS expression was upregulated in cervical cancer and was significantly associated with age, clinical stage, PLNM, SCC antigen, tumor recurrence, vital status, differentiation grade, radiation therapy, property of surgical margin and lymphovascular space involvement. Furthermore, TIMELESS protein overexpression was identified as an independent prognostic biomarker for predicting the poor clinical outcome of early-stage cervical cancer patients. However, further investigation into the molecular mechanisms behind the TIMELESS-mediated potential tumorigenic effects in cervical cancer are required.

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