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Upregulation of LncRNA PANDAR predicts poor prognosis and promotes cell proliferation in cervical cancer

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Abstract. – OBJECTIVE: To investigate the expression level of long non-coding RNA promoter of CDKN1A antisense DNA damage activated RNA (lncRNA PANDAR) in cervical cancer and to analyze the effects of PANDAR on biological behaviors of cervical cancer and the clinical significance of PANDAR.

PATIENTS AND METHODS: A total of 68 pairs of cervical cancer and paired cancer-adjacent tissue specimens were collected. The relative expression of PANDAR in tissues was detected by quantitative Real-time polymerase chain reaction (qRT-PCR) so as to analyze the relationship between PANDAR and clinicopathological factors of cervical cancer and evaluate its clinical significance. qRT-PCR was used to detect the relative expression of PANDAR in cervical cancer cells. Interference in PANDAR was conducted. Cell counting kit-8 (CCK-8) assay was used to detect the changes in cell proliferation capacity.

RESULTS: The results of qRT-PCR showed that the expression level of PANDAR was significantly up-regulated in cervical cancer tissues compared with that in cancer-adjacent tissues. Statistical analysis showed that PANDAR expression was correlated with International Federation of Gynecology and Obstetrics (FIGO) staging, tumor size and tumor invasion degree. Kaplan-Meier survival analysis showed that the survival time of patients with highly expressed PANDAR was shorter than that of patients with lowly expressed PANDAR. Cox multivariate regression analysis showed that PANDAR could be used as an independent prognostic factor for patients with cervical cancer. CCK-8 results showed that interference in PANDAR significantly inhibited tumor cell proliferation.

CONCLUSIONS: The expression level of PANDAR is up-regulated in cervical cancer tissues and cells, and PANDAR promotes tumor growth. PANDAR may be a biomarker for the early diagnosis of cervical cancer and a potential therapeutic target for reversing the malignant phenotype of tumors.

Key Words:
Cervical cancer, lncRNA PANDAR, Clinical significance, Biomarkers.

Introduction

The incidence rate of cervical cancer ranks second among female malignant tumors in the world, which is one of the main causes of cancer-related deaths in females at present[1]. Cervical cancer in the early stage can be cured by radical operations assisted by radiotherapy and chemotherapy, but the overall prognosis is still poor. The present studies show that factors affecting the prognosis of cervical cancer mainly include tumor infiltration degree, whether there is vascular invasion or not, parametrium infiltration degree, whether there is pelvic lymph node metastasis or not, etc.[2], in which the most important risk factor affecting the postoperative efficacy is parametrium infiltration degree[3]. Compared with patients without parametrium infiltration, the 5-year survival rate is decreased by 20% to 30% in patients with parametrium infiltration[4]. Therefore, the early diagnosis of cervical cancer and the implementation of effective interventions can help improve the prognosis of patients with cervical cancer.

Human whole genome sequencing results show that about 93% of the sequences in the genome can be transcribed, of which only 2% transcripts have protein coding functions, whereas 98% transcripts are non-coding RNAs (ncRNAs) without protein coding functions[5,6]. Recent studies show a new class of ncRNAs with over 200 nt transcripts, namely, the long non-coding RNA (lncRNA). They do not have the function of coding proteins but regulate gene expression in the form of RNA at various levels, including transcription regula-

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tion, post-transcription regulation, chromatin modification, genomic imprinting, protein translation regulation, etc[7-10]. With the deepening of lncRNA research, more and more evidence shows that lncRNA participates in the occurrence and development of tumors and can be clinically used as a potential diagnostic marker or therapeutic target.

LncRNA promoter of CDKN1A (Cyclin Dependent Kinase Inhibitor 1A) antisense DNA damage activated RNA (PANDAR) is 1506bp in total length, located at the chromosome 6p21.2 position11. Initially, Hung et al[12] found from DNA injury-induced sequencing that in fibroblasts, PANDAR regulates the nuclear transcription factor Y (NFYA) to inhibit cell apoptosis. More and more evidence shows that PANDAR can play dual "roles" in various stages of human tumors to participate in different stages of tumor growth. Han et al[13] found that in non-small cell lung cancer, lowly expressed PANDAR suggest that the prognosis of patients is poor, and it regulates apoptosis by regulating B-cell lymphoma 2 (Bcl-2) genes. In colorectal cancer, highly expressed PANDAR can promote tumor metastasis by regulating epithelial-mesenchymal transition (EMT)[14]. However, the relationship between PANDAR and cervical cancer has not yet been reported.

It was found for the first time in this study that PANDAR was relatively highly expressed in cervical cancer tissues and cells, and highly expressed PANDAR was related to International Federation of Gynecology and Obstetrics (FIGO) clinical staging, tumor size and tumor infiltration degree, and can be used as an independent factor for predicting prognosis of patients. Knocking down PANDAR significantly inhibited tumor cell proliferation, and the detection of PANDAR expression was of important clinical value for predicting the prognosis of patients and developing the therapeutic target for PANDAR.

Patients and Methods

Tissues and Cells

A total of 68 patients aged 30 to 72 years old with cervical squamous cell carcinoma who received radical hysterectomy and pelvic lymphadenectomy in the Department of Obstetrics and Gynecology from Zhangjiagang First People's Hospital from January 2013 to January 2017 were selected. The postoperative staging of each case was consistent with the diagnostic criteria of FIGO. 68 patients did not receive radiotherapy and chemotherapy before operation, and specimens were products of the surgical excision, which were supported by histopathology. 68 pairs of specimens were collected from tumor tissues and paired cancer-adjacent normal tissues (more than 5 cm from the tumor tissues) within 15 min after the excision, and then stored at -80°C. All the applied specimens received the informed consent signed by patients or their clients, and all the studies involved the human body were approved by the Ethics Committee of Zhangjiagang First People's Hospital in advance.

Human cervical cancer cell lines HeLa and C33-A and normal human skin epithelial cell line HaCaT were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. HeLa and HaCaT cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) Gibco (Rockville, MD, USA) containing 10% fetal bovine serum (FBS), and C33-A cells were cultured in the 1640 Gibco (Rockville, MD, USA) medium containing 10% FBS. The media were added with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and the cell suspension was placed in a constant temperature incubator with 5% CO_2 at 37°C.

Detection of the Expression Level of PANDAR in Tissues and Cells

The total RNA was extracted from cervical cancer cells by RNA extraction kits, and RNAs were quantified by quantitative spectrophotometry (D 260/D 280). The complementary DNA (cDNA) (PrimeScriptTM RT Master Mix) was produced by reverse transcription according to the instructions of reverse transcription kit, and the reverse transcription system was 20 μL. Quantitative Real-time polymerase chain reaction (qRT-PCR) was conducted according to the instructions of qRT-PCR kits. The reaction system (20 μL): 2 μL cDNAs, 2 μL forward primers, 2 μL reverse primers, 10 μL 2 s mix and 4 μL diethyl pyrocarbonate (DEPC) water. The process: the reaction lasted for 30 s at 95°C, 5 s at 95°C and 30 s at 60°C, respectively, and the whole process was repeated for 40 cycles. Each specimen was tested for 3 times. The relative expression level of genes was represented by 2-ΔΔCT.

Synthesis of PANDAR Small Interfering RNA (siRNA) and Primers

The primer sequences for PANDAR and glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) were designed by polymerase chain reaction (PCR) primer design software Primer Premier 5.0. The specific sequences were shown as follows: PANDAR-F TGCACACATTTAACCCGAAG, PANDAR-R CCCCA AAGCTACATCTATGACA; GAPDH-F AGCCACATCGCTCAGACAC, GAPDH-R GCCCAATACGACCAAATCC. Primer dry powders were prepared into 20 μmol/L solution with diethyl pyrocarbonate (DEPC) water after centrifugation, and then the solution was stored in refrigerator at -20°C. The effective interference sequence of PANDAR was designed by Invitrogen (Carlsbad, CA, USA) siRNA1# (5'-AAUGUGUGCACGUAACAGAUU-3'), siRNA2# (5'-GGGCAUGUU UUCACAGAGGUU-3') and siRNA3# (5'-AAUGUGU GCACGUAACAGAUU-3').

Detection of the Cell Proliferation Capacity by CCK-8 Assay

si-RNA and control sequences were transfected into cervical cancer cells. The cell suspension was collected, and the cell concentration was adjusted to 3×104/mL. The cells were uniformly inoculated into a 96-well culture plate with 100 μL per well. 3 repeatedly used wells were set in the experimental group and the control group. After the incubation at 0 h, 24 h, 48 h, 72 h, 96 h, the medium was removed and washed twice with phosphate-buffered saline (PBS). The CCK-8 and serum-free medium (1:10) mixtures were prepared, and 110 mL mixtures were added to each well. The optical density (OD) value was measured at the wavelength 450 nm using a microplate reader. Finally, the cell growth curve was drawn.

Statistical Analysis

Statistical Product and Service Solutions (SPSS Version X; IBM, Armonk, NY, USA) 17.0 was used for statistical analysis. The difference in the expression level of PANDAR between cervical cancer tissues and cancer-adjacent tissues was analyzed by t-test. The relationship between PANDAR expression and clinicopathological features of patients with cervical cancer was analyzed by χ^2 test. The relationship between PANDAR expression and patient survival time was analyzed by Kaplan-Meier analysis, and the multivariate analysis of the prognosis of cervical cancer was performed using the Cox proportional-hazards regression model. $p<0.05$ represented the difference was statistically significant.

Results

Detection of the Relationship of PANDAR Expression and Clinicopathological Features

The results of qRT-PCR showed that PANDAR was up-regulated in cervical cancer tissues of 52 patients (fold change >1.0) (Figure 1A). The average number of fold change was taken as the cut-off point for dividing the patients with cervical cancer into PANDAR high expression group (n=38, fold change>4.7) and low expression group (n=30, fold change<4.7) (Figure 1B). Statistical analysis showed that PANDAR expression was correlated with the FIGO clinical staging, tumor size and tumor infiltration degree, and the difference was statistically significant ($p<0.05$) (Table I).

Correlation Between PANDAR Expression and Patient Survival Time

Kaplan-Meier survival analysis was used to study the correlation between PANDAR expression and the prognosis of patients with cervical cancer. The results showed that the survival time of the patients with highly expressed PANDAR was shorter than that in patients with lowly expressed PANDAR (Figure 1C). The single-factor analysis of the survival data of 68 patients with cervical cancer was performed using the Cox proportional-hazards regression model. The results showed that there were statistical significances in the highly expressed PANDAR ($p=0.016$), FIGO staging ($p=0.004$). The multivariate analysis of the survival data of 68 patients with cervical cancer was further performed using the Cox proportional-hazards regression model. The results showed that the highly expressed PANDAR ($p=0.03$), FIGO staging ($p=0.006$) could be used as independent prognostic factors for patients with cervical cancer (Table II).

Effects of PANDAR on Cervical Cancer Cell Proliferation

First, the relative expression of PANDAR in human cervical cancer cell lines HeLa and C33-A and the human normal skin epithelial cell line HaCaT cell line were detected by qRT-PCR (Figure 1D). The results showed that the relative expression of PANDAR was up-regulated. The specific interference sequences of PANDAR were designed and synthesized, and then were transiently transfected into tumor cells. 48 h later, the transfection efficiency was detected by qRT-PCR, and

Table I. Correlation between PANDAR expression and clinicopathological characteristics of cervical cancer patients (n = 68). * Overall $p < 0.05$.

Characteristics	PANDAR Low no. Case (%)	PANDAR High no. Case (%)	p x^2-test p-value
Age (years)			
>40	12	18	0.626
≤40	18	20	
Tumour size (cm)			
<4	22	13	0.002*
≤4	8	25	
Differentiation			
Well+moderate	15	10	0.075
Poor	15	28	
FIFO staging			
I+II	18	12	0.027*
III+IV	12	26	
Lymph lode metastasis			
No	16	16	0.464
Yes	14	22	
Depth of cervical invasion			
≤2/3	20	14	0.027*
>2/3	10	24	
Gynecological infection			
No	23	30	1.0
Yes	7	8	

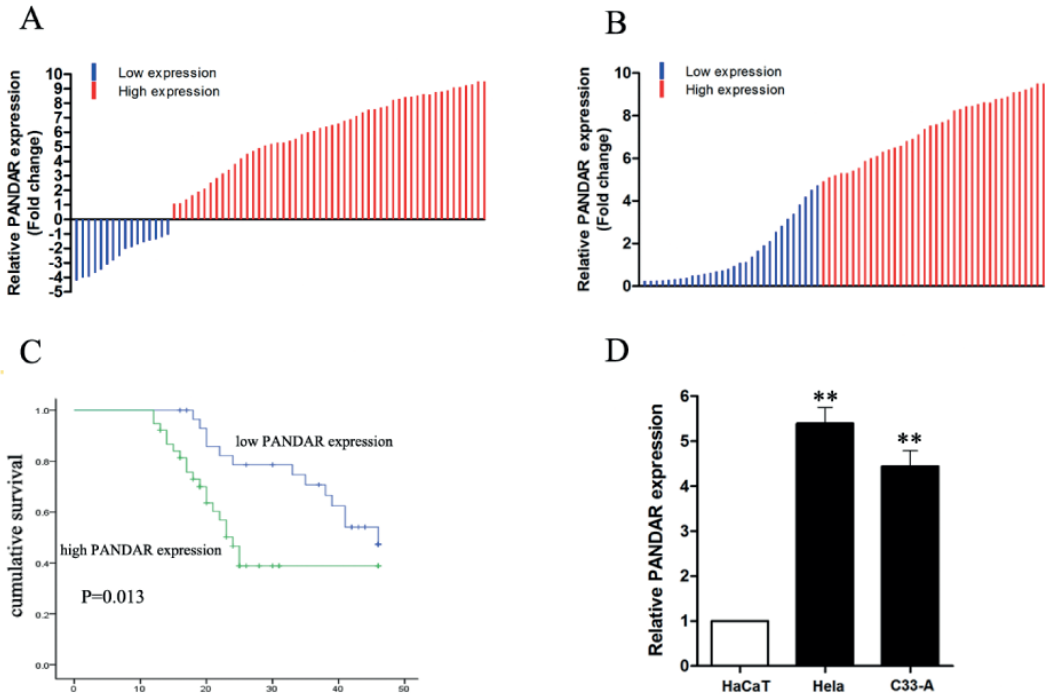

Figure 1. The expression level of PANDAR in cervical cancer and its clinical significance. ***A,*** The relative expression of PANDAR in cervical cancer tissues and cancer-adjacent tissues is detected by qRT-PCR assay with GAPDH as the internal reference. ***B,*** Patients with cervical cancer is divided into two groups according to the fold change in PANDAR expression: PANDAR high expression group (n=38) and PANDAR low expression group (n=30). ***C,*** Kaplan-Meier survival analysis shows that the survival time of patients in the PANDAR high expression group is lower than that in patients in the PANDAR low expression group. ***D,*** The relative expression level of PANDAR in cervical cancer cells compared with in human normal skin epithelial cells by qRT-PCR assay. $^{*}p< 0.05$, $^{**}p< 0.01$.

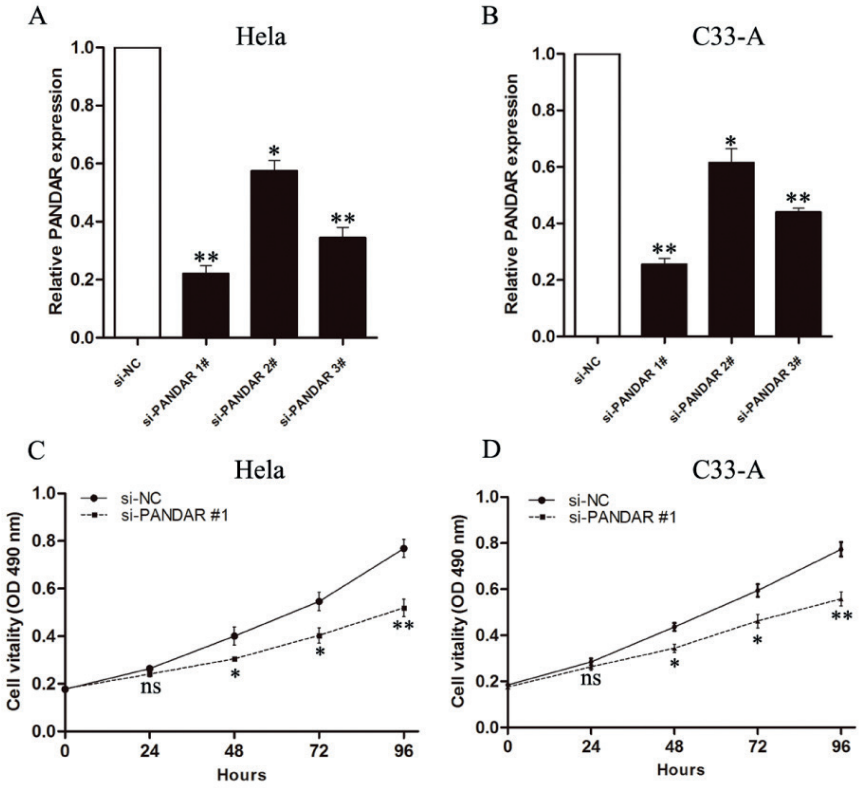

Figure 2. Effects of PANDAR on the proliferation of cervical cancer cells. ***A-B,*** The interference efficiency of cervical cancer cells HeLa and C33-A 48 hours after the transfection with siRNA is detected. ***C-D,*** After the transfection with siRNA in HeLa and C33-A cells, CCK-8 assay is used to detect changes in cell viability. All experiments are repeated for three times independently. $^{*}p< 0.05$, $^{**}p< 0.01$.

the sequences with the highest interference efficiency were used for subsequent experiments (Figure 2A-B). CCK-8 experiments showed that the knockdown of PANDAR expression significantly inhibited the proliferation of cervical cancer cells (Figure 2C-D).

Discussion

Cervical cancer is the second largest malignant tumor in women, and its incidence rate in young women has been increased year by year, so it is one of the risk factors seriously threatening wo-

Table II. Univariate and multivariate analysis of over-survival in cervical cancer patients (n=68).

	Univariate analysis			Multivariate analysis		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Age	0.708	0.356-1.407	0.324			
Tumour size (cm)	1.762	0.884-3.512	0.108			
Differentiation	0.935	0.464-1.881	0.850			
Gynecological infection	0.829	0.360-1.911	0.660			
Depth of cervical invasion	1.779	0.885-3.573	0.106			
Lymph lode metastasis	1.024	0.517-2.028	0.945			
FIFO staging	3.268	1.469-7.268	0.004*	3.089	1.383-6.898	0.006*
PANDAR expression	2.461	1.180-5.136	0.016*	3.194	1.085-4.870	0.030*

HR, hazard ratio; 95 % CI, 95% confidence interval, *overall $p < 0.05$.

men's physical and mental health[2]. According to the statistics of the World Health Organization (WHO) (www.who.int/en/), in 2013, more than 270,000 women died for cervical cancer, of which 85% came from developing countries. Similar to other malignant tumors, cervical cancer is difficult to be diagnosed with poor prognosis, which comes down to the unclear molecular mechanism of the occurrence and development of cervical cancer. With the deepening of research, research on lncRNA in cervical cancer may provide new molecular diagnostics and targets for the diagnosis and treatment of cervical cancer.

LncRNA plays a variety of roles in the form of RNA in the body of creatures, and its abnormal expression is closely related to a variety of human diseases, including diabetes mellitus, Alzheimer's disease (AD), etc.[15,16]. Especially, lncRNA can play dual roles in regulating the activation or silencing of genes and signaling pathways[17,18]. The first lncRNA BC200 cell line in cervical cancer was found in the study of Chen et al[19] in 1997. Many successive studies of scholars have shown that other lncRNAs are abnormally expressed in cervical cancer, including lncRNA PVT1, HOTAIR, etc[20]. However, the research on the correlation between PANDAR and cervical cancer has not been reported. Recent studies have shown that PANDAR is associated with the occurrence and development of bladder cancer, breast cancer and gastric cancer[21-23].

Conclusions

This study shows that PANDAR is highly expressed in cervical cancer tissues and cells and related to FIGO clinical staging, lymph node metastasis, tumor infiltration degree of cervical cancer, suggesting that PANDAR is also a cancer-promoting gene of cervical cancer. *In vitro* cell experiments further confirm that interference in PANDAR expression can significantly inhibit the proliferation of cervical cancer cells.

Conflict of interest

The authors declare no conflicts of interest.

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