Int J Clin Exp Pathol 2017;10(4):4522-4529
www.ijcep.com /ISSN:1936-2625/IJCEP0048558

Original Article

Long non-coding RNA PVT1 facilitates cervical cancer progression through negative modulation of miR-128-3p

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Received January 10, 2017; Accepted March 9, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Long non-coding RNAs (lncRNAs) play critical roles in tumor progression, including in cervical cancer. However, the potential biological roles and regulatory mechanisms of lncRNA PVT1 in cervical cancer are still unclear. In the present study, we showed that lncRNA PVT1 was significantly upregulated in cervical cancer tissues. Increased expression of lncRNA PVT1 was closely associated with tumor progression and poor overall survival in patients with cervical cancer. In vitro assays, we showed that lncRNA PVT1 reduction remarkably inhibited cervical cancer cell proliferation, migration and invasion. In addition, bioinformatics analysis and luciferase reporter assay indicated that lncRNA PVT1 was directly bound to miR-128-3p, which has been reported to act as a tumor suppressor in diverse cancers. Furthermore, we found that the oncogenic effects of lncRNA PVT1 in cervical cancer was at least partly by negative modulation of miR-128-3p. Taking together, we demonstrated that lncRNA PVT1 could function as an oncogenic lncRNA in cervical cancer progression, which could be an important therapeutic target in the treatment of cervical cancer.

Keywords: Long non-coding RNAs, PVT1, cervical cancer, miR-128-3p, progression

Introduction

Cervical cancer is the third most common cancer and the fourth leading cause of malignancy related mortality in women worldwide [1]. Despite scientific efforts and systemic therapy for cervical cancer in recent years, the overall survival rates is still poor following cancer progression to an advanced stage or recurrence [2, 3]. Cervical cancer formation is the result of multiple factors involving many kinds of oncogene activation and anti-oncogene inactivation [4]. Thus, recent studies were focused on resolving the molecular mechanisms in the progression of cervical cancer.

Long non-coding RNAs (lncRNAs), which represent a subgroup of non-coding RNAs that are longer than 200 nucleotides without protein coding functions [5, 6]. LncRNAs have been implicated in the regulation of a variety of cellular functions, disease processes and cancer progression [7]. Recent studies showed that lncRNAs might function as a competing endogenous RNA (ceRNA) or a molecular sponge in modulating miRNA [8]. For example, Liu et al found that lncRNA GAS5 acted as a molecular sponge to regulate miR-23a in gastric cancer [9]. Lv et al suggested that lncRNA Unigene 56159 promoted epithelial-mesenchymal transition by acting as a ceRNA of miR-140-5p in hepatocellular carcinoma cells [10]. Wang et al showed that lncRNA Malat1 promoted gallbladder cancer development by acting as a molecular sponge to regulate miR-206 [11].

PVT1, which maps to chromosome 8q24, encodes a long non-coding RNA. PVT1 was originally identified as a common retroviral integration site in murine leukemia virus (MLV)-induced T lymphomas [12]. Previous study showed that PVT1 dysregulation promoted tumorigenesis in a variety of human cancer. For example, Huang et al found that lncRNA PVT1 overexpression was a poor prognostic biomarker and regulated migration and invasion in small cell lung cancer [13]. Zheng et al showed that lncRNA PVT1 promoted invasion by inducing epithelial-to-mesenchymal transition in esophageal cancer [14]. Liu et al indicated that lncRNA PVT1 regulated

Table 1. Clinicopathological features associated with lncRNA PVT1 expression in 87 cervical cancer patients

Clinicopathological features	Total	lncRNA PVT1 expression		*P value*
		Low	High	
Age				
<45	37	18	19	0.901
≥45	50	25	25	
Tumor size (cm)				
<4.0	38	21	17	0.338
≥4.0	49	22	27	
Histology				
Squamous	71	34	37	0.546
Adenocarcinoma	16	9	7	
FIGO stage				
Ib~IIa	35	22	13	0.040
IIb~IIIa	52	21	31	
Lymph node metastasis				
No	43	29	14	0.001
Yes	44	14	30	
Depth of cervical invasion				
<2/3	45	28	17	0.013
≥2/3	42	15	27	

prostate cancer cell growth by inducing the methylation of miR-146a [15].

In the current study, our data showed that lncRNA PVT1 was increased in cervical cancer tissues, upregulated expression of lncRNA PVT1 was closely associated with tumor progression and shorter overall survival of patients with cervical cancer. In vitro assays showed that lncRNA PVT1 inhibition significantly suppressed cervical cancer cell proliferation, migration and invasion. Furthermore, subsequent mechanistic studies showed that lncRNA PVT1 exerted its function by competitively sponging and then inhibiting miR-128-3p. Therefore, these findings suggested that lncRNA PVT1 could serve as an important molecular marker for predicting prognosis and an important therapeutic target for the treatment of cervical cancer.

Materials and methods

Patients and specimens

Tissue samples were obtained from a total of 87 patients with cervical cancer who underwent radical resections at Department of Obstetrics and Gynecology, Huaihe Hospital of Henan University. None of the patients had received chemotherapy or radiotherapy prior to surgery. The detailed clinical pathological features in this study are summarized in **Table 1**. Tumor tissues were obtained and stored immediately in liquid nitrogen after surgical resection. All samples were confirmed by a senior pathologist and were staged according to the Federation International of Gynecology and Obstetrics (FIGO) staging system for cervical cancer. The study was performed with the approval of the Ethic and Research Committees of Huaihe Hospital of Henan University and was conducted in accordance with the Declaration of Helsinki Principles.

Cell culture and transfection

Human cervical cancer cell lines (HeLa, CaSki) were purchased from the American Type Culture Collection. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with the addition of 10% fetal bovine serum (FBS, Gibco) which was contained in a humidified atmosphere with 5% CO_2 at 37°C.

Small interfering RNA specifically targeting lncRNA PVT1 (si-PVT1) and a corresponding scrambled siRNA negative control (si-NC) were purchased from Shanghai GenePharma (Shanghai). miR-128-3p inhibitor, mimics and their parental negative control were prepared by RiboBio (Guangzhou). Cells were cultured until density in growth media reached 70% prior to transfection using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The knockdown efficiency at the mRNA level was assessed using qRT-PCR assay.

RNA extraction and quantitative realtime-PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was reversed transcribed into cDNAs using the Primer-Script one step RT-PCR kit (TaKaRa). The cDNA template was amplified by real-time RT-PCR using the SYBR Premix Dimmer Eraser kit (TaKaRa). Gene expression in each sample was normalized to GADPH expression. The primer sequences used were as follows: PVT1-forward 5'-TGAGAACTGTCCTTACGTGACC-3 and PVT1-reverse 5'-AGAGCACCAAGACTGGCTCT-3'; GAPDH-forward 5'-GTCAACGGATTTGGTCTGTATT-3' and GA-

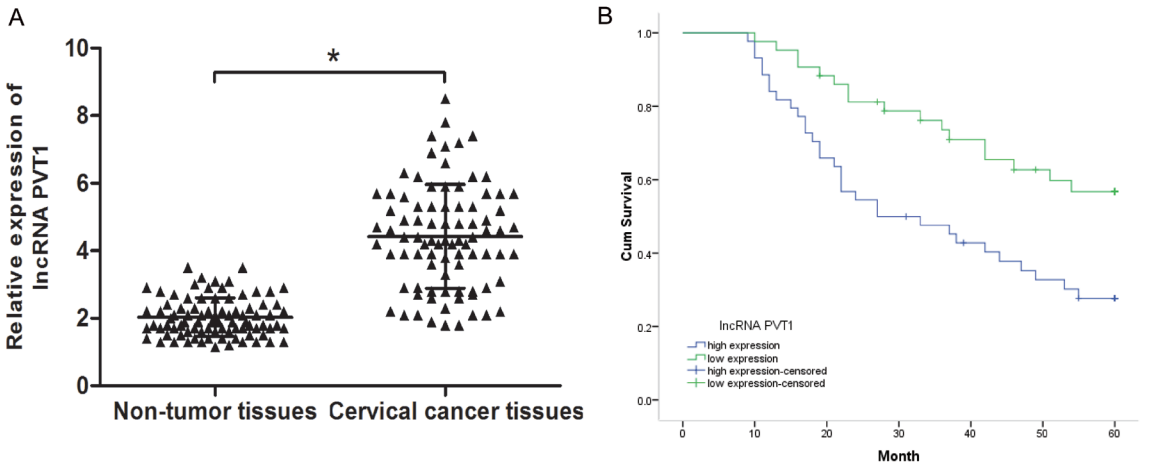

Figure 1. Relative expression of lncRNA PVT1 in cervical cancer and its clinical significance. A. Relative expression of PVT1 in cervical cancer tissues and adjacent non-tumor tissues was analyzed by qRT-PCR. B. Kaplan-Meier analysis revealed that high expression of PVT1 was associated with a poor overall survival of patients with cervical cancer (long-rank test: $P<0.05$).

PDH-reverse 5'-AGTCTTCTGGGTGGCAGTGAT-3'. Real-time PCR reactions were performed by the ABI7500 system (Applied Biosystems). The relative expression fold change of mRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation was assayed by the Cell Counting Kit-8 (CCK-8) assay (Dojindo) according to the manufacturer's protocol. A total of approximately 5000 transfected cells were plated in 96-well plates in triplicate and cultured in 90 μl medium containing 10% FBS. Then 10 μl CCK-8 solution was added to each well and incubated for 2 h at 37°C at each indicated time points. Absorbance was then recorded at 450 nm using Multifunctional microplate reader SpectraMax M5 (Molecular Devise).

Transwell migration and invasion assays

Briefly, 24-well transwell chambers (Costar) with uncoated or Matrigel-coated membranes were used for migration and invasion assays in this study, respectively. The post-transfected cervical cancer cells were seeded on the upper chamber which contained the serum-free DMEM medium, while the DMEM medium combined with 10% FBS which acted as chemoattractant was supplemented to the lower chamber. After incubation for 24 h (migration assay) or 48 h (invasion assay), cells that did not migrate across the upper surface were removed using a cotton swab, while cells that adhered to the lower surface of the inserts were stained with crystal violet of 0.1% for 20 min. Finally, the complete filters were washed in water twice before being observed.

Dual-luciferase reporter assay

The assay was performed as previously described [18]. Briefly, the wild-type (wt) and mutant (mut) miR-128-3p binding site in the 3'-UTR of PVT1 were synthesized and subcloned into the pGL3 Basic vector (Promega). For reporter assays, pLUC-wt-PVT1 or pLUC-mut-PVT1 were transiently transfected into HeLa cells, together with miR-128-3p mimics or miR-NC using Lipofectamine 2000. After incubation for 48 h, Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega). The renilla luciferase readings were normalized to the firefly luciferase activity in the corresponding well.

Statistical analysis

All statistical analysis of this research was carried out using SPSS 17.0 statistical software. Data are presented as mean ± SD (standard deviation). The results were analyzed using ANOVA or a two-tailed Student's t test. $P<0.05$ was considered to be significant.

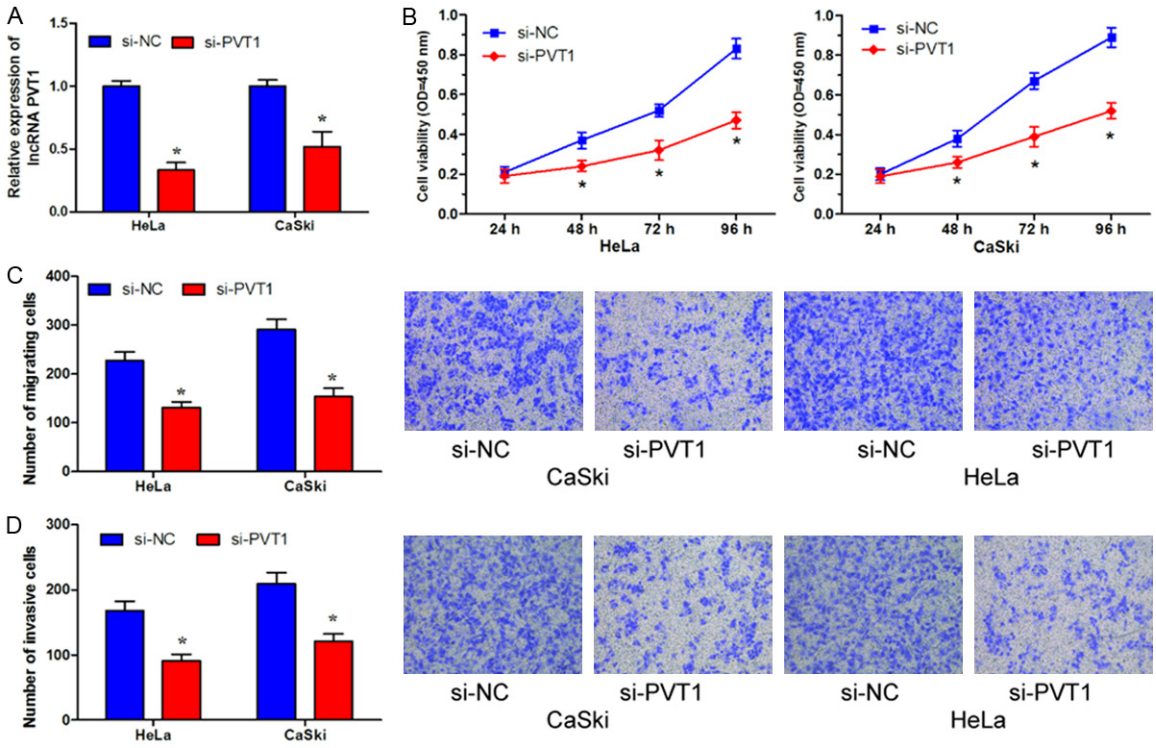

Figure 2. LncRNA PVT1 knockdown suppresses cell proliferation, migration and invasion of cervical cancer cells. (A) Relative expression of PVT1 in cervical cancer cells was decreased by si-PVT1 compared to si-NC group. (B) CCK-8 assay revealed PVT1 inhibition suppressed cell proliferation of HeLa and CaSki cells. Migration (C) and invasion (D) cell numbers of PVT1 suppressing HeLa and CaSki cells were less than cells transfected with si-NC. *P<0.05.

Results

LncRNA PVT1 was upregulated in cervical cancer

To determine the function of lncRNA PVT1 in cervical cancer, we first explored the expression level of PVT1 in cervical cancer tissues by qRT-PCR. We found that PVT1 expression was significantly elevated in cervical cancer tissues compared to adjacent non-tumor tissues (**Figure 1A**; P<0.05). Next, we explore the relationship between clinicopathological features and PVT1 expression in patients with cervical cancer. Our data showed that PVT1 expression levels were significantly positively correlated with advanced FIGO stage, lymph node metastasis and depth of cervical invasion. However, there was no significant association between PVT1 expression levels and other clinical factors, such as age, tumor size, and histology (**Table 1**). We then asked whether PVT1 expression was associated with cervical cancer patients' overall survival. Kaplan-Meier survival curves showed that the overall survival of patients with high PVT1 expression was significantly poor than patients with low PVT1 expression (**Figure 1B**; P<0.05).

LncRNA PVT1 inhibition suppressed cervical cancer cell proliferation and invasion

To investigate the potential biological function of lncRNA PVT1 in cervical cancer progression. We decreased PVT1 expression in cervical cancer cells by transfection PVT1 siRNA. As shown in **Figure 2A**, HeLa and CaSki cells transfected with si-PVT1 presented an obviously decreased mRNA expression level of PVT1 compared with the si-NC group in both cells (P<0.05). CCK-8 assay revealed that the PVT1 inhibition significantly reduced the proliferation rate of HeLa and CaSki cells compared with the si-NC group (**Figure 2B**; P<0.05). To determine whether PVT1 increased migration and invasion in cervical cancer cells, we performed transwell migration and invasion assays. Transwell migration assay showed that the migration ability of si-PVT1 transfected HeLa and CaSki cells was significantly decreased compared to si-NC transfected cells (**Figure 2C**; P<0.05). Transwell invasion assay also showed that the invasion ability

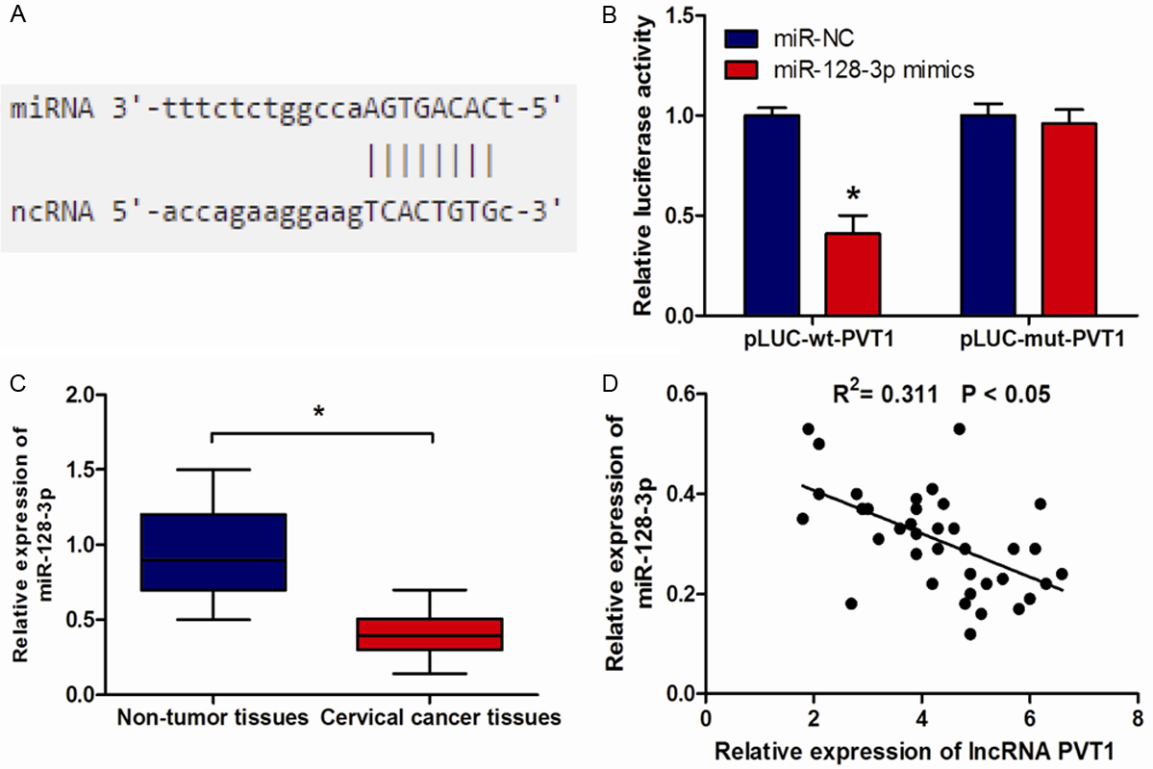

Figure 3. miR-128-3p is a target of lncRNA PVT1. A. Putative miR-128-3p binding sequence of PVT1 RNA. B. Co-transfection of miR-128-3p mimics and PVT1-wt reduced the luciferase activity. However, co-transfection of miR-NC and PVT1-wt or miR-128-3p and PVT1-mut did not change the luciferase activity. C. Relative expression of miR-128-3p in cervical cancer tissues and adjacent non-tumor tissues was analyzed by qRT-PCR. D. The correlation analysis was performed between PVT1 expression and miR-128-3p expression in cervical cancer tissues. *P<0.05.

of si-PVT1 transfected HeLa and CaSki cells was significantly decreased compared to si-NC transfected cells (**Figure 2D**; P<0.05). These data suggested that knockdown of PVT1 exerted tumor suppressive effects in human cervical cancer progression.

Identification of miR-128-3p as a target of PVT1

Previous studies have shown that lncRNAs function as ceRNA or "molecular sponges" to modulate miRNAs [16]. As expected, bioinformatics tools (starBase 2.0) for the predicted lncRNA-miRNA interactions revealed potential binding domains within PVT1 and miR-128-3p (**Figure 3A**). To further investigate whether PVT1 was a functional target of miR-128-3p, dual-luciferase reporter assay was performed. Our data showed that the luciferase activity was significantly decreased by the co-transfection of miR-128-3p mimics and pLUC-wt-PVT1 rather than the co-transfection of miR-NC and pLUC-wt-PVT1, suggesting that PVT1 was the target of miR-128-3p. Meanwhile, co-transfection of miR-128-3p and pLUC-mut-PVT1 did not change the luciferase activity, suggesting that the miR-128-3p binding site within PVT1 was functional (**Figure 3B**; P<0.05). In addition, we determined the expression miR-128-3p in 38 cervical cancer tissues by qRT-PCR. We found that miR-128-3p was decreased in cervical cancer tissues compared to adjacent non-tumor tissues (**Figure 3C**; P<0.05). Furthermore, our data showed that the expression of miR-128-3p was negatively correlated with the expression of PVT1 in cervical cancer tissues (**Figure 3D**; P<0.05). These data strongly suggested that miR-128-3p could directly bind to PVT1 at the miRNA recognition site in cervical cancer.

The oncogenic function of PVT1 in cervical cancer was dependent on miR-128-3p

To determine whether the tumor-suppressive effects of PVT1 knockdown were mediated by miR-128-3p, we co-transfected miR-128-3p inhibitors and si-PVT1 to the assessment of relative miR-128-3p expression, cell proliferation,

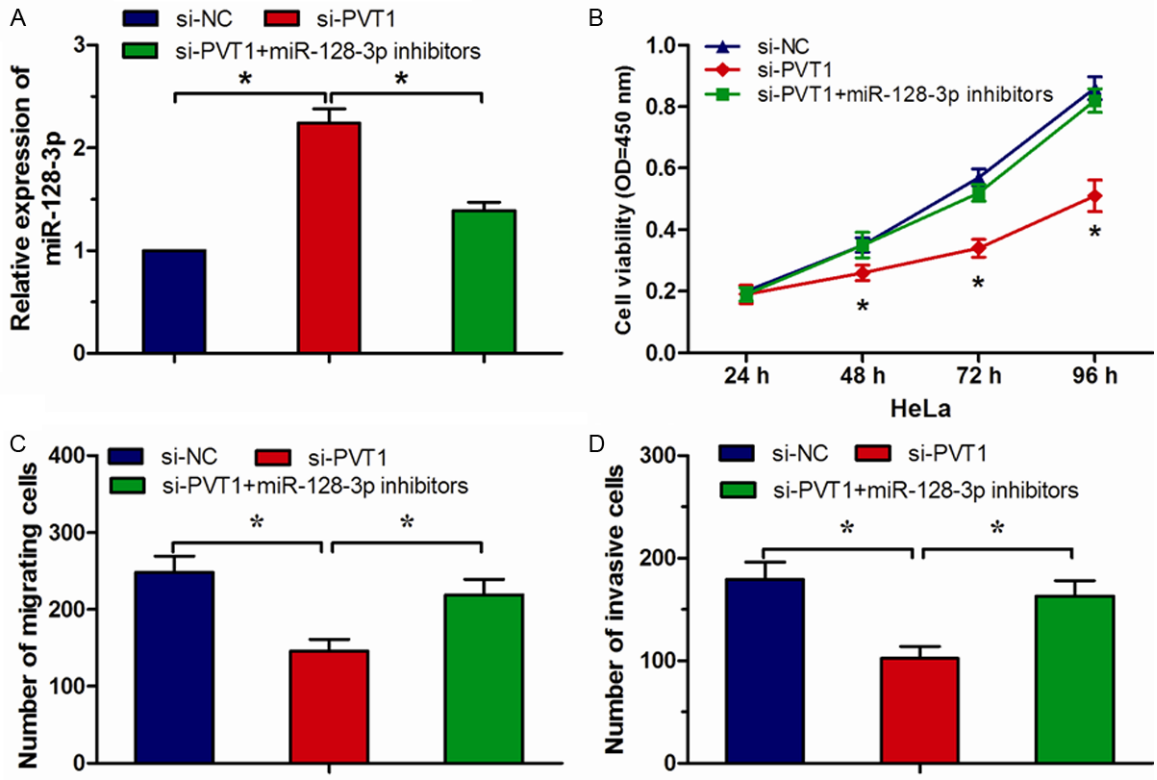

Figure 4. The tumor-suppressive effects of PVT1 knockdown in cervical cancer cells was partially reversed by miR-128-3p inhibitor. (A) Relative expression of miR-128-3p in HeLa cells, which were transfected with si-PVT1 in the presence of miR-128-3p inhibitor or si-NC. (B) CCK-8 assay was used to detect the proliferation ability of HeLa cells transfected with si-PVT1 in the presence of miR-128-3p inhibitor or si-NC. (C, D) Transwell migration and invasion assay were used to determine the migration (C) and invasion (D) capability of HeLa cells transfected with si-PVT1 in the presence of miR-128-3p inhibitor or si-NC. *P<0.05.

migration and invasion. As shown in **Figure 4A**, the expression level of miR-128-3p was significantly higher in si-PVT1 transfected HeLa cells compared to si-NC or si-PVT1+miR-128-3p inhibitors transfected HeLa cells (P<0.05). CCK-8 assay showed that HeLa cells co-transfected with miR-128-3p inhibitors rescued inhibitory effect of si-PVT1 on cell proliferation ability (**Figure 4B**; P<0.05), Moreover, transwell assay indicated that HeLa cells co-transfected with miR-128-3p inhibitors rescued cell migration and invasion ability induced by si-PVT1 (**Figure 4C** and **4D**; P<0.05). Based on the above results, we demonstrated that the tumor-suppressive effect of PVT1 knockdown in cervical cancer is at least partly through modulation of miR-128-3p.

Discussion

LncRNAs are non-coding RNAs that are important in tumor growth and metastasis, including cervical cancer. For example, Yang et al showed that lncRNA CCHE1 promoted cervical cancer cell proliferation via upregulating PCNA [17], Zhang et al indicated that increased expression of lncRNA ANRIL indicated a poor prognosis of cervical cancer and promoted cervical cancer growth and metastasis via PI3K/Akt pathways [18]. Sun et al showed that lncRNA EBIC promoted tumor cell invasion by binding to EZH2 and repressing E-cadherin in cervical cancer [19]. In the current study, we found that the expression of PVT1 was significantly upregulated in cervical cancer tissues. High expression of PVT1 was correlated with advanced FIGO stage, lymph node metastasis, depth of cervical invasion and poor overall survival of patients with cervical cancer. Moreover, in vitro assays, our data showed that PVT1 inhibition significantly suppressed cervical cancer cell proliferation, migration and invasion. These findings indicated that PVT1 played an oncogenic role in cervical cancer progression.

Recently, studies showed that there is a novel regulatory mechanism between lncRNAs and miRNAs [20]. LncRNAs might function as a ceRNA or a molecular sponge in modulating miRNA [16]. For example, Xie et al showed that lncRNA TUG1 contributed to tumorigenesis of human osteosarcoma by sponging miR-9-5p and regulating POU2F1 expression [21]. Luan et al suggested that lncRNA MALAT1 acted as a competing endogenous RNA to promote malignant melanoma growth and metastasis by sponging miR-22 [22]. Huang et al indicated that lncRNA CASC2 functioned as a competing endogenous RNA by sponging miR-18a in colorectal cancer [23]. In the present study, we explored whether miR-128-3p mediated the oncogenic roles of PVT1 in cervical cancer progression. Dual-luciferase reporter assay suggested that PVT1 was the target of miR-128-3p. In addition, qRT-PCR showed that miR-128-3p was decreased and negatively correlated with the expression of PVT1 in cervical cancer tissues. Furthermore, we found that PVT1 inhibition suppressed cervical cancer cell proliferation, migration and invasion ability, reduced expression of miR-128-3p reversed the effects that knockdown of PVT1 exerted. Therefore, we demonstrated that lncRNA PVT1 promoted cervical cancer cell growth and metastasis might at least partly by suppressing the expression of miR-128-3p.

In conclusion, our data showed that knockdown of PVT1 suppressed cervical cancer cell proliferation, migration and invasion by modulating miR-128-3p expression. On the basis of these findings, we demonstrated that lncRNA PVT1 might be used as a prognostic factor and a potential therapeutic target for the treatment of cervical cancer patients.

Acknowledgements

This study was supported by grants from the Key Research Projects of Institutions of Higher Education in Henan Province (No. 17A320023) and the Development of Social Science and Technology Research Projects of Kaifeng (No. 1603053).

Disclosure of conflict of interest

None.

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