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Original Article

High expression of prostate tumor overexpressed 1 (PTOV1) is a potential prognostic biomarker for cervical cancer

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Abstract: Background: To investigate the role of prostate tumor overexpressed 1 (PTOV1) in the development and progression of human cervical cancer. Methods: Real-time quantitative PCR, Western blot, and immunohistochemistry were used to explore PTOV1 expression in cervical cancer tissues and cell lines. Cell proliferation capability was examined by MTT assay. Statistical analyzes were applied to evaluate the correlation of PTOV1 expression with clinical parameters and prognosis. Results: The expression level of PTOV1 was markedly higher in cervical cancer tissues and cell lines than that in adjacent noncancerous tissues and the normal cervical epithelial cells. PTOV1 overexpression was correlated with higher tumor stage (P = 0.001), larger tumor size (P = 0.004), and lymph node involvement (P = 0.036). Moreover, patients with high PTOV1 expression showed shorter overall and recurrence-free survival time (P = 0.013 and P = 0.010, respectively). PTOV1 knockdown by short hairpin RNAi inhibited cancer cell growth in vitro. Conclusion: PTOV1 may be an important factor associated with proliferation of cervical cancer.

Keywords: PTOV1, cervical cancer, proliferation

Introduction

Cervical cancer is one of the most common gynecologic cancers worldwide, with more than 527,000 diagnosed new cases and 265,000 deaths every year [1]. Most of the cervical cancer patients have favorable prognosis if detected in early stage and treated appropriately. The 5-year overall survival rate of patients with stage IB1-IIA1 cervical carcinoma was more than 80% [2]. However, while tumor recurrence following primary surgery or radiotherapy accounts for only 10%-20% of all those cases without lymph node involvement, 70% of the cases with nodal metastasis will have recurrent diseases and shortened clinical survival [3]. Therefore, novel reliable biomarkers are needed to predict tumor metastasis and patients' outcome.

Prostate tumor overexpressed 1 (PTOV1) was first identified in prostate cancer [4]. The PTOV1 gene comprises 12 exons, and is located on a region of chromosome 19 (19q13), which encoded protein with two similar, tandemly-arranged domains [5]. PTOV1 is overexpressed both in prostate carcinomas and prostate intraepithelial neoplasia, while it is hardly detectable in normal prostatic epithelium, suggesting its potential value in the early diagnosis of prostate carcinoma [6]. Functional assays showed that overexpression of PTOV1 can promote the entry of cells into the S phase, and contribute to the proliferative status of prostate tumor cells [6]. Moreover, PTOV1 expression in metastatic primary prostatic tumors and metastatic lesions was higher than that in non-metastatic tumors [7]. In subsequent study, PTOV1 was found to promote c-Jun expression level and the invasiveness and metastasis of prostate cancer cells [8]. Recent studies indicated that PTOV1 may be of importance in the carcinogenesis of several cancers. However, its role in cervical cancer needs to be further elucidated.

In this study, we reported for the first time the characterization of PTOV1 expression in cervi-

cal cancer tissues, and its relationship with clinicopathologic parameters and prognosis in cervical cancer patients. Moreover, we evaluated the role of PTOV1 in cervical cancer cell proliferation. Taken together, our results suggest that PTOV1 plays a significant role in cervical cancer development and progression.

Materials and methods

Patients and tissue samples

A total of 110 cervical cancer tissues were collected from archival paraffin-embedded specimens at Guangzhou Women and Children's Medical Center between February 2005 and November 2009. Besides, 4 pairs of snap-frozen tissues, including cervical cancer and normal adjacent-noncancerous tissue specimens, were obtained for real-time PCR and Western blot. All the patients had been diagnosed and graded according to the principles laid down in the latest International Federation of Gynecology and Obstetrics criteria [9]. This study was performed according to the Declaration of Helsinki and was approved by the ethics committee of Guangzhou Women and Children's Medical Center. Informed written consent was obtained from all the participants.

Cell lines

Cervical cancer cell lines (Hela, ME-180, SiHa, C33A, CaSKi, MS751, and HT-3) were grown in RPMI-1640 medium (Gibco, NY) supplemented with 10% fetal bovine serum (HyClone, UT). Primary normal cervical epithelial cells (NCEC) were obtained from adjacent non-cancerous cervical tissue and cultured in keratinocyte serum-free medium (Invitrogen, CA) supplemented with epithelial growth factor, bovine pituitary extract and antibiotics (120 mg/ml streptomycin and 120 mg/ml penicillin).

RNA extraction and reverse transcription-PCR

Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and used for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using the ABI Prism 7900 system. This study used the following primers: PTOV1 F: 5'-CGAGTACAGGAGCATGAGCA-3', R: 5'-CTTCACCAACAGAGACTGCG-3'; a-catenin F: 5'-CAACCCTTGTAAACACCAAT-3', R: 5'-CCTTCTCCAAGAAATTCTCA-3'; Vimentin F: 5'-AGGAAATGGCTCGTCACCTTCGTGAATA-3', R: 5'-GGAGTGTCGGTTGTTAAGAACTAGAGCT-3'; Fibronectin F: 5'-TTATGACGACGGGAAGACCT-3', R: 5'-GCTGGATGGAAAGATTACTC-3'; Snail F: 5'-ACCACTATGCCGCGCTCTT-3', R: 5'-GGTCGTAGGGCTGCTGGAA-3'; Twist F: 5'-CGGGAGTCCGCAGTCTTA-3', R: 5'-TGAATCTTGCTCAGCTTGTC-3'; GAPDH F: 5'-GACTCATGACCACGTCCATGC-3', R: 5'-AGAGGCAGGGATGATGTTCTG-3'. PTOV1 expression data was normalized to GAPDH expression.

Western blotting

Total proteins from cells and tissue specimens were extracted and separated in SDS PAGE gels, and subjected to a Western blot analysis according to standard procedures, using monoclonal antibody against human PTOV1 (1:1000, Abcam, 5 g/ml in blocking buffer). Reactivity was detected with a chemiluminescent substrate (ECL, Amersham Biosciences). GAPDH was used as a loading control on the same membrane.

Immunohistochemistry

Paraffin-embedded cervical cancer tissue specimens were cut in 5 mm sections, which were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min, and 10% goat serum solution was used to avoid nonspecific staining. Then sections were incubated with anti-PTOV1 antibody (1:100 Abcam) for 1 h at room temperature, followed by incubation with peroxidase-labeled secondary antibody and streptavidin-horseradish peroxidase complex.

The PTOV1 immunoreactivity result was assessed by 2 independent observers blinded to the survival data. PTOV1 expression levels were classified semi-quantitatively combining the proportion of positively stained tumor cells and the intensity of staining. Staining intensity was scored as follows: 0: no staining; 1: weak staining; 2: positive staining; and 3: strong staining. The percentage of positive staining tumor cells was scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (11-50%% positive tumor cells); 3 (51-80% positive tumor cells); 4 (>80% positive tumor cells). The sum of the percentage score and the staining intensity score was used to define the PTOV1

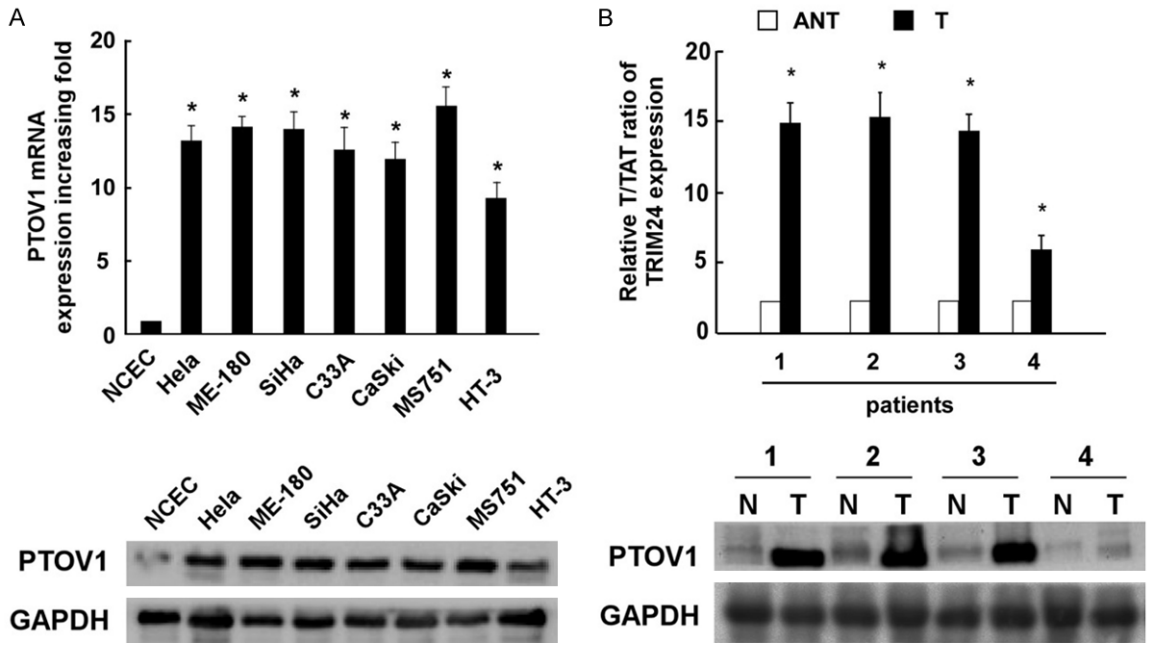

Figure 1. PTOV1 is over expressed in cervical cancer. A. Expression of PTOV1 mRNA and protein in normal cervical epithelial cells (NCECs) and 6 cervical cancer cell lines. B. Real-time PCR and Western blot analysis of PTOV1 expression in primary cancer tissues (T) and the matched adjacent non-cancerous tissues (ANT). *P<0.05.

Table 1. Correlation between PTOV1 expression and clinicopathologic parameters of in 110 cervical cancer patients

Parameters		N	PTOV1 expression		*p*
			High	Low	
Age (y)	≤50	45	28	17	0.814
	>50	65	39	26	
FIGO stage	IB1-IB2	48	21	27	0.001
	IIA1-IIA2	62	46	16	
Grade	1/2	72	46	26	0.378
	3	38	21	17	
Tumor size	≤4 cm	53	25	28	0.004
	>4 cm	57	42	15	
Histological type	SCC	70	45	25	0.337
	AC	40	22	18	
LN metastasis	No	89	50	39	0.036
	Yes	21	17	4	

SCC: squamous cell cancer; AC: Adenocarcinoma.

protein expression levels: scores <6 was defined as low expression and scores ≥6 was defined as high expression.

Small RNA transfection

The siRNA-mediated PTOV1 knockdown was performed by transfecting the synthetic siRNA duplexes for 24-96 h. The siRNA sequences were designed by RiboBio (RiboBio, Guangzhou, China). Cells were transfected with siRNA with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

MTT assay

Cell viability was measured using MTT assay. After PTOV1 knockdown, 2×10^3 Hela and Siha cells/well were seeded in 96-well plates. At each time point, 100 μl of MTT solution (0.5 mg/ml, Sigma) was added to each well to incubate for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μl of DMSO (Sigma). Absorbance was determined at 570 nm immediately. All experiments were performed in triplicates.

Statistical analysis

The statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, IL, USA). The correlation between PTOV1 expression and clinicopathologic features was evaluated by chi-square test. Survival curves were plotted using the Kaplan-Meier method and log-rank test. The Cox proportional hazard model was used for multivariate analysis of the prognostic factors for overall and recurrence-free survival. P<0.05 was considered to be statistically significant.

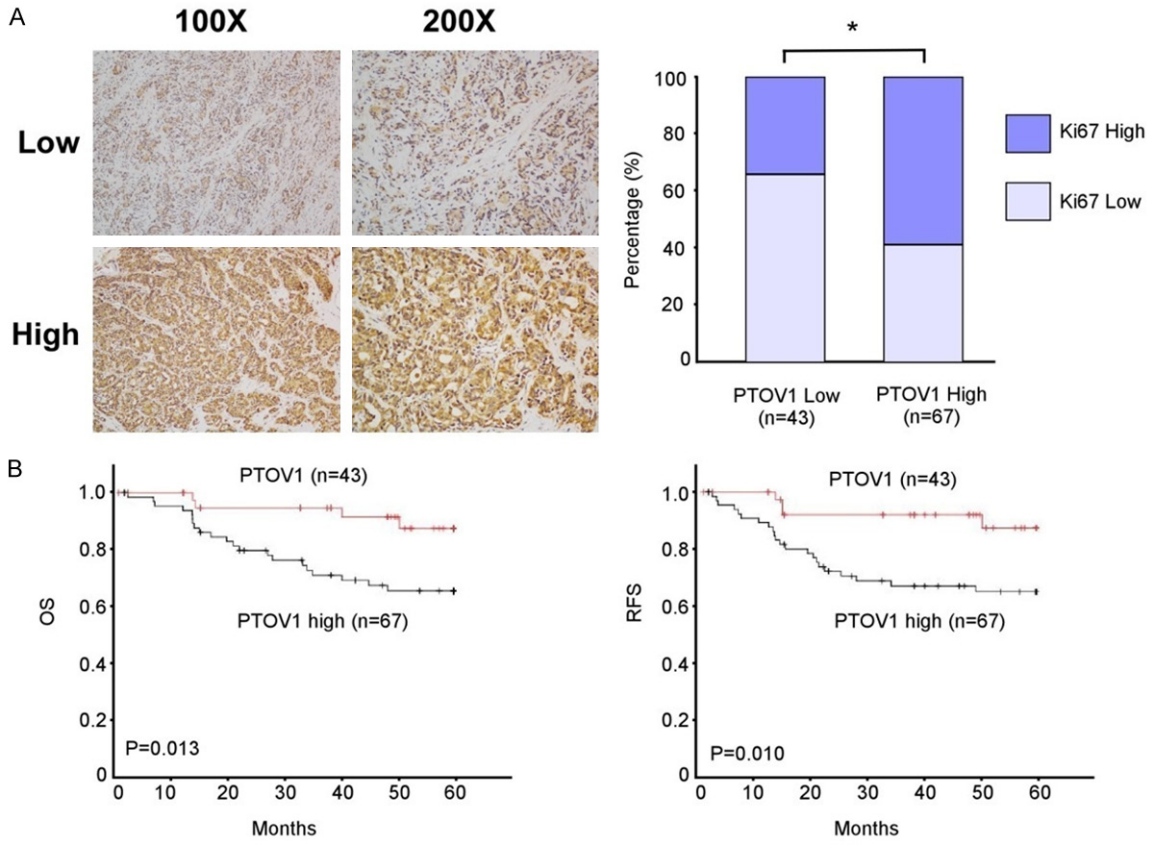

Figure 2. Expression levels of PTOV1 in archived paraffin-embedded cervical cancer. A. Left, Representative IHC staining images of PTOV1 expression levels in cervical cancer tissues; Right, correlations between PTOV1 and Ki67 expression based on immunostaining data. B. Kaplan-Meier survival curves for overall and recurrence-free survival of cervical cancer patients with high PTOV1 versus low PTOV1. *P<0.05.

Results

PTOV1 expression is upregulated in cervical cancer

To investigate the role of PTOV1 in the development of cervical cancer, we determined the expression levels of PTOV1 mRNA and protein by real time-PCR and Western blotting. These results indicated that PTOV1 is upregulated at both mRNA and protein levels in cervical cancer cell lines compared with those in NCEC (**Figure 1A**). Consistent with the finding, higher levels of PTOV1 expression was found in cervical cancer tissues in compare to that in adjacent noncancerous tissues (**Figure 1B**).

Association between PTOV1 expression and clinicopathological parameters

The association between PTOV1 expression and clinicopathologic parameters was assessed, as shown in **Table 1**. The results implied that high PTOV1 expression correlated with advanced tumor stage (P = 0.001), larger tumor size (P = 0.004), and lymph nodes metastasis (P = 0.036). However, no significant association was observed between PTOV1 expression and age, tumor grade, or histological type.

Association between PTOV1 expression and clinical prognosis

PTOV1 expression in 110 cervical cancer tissue specimens was determined by immunohistochemistry. The representative immunostaining profiles of PTOV1 were shown in **Figure 2A**. Kaplan-Meier analysis and log-rank test indicated that patients with high recurrence-free survival expression had worse overall and recurrence-free survival than patients with low PTOV1 expression (P = 0.013 and P = 0.010, respectively; **Figure 2B**). Multivariate Cox analysis showed that low PTOV1 expression of is an

Table 2. Multivariate cox regression analysis of OS and RFS

Prognostic variables	OS		RFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (>50 vs ≤50)				
FIGO Stage (IIA1-IIA2 vs IB1-IB2)	2.965 (0. 627-6.547)	0.021		
Grade (3 vs 1/2)				
Tumor size (>4 cm vs ≤4 cm)	2.784 (0.254-5.137)	0.025		
Histological type (SCC vs AC)				
LN metastasis (+ vs -)			2.463 (0.943-7.297)	0.029
PTOV1 expression (high vs low)	2.581 (0.365-7.735)	0.007	2. 251 (0.136-8.127)	0.009

SCC: squamous cell cancer; AC: Adenocarcinoma.

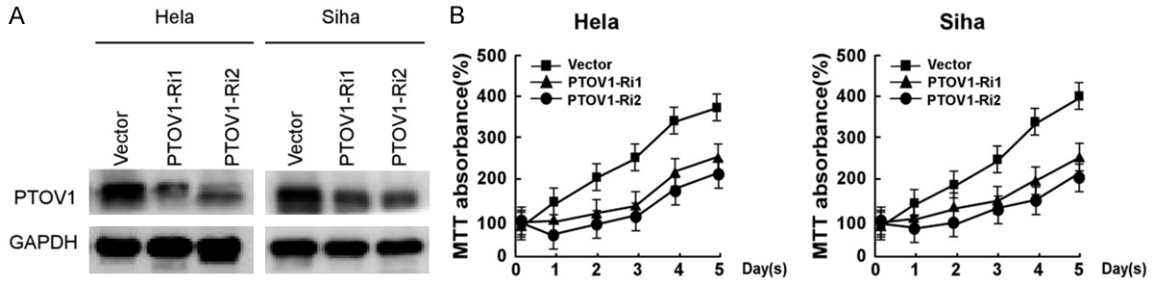

Figure 3. Depletion of PTOV1 inhibits cervical cancer cell proliferation. A. Western blotting analysis of PTOV1 expression in vector and in cervical cancer cell lines stably silencing PTOV1. B. Inhibition of PTOV1 reduces growth rate of cervical cancer cells, determined by MTT assay. *P<0.05.

independent prognostic factor for both overall and recurrence-free survival of cervical cancer patients (P = 0.007 and P = 0.009, respectively; **Table 2**).

PTOV1 knockdown inhibits cervical cancer cells proliferation

To assess the biological roles of PTOV1 in cervical cancer cells, we knocked down the expression of PTOV1 using its specific siRNA(s) in Hela and Siha cell lines (**Figure 3A**), and measure the cell viability by MTT assay. The results showed that the decreased PTOV1 expression significantly inhibited proliferation of both Hela and Siha cells (**Figure 3B**), indicating that PTOV1 could promote the proliferation of cervical cancer cells.

Discussion

Cervical carcinoma is one of the most common cancers worldwide. It is highly preventable and curable if detected early, but the death rate among patients with advanced stage disease is still high, due in large part to uncontrolled cancer metastasis and recurrence [10]. Currently, there is lack of reliable biomarkers to predict cervical cancer metastasis and patients' outcome. In the current study, we examined the expression level of PTOV1 in cervical cancer and then investigated its clinical significance. Moreover, we evaluated its role in tumorigenic process and progression of cervical cancer by in vitro assays. These data provided evidence that PTOV1 might contribute to the progression of cervical cancer.

PTOV1 was originally identified as a novel gene that was part of the abnormal transcriptional repertoire in the early stages of the development of prostate cancer [4]. PTOV1 may interact with the lipid-raft associated protein flotillin-1 in its nuclear translocation and promote cell proliferation [11]. Additionally, PTOV1 was shown to differentially modulate retinoic acid (RA) sensitivity in cancer cells depending on their expression levels [12]. It could also cooperate with Zyxin for the negative regulation of RA signaling [13], implying a potential molecular mechanism underlying RA resistance. Recent studies suggested that PTOV1 functions as an oncogene in several human cancers, including bladder, ovary, endometrium,

lung and kidney cancer [14]. However, the clinical significance of PTOV1 in cervical cancer has not been classified. The present study demonstrated that PTOV1 expression is elevated in cervical cancer tissue specimens and cell lines compared with adjacent normal cervical tissues and normal cervical epithelial cells at both the mRNA and protein levels, respectively. Furthermore, we analyzed 110 archived cervical cancer samples and found that PTOV1 expression is positively correlated with tumor stage, tumor size, and lymph nodes metastasis. Consistently, elevated PTOV1 expression is closely correlated with high Ki67 immunoreactivity. The results suggested a functional relationship between PTOV1 overexpression and cervical cancer progression.

The prognostic role of PTOV1 has been investigated in human cancers. Lei et al. revealed that the expression of PTOV1 was positively associated with aggressiveness and tumor progression of breast cancer [15]. Guo et al. reported that a high expression level of PTOV1 was significantly associated with poor prognosis of epithelial ovarian cancer patients [16]. Yang et al. revealed that PTOV1 was an independent prognostic indicator of overall survival and progression-free survival for laryngeal squamous cell carcinoma [17]. In contrast, Rausch et al. found PTOV1 nuclear localization was frequent in invasive urothelial carcinoma tissue, while the expression pattern of PTOV1 did not show correlation to survival data [18]. Our results showed that patients with PTOV1 overexpression have a significant worse overall survival and recurrence-free survival than that with low PTOV1 expression. Together, these studies indicate that PTOV1 may perform different functions in different cancers, and that the prognostic role of PTOV1 might be diverse in different cancers.

To further investigate the effect of PTOV1 on the biological behavior of cervical cancer cells, we downregulated PTOV1 expression via RNAi in Hela and Siha cells. The results demonstrated that PTOV1-shRNA effectively suppressed the proliferation of the cancer cells. Although studies have unraveled the significance of PTOV1 in a several human cancers, this is the first study to show that PTOV1 could contribute to the proliferative status and progression of cervical cancer.

In conclusion, we reported for the first time that PTOV1 expression was increased in cervical tissues and high PTOV1 expression was associated with tumor progression, metastasis, and poor prognosis in cervical cancer patients. Moreover, downregulation of PTOV1 expression suppresses the malignant biological behavior of cervical cancer cells. However, more detailed molecular mechanisms of PTOV1 promoted cervical cancer progression still require further investigation.

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Disclosure of conflict of interest

None.

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