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Original Article

LncRNA PVT1 overexpression is a poor prognostic biomarker and regulates migration and invasion in small cell lung cancer

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Abstract: PVT1 has been suggested as playing important roles in diverse biological processes including tumorigenesis. However, the clinical significance and biological function of PVT1 in small cell lung cancer (SCLC) is still unclear. The purpose of this study is to identify the role of PVT1 in SCLC. The expression of PVT1 was examined in SCLC tissues and cell lines through real-time PCR. Meanwhile, the relationship of PVT1 expression levels with clinical characteristics of 120 SCLC patients was analyzed. Univariate and multivariate analyses were performed to determine the association between PVT1 expression and prognosis of SCLC patient. The biological function of PVT1 on tumor cell growth and mobility were explored through MTT, colony formation, Transwell migration and invasion assays in vitro. In our results, PVT1 expression was markedly higher in SCLC tissues and cell lines than in normal lung tissues and normal bronchial epithelial cell lines (both $P<0.001$). High levels of PVT1 were positively associated with the status of clinical stage (Limited vs. Extensive, $P<0.001$), lymph node metastasis (No vs. Yes, $P<0.001$), and distant metastasis (No vs. Yes, $P<0.001$) in SCLC patients. Patients with higher PVT1 expression had a significantly poorer overall survival time than did patients with low PVT1 expression ($P<0.001$). Multivariate analysis showed that PVT1 overexpression was an independent prognostic indicator ($P=0.024$) for the survival of patients with SCLC. Knocking down PVT1 expression significantly inhibited the SCLC cell migration and invasion in vitro (both $P<0.001$), but has no effect on the growth of SCLC cells (both $P>0.05$). In conclusion, PVT1 could serve as a new biomarker and a potential therapeutic target for SCLC patients.

Keywords: PVT1, SCLC, lncRNA, biomarker

Introduction

The incidence and mortality of lung cancer are the highest in the malignant tumor in our country [1]. In the America, a total of estimated 221,200 new lung cancer patients and 158,040 lung cancer patient deaths occur in 2015 according to 2015 Cancer Statistics [2]. Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are main subtypes of lung cancer, and SCLC represents 15-20% of all lung cancer cases which is characterized by its aggressive nature and poor prognosis [3]. In the recent ten years, NSCLC has made great progress on the target therapy, such as EGFR-TKI (Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors) and ALK (Anaplastic lymphoma kinase) inhibition [4, 5]. Although the effective rate of front-line chemotherapy is acceptable, the 5-year survival of SCLC remains 15-25% for patients with limited stage [6, 7] and 7.8% for patients with extensive stage [8]. Thus, it is urgent to identify reliable prognostic biomarkers and develop targeted molecular therapies for SCLC.

Long non-coding RNAs (lncRNAs) are a group of non-protein-coding RNAs that regulate gene expression at the transcriptional or posttranscriptional level [9]. Benefiting from the fast

development of sequencing technique and bioinformatics methods, more and more new lncRNAs are discovered and identified as oncogene or anti-oncogene in lung cancer, such as MALAT1 [10-12], HOTAIR [13-15], H19 [16], MEG3 [17], and GAS6-AS1 [18].

PVT1, which maps to chromosome 8q24, encodes a long noncoding RNA. PVT1 was originally identified as a common retroviral integration site in murine leukemia virus (MLV)-induced T lymphomas [19]. Recently, PVT1 has been identified as a candidate oncogene. Increased copy number and overexpression of PVT1 have been found in many types of human cancers including ovarian cancer, breast cancer, hepatocellular carcinoma, bladder cancer and gastric cancer [20]. In NSCLC, PVT1 has been found significantly upregulated in NSCLC tissues and cell lines compared with normal lung tissues and cell line, and might serve as a promising biomarker for diagnosis and prognosis of NSCLC [21, 22]. However, the significance of PVT1 in SCLC is still unclear. The goal of our study was to identify the clinical significance and biological function of PVT in SCLC.

Materials and methods

Patients and samples

One hundred and twenty freshly-frozen SCLC samples and twenty paired adjacent normal gastric tissue samples were collected, and the pathological information was retrieved from the archives of the Pathology Department of Shandong Cancer Hospital Affiliated to Shandong University. The histopathological diagnosis of all samples was respectively diagnosed by two pathologists. Patients with complete clinical data who underwent any form of preoperative chemotherapy and/or radiation therapy were excluded. None of the patients enrolled in this study suffered from other cancers. TNM classification was determined by UICC/AJCC 7th edition for the lung [23]. The system treatments were performed according to NCCN guideline. Non-smokers were defined as patients who smoked less than 100 cigarettes in their lifetime, whereas smokers were those who smoked more than 100 cigarettes in their lifetime. Before the use of these clinical samples, prior consents from the patients and approval from the Institutional Ethics Committee of Shandong Cancer Hospital Affiliated to Shandong University were obtained.

Cell lines and cell cultures

Two SCLC cell lines (H446, H2227) and normal bronchial epithelial cell lines (16HBE, BEAS-2B) were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified CO_2 (5%) atmosphere.

Real-time PCR

Total RNA was extracted from clinical samples or cell lines with RNAiso Plus (Takara, Japan). The isolated total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara, Japan) for PVT1, according to manufacturer instructions. Relative expression was calculated via the comparative cycle threshold method and was normalized to the expression of GAPDH. The sequence-specific forward and reverse primers sequences for PVT1 were 5'-TGAGAACTGTCCTTACGTGACC-3 and 5'-AGAGCACCAAGACTGGCTCT-3' respectively. Forward and reverse primers sequences for GAPDH mRNA were 5'-TTGGTATCGTGGAAGGACTCA-3'; and the reverse primer was 5'-TGTCATCATATTTGGCAGGTT-3' respectively. The reactions were performed using SYBR Premix Ex TaqTM II (Takara, Japan) on a LightCycler (Roche, USA). Relative quantification was calculated by using the $2^{-\Delta\Delta}$Ct method. All qRT-PCR reactions were performed in triplicate.

Cell transfection

PVT1 siRNA (si-PVT1) and non-targeting siRNA (Control) were purchased from RiboBio (China) and used at 20 mM Opti-MEM transfection media (Invitrogen, USA) and Lipofectamine 2000 reagent (Invitrogen, USA) were used to transfect the cells once they reached 60% confluency. Knockdown was assessed by Real-time PCR after 48 hours of transfection.

Cell proliferation analysis

Cell proliferation was analyzed using MTT assay. Briefly, 1×10^3 cells were seeded into a 96-well plate with quadruplicate repeat for each condition. The cells were incubated for 1, 2, 3, and 4 days. Twenty microliters of MTT (5 mg/ml) (MP Biomedicals, USA) was added to each well and incubated for 4 h. At the end of incubation, the supernatants were removed and 150 μl of DMSO (MP Biomedicals, USA) was added to each well. The absorbance value

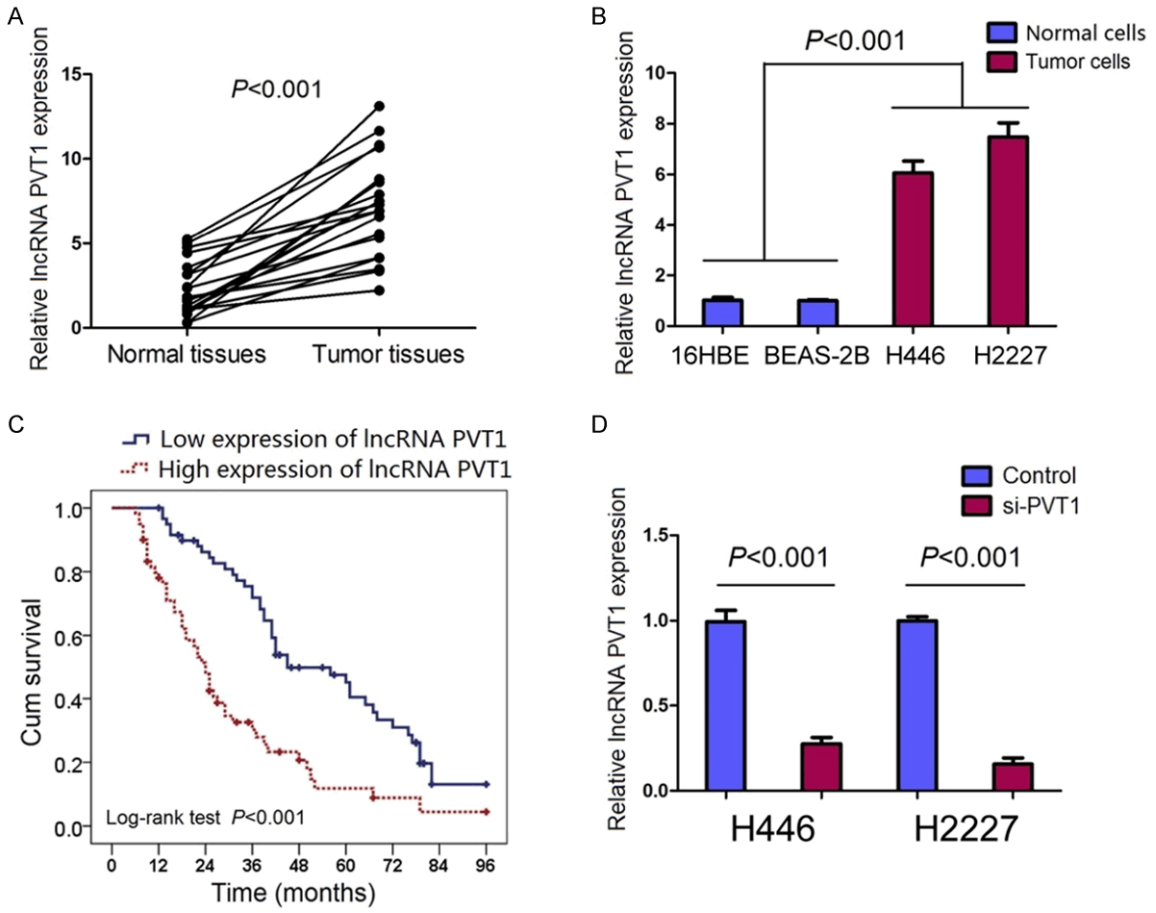

Figure 1. PVT1 expression is increased in SCLC tissues and cell lines, and associates with overall survival in SCLC patients. A. Expression of PVT1 is increased in SCLC tissues compared with paired normal lung tissues (P<0.001). B. PVT1 is overexpressed in SCLC cell lines compared with normal bronchial epithelial cell line (P<0.001). C. High expression of PVT1 predicts an unfavorable prognosis in SCLC patients. The relationship between PVT1 and SCLC patient survival was estimated using the Kaplan-Meier method and the log-rank test (P<0.001). D. PVT1 expression is decreased by small interfering RNA (si-PVT1) in H446 and H2227 cells.

(OD) of each well was measured at 490 nm. Experiments were performed three times.

Colony formation assay

Briefly, Cells (0.5×10^3) were plated into six well plates and cultured for ten days. Colonies were then fixed for 5 min with 10% formaldehyde and stained with 1.0% crystal violet for 30 s. The number of colonies containing ≥50 cells was counted under a microscope. Experiments were performed three times.

Cell migration and invasion assays

Briefly, 1×10^5 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Corning, USA). After the cells were incubated for 12 h, Giemsa-stained cells adhering to the lower surface were counted under a microscope in five predetermined fields (100×). For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were pre-coated with 24 mg/ml Matrigel (Corning, USA). Experiments were performed three times.

Statistical analysis

All data were analyzed for statistical significance using SPSS 18.0 software and GraphPad Prism 5. The chi-square test was applied to the examination of correlation between PVT1 expression and clinicopathological characteristics. Survival curves were plotted using the Kaplan-Meier method and the log-rank test. Cox regression was used for univariate analysis. The significance of survival variables

Table 1. Correlation between the clinicopathologic characteristics and expression of lncRNA PVT1 protein in SCLC

Characteristics	n	lncRNA PVT1 (%)		*P*
		High expression	Low expression	
Gender				
Female	46	26 (56.5)	20 (43.5)	0.260
Male	74	34 (45.9)	40 (54.1)	
Age (y)				
<50	47	21 (44.7)	26 (55.3)	0.350
≥50	73	39 (53.4)	34 (46.6)	
Smoking				
No	67	35 (52.2)	32 (47.8)	0.581
Yes	53	25 (47.2)	28 (52.8)	
Clinical stage				
Limited	57	14 (24.6)	43 (75.4)	<0.001
Extensive	63	46 (73.0)	17 (27.0)	
Tumor size				
<5 cm	72	33 (45.8)	39 (54.2)	0.264
>5 cm	48	27 (56.3)	21 (43.8)	
Lymph node metastasis		60	60	
No	60	15 (25.0)	45 (75.0)	<0.001
Yes	60	45 (75.0)	15 (25.0)	
Distant metastasis				
No	89	33 (37.1)	56 (62.9)	<0.001
Yes	31	27 (87.1)	4 (12.9)	

(*P*<0.05) in univariate analysis were included into the final multivariable Cox proportional hazards model. Two-tailed Student's t test was used for comparisons of two independent groups. One-way ANOVA was used to determine the differences between groups or all in vitro analyses. A *P*-value of less than 0.05 was considered statistically significant.

Results

PVT1 expression is increased in SCLC tissues and cell lines

We measured the expression levels of PVT1 in SCLC tissues and cell lines, and normal lung tissues and normal bronchial epithelial cell lines. Real time-PCR analysis showed that the PVT1 expression level was significantly increased in SCLC tissues compared with paired adjacent normal lung tissues (**Figure 1A**, *P*<0.001). Meanwhile, the expression of PVT1 was also elevated in SCLC cell lines (H446, H2227) compared with normal bronchial epithelial cell lines (16HBE, BEAS-2B) (**Figure 1B**, all *P*<0.001).

Overexpression of PVT1 is associated with malignant status in SCLC patients

We next analyzed the association between the expression of PVT1 and clinicopathological characteristics of SCLC patients. SCLC tissue samples were classified into the high expression group (n=60) and the low expression group (n=60) according to the median expression level of all SCLC samples. This classification was based on published study [24]. As summarized in **Table 1**, there were no significant associations between PVT1 expression and gender (Female vs. Male, *P*=0.260), age (<50 vs. ≥50, *P*=0.350), smoking (No vs. Yes, *P*=0.581) and tumor size (<5 cm vs. >5 cm). However, high expression of PVT1 was significantly associated with clinical stage (Limited vs. Extensive, *P*<0.001), lymph node metastasis (No vs. Yes, *P*<0.001), and distant metastasis (No vs. Yes, *P*< 0.001).

PVT1 expression is associated with overall survival in SCLC patients

In SCLC patients with prognosis information, we found that the level of PVT1 expression was significantly associated with the overall survival of SCLC patients, as patients with lower levels of PVT1 expression had better survival than those with higher levels of PVT1 expression (*P*<0.001, **Figure 1C**). Furthermore, we also found that high expression of PVT1 showed poor prognosis in SCLC patients (*P*<0.001, **Table 2**), regardless of clinical stage, lymph node metastasis, and distant metastasis. Multivariate cox regression analyses showed that high expression of PVT1 was a poor independent prognostic factor for SCLC patients (*P*=0.024, **Table 2**).

Decreased PVT1 expression has no effect on the growth of SCLC cells

We observed that PVT1 expression was relatively higher in H446 and H2227 SCLC cell lines than normal bronchial epithelial cell line (16HBE, BEAS-2B). Therefore, we chose H446 and H2227 cell lines for the following loss-of-function studies. To study the biological functions of PVT1 in SCLC lines, we induced down-regulation of PVT1 expression induced by siRNA in H446 and H2227, and these efficiencies were confirmed by qRT-qPCR (**Figure 1D**).

Table 2. Summary of univariate and multivariate Cox regression analyses of overall survival duration

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Gender (Female vs. Male)	0.572	0.884	0.576-1.356			
Age (years) (<50 vs. ≥50)	0.642	0.904	0.592-1.382			
Smoking (No vs. Yes)	0.972	0.993	0.649-1.518			
Clinical stage (Limited vs. Extensive)	<0.001	2.302	1.487-3.563	0.336	0.497	0.120-2.060
Tumor size (<5 cm vs. >5 cm)	0.078	1.469	0.957-2.255			
Lymph node metastasis (No vs. Yes)	<0.001	3.085	1.930-4.931	0.200	2.581	0.594-12.098
Distant metastasis (No vs. Yes)	<0.001	6.156	3.586-10.568	<0.001	3.988	2.091-7.606
LncRNA PVT1 (Low vs. High)	<0.001	2.634	1.712-4.053	0.024	1.782	1.078-2.945

HR, hazard ratio; 95% CI, 95% confidence interval.

Subsequently, we explored the effect of decreased PVT1 expression on SCLC cell growth in vitro. The growth curves detected by MTT assay showed that Knocking down PVT1 has no effect on SCLC cell viability (**Figure 2A**, *P*>0.05). Futhermore, the results of MTT assay were also consistent with clonogenicity tests as suppressing PVT1 has no effect on the number of colonies compared to Control group over a ten days period (**Figure 2B**, *P*>0.05).

Knock-down of PVT1 suppresses SCLC cells migration and invasion

To examine the effect of PVT1 on cell migration, After 24 hours of transfection, the number of migrated cells in both si-PVT1 SCLC cells groups were significantly less than that in the Control SCLC cells (for both *P*<0.001, **Figure 3A**). Using a boyden chamber coated with matrigel, we determined changes in cell invasiveness after 24 h hours of transfection. Compared with the Control SCLC cells, si-PVT1 SCLC cells showed significantly decreased invasiveness (for both *P*<0.001, **Figure 3B**).

Discussion

Long noncoding RNAs (lncRNAs) are broadly defined as RNA longer than 200 nucleotides lacking extended open reading frames [25]. In mid-term of 1980s, the mouse plasmacytoma variant translocation gene (Pvt1) has been first discovered in mouse as frequently involved in a variant translocation in plasmacytomas [26, 27]. The human PVT1 gene is a long intergenic noncoding RNA (lincRNA) homologous to the mouse Pvt1 [20]. Recently, PVT1 has been suggested to play critical roles in tumor development and progression through regulating cell proliferation, metastasis, cell cycle, apoptosis, stemness, and drug resistance [20].

In past five years, high level expression of PVT1 was gradually suggested in several types of human cancer such as acute promyelocytic leukemia [28], hepatocellular carcinoma patients [29, 30], thyroid cancer [31], colorectal cancer [32], bladder cancer [33], gastric cancer [34], malignant pleural mesothelioma [35] and non-small cell lung cancer (NSCLC) [21, 22]. In NSCLC, Yang et al reported that the expression of PVT1 was significantly increased in NSCLC tissues and cell lines compared with corresponding adjacent normal tissues and normal bronchial epithelial cell line [21]. Meanwhile, Cui et al also found PVT1 expression was obviously elevated in NSCLC tissues and cell [22]. However, the expressive status of PVT1 in small cell lung cancer (SCLC) is still unknown. Our study first showed that the expressions of PVT1

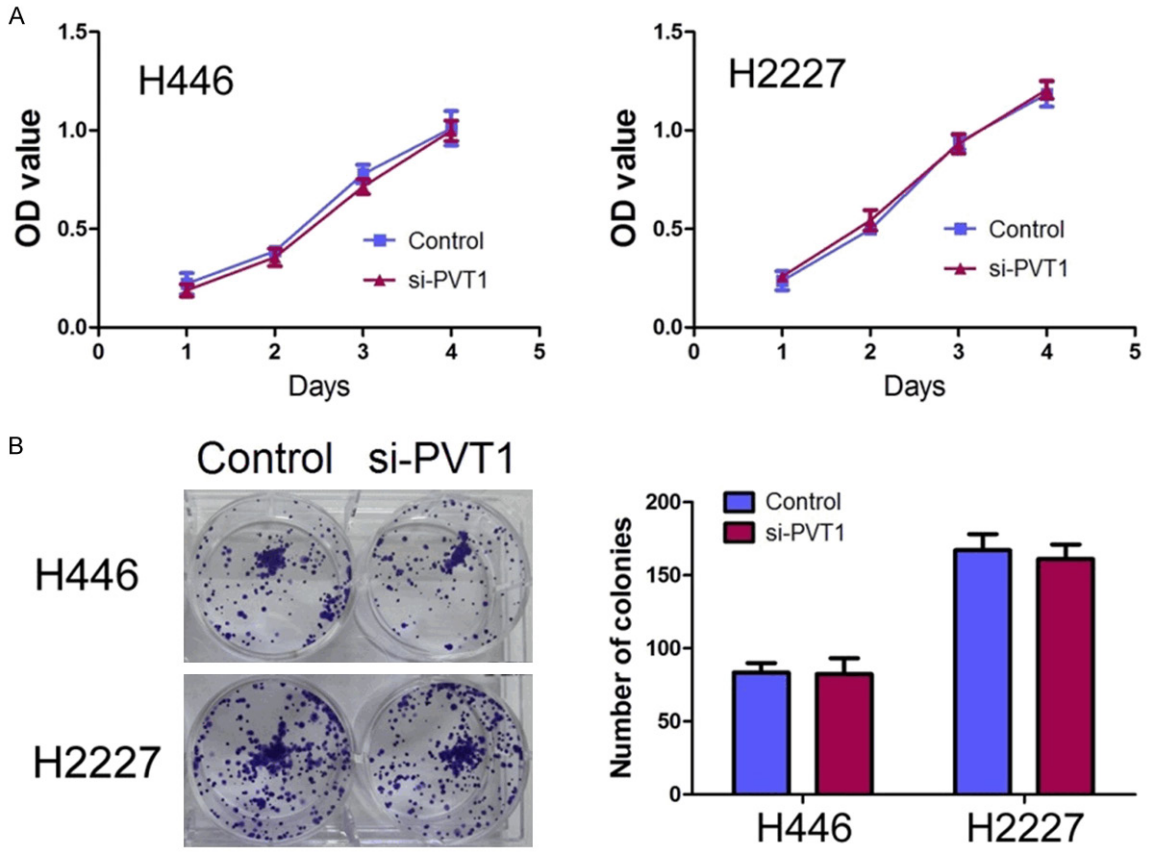

Figure 2. Decreased PVT1 expression has no effect on the growth of SCLC cells. A. In vitro viability of H446 and H2227 cells did not affected by si-PVT1 through MTT assay (both $P>0.05$). B. Knocking down PVT1 expression has no effect on the proliferative ability of H446 and H2227 cells (both $P>0.05$).

were obviously increased in SCLC tissues and cell lines compared with paired adjacent normal lung tissues and normal bronchial epithelial cell lines, which was coincident with the status of PVT1 in other cancers. In order to explore the clinical significance of PVT1 in SCLC patients, we measured the levels of PVT1 expression in 120 SCLC samples, and analyzed the correlation between the expression of PVT1 and clinicopathological characteristics. We found that high expression of PVT1 was significantly associated with clinical stage, lymph node metastasis, and distant metastasis. Similar results in NSCLC also been reported by Yang et al [21] and Cui et al [22], they showed that PVT1 overexpression was significantly correlated with clinical stage, histological grade, and lymph node metastasis. In colorectal cancer, Takahashi et al demonstrated that PVT-1 expression levels in cancerous tissues were significantly higher than those in non-cancerous tissues, and The high PVT-1 expression group showed greater lymph node metastasis and venous invasion compared with the low PVT-1 expression group [32]. Wang et al suggested that the levels of PVT1 were significantly up-regulated in hepatocellular carcinoma tissues compared with the corresponding non-cancerous hepatic tissues from the same patient, and high levels of PVT1 expression were associated with larger tumor size, HBV infection, and tumor stage [30]. These studies consistently implied that PVT1 overexpression may serve as a poor prognostic biomarker for cancer patients.

Recent years, PVT1 overexpression has identified as an independent predictor for overall survival in various human cancers, such as hepatocellular carcinoma [29, 30], colorectal cancer [32], gastric cancer [34], and NSCLC [21, 22]. However, the association of PVT1 expression with the overall survival in SCLC patients has been seldom reported. In our study, we first

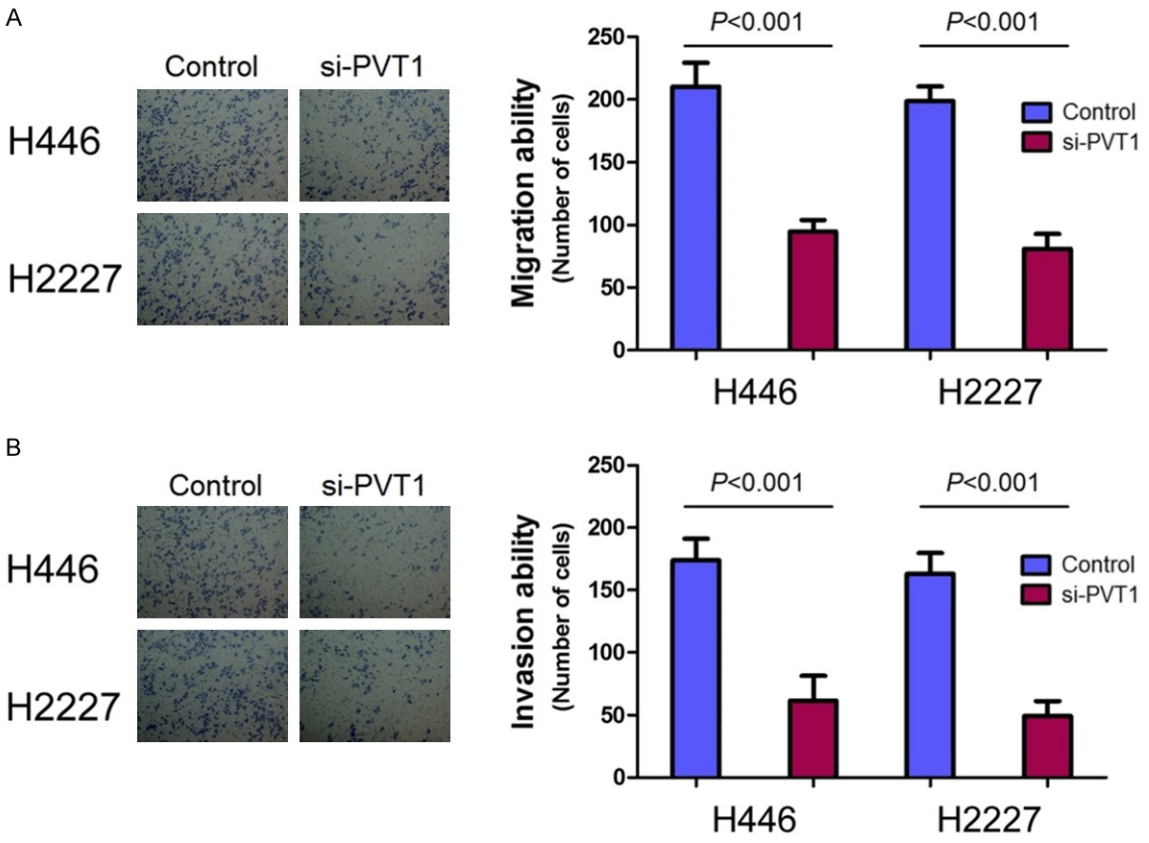

Figure 3. Knock-down of PVT1 suppresses SCLC cells migration and invasion. A. Down-regulated PVT1 expression dramatically decreased the ability of H446 and H2227 cells migration in vitro (both $P<0.001$). B. Suppressed PVT1 expression inhibited invasiveness of H446 and H2227 cells (both $P<0.001$).

showed that high expression of PVT1 obviously correlated with overall survival in SCLC patients. The SCLC patients with overexpression of PVT1 had poorer overall survival time. According to multivariate analysis, PVT1 overexpression was an independent poor prognostic factor for SCLC patients. Similarly, NSCLC patients with PVT1 overexpression have shown markedly shorter overall survival than those with low levels of PVT1 expression, and PVT1 expression was an independent prognostic factor for overall survival in a multivariate analysis [21, 22]. In gastric cancer, Kong et al indicated that PVT1 overexpression was obviously associated with deeper invasion depth and advanced TNM stage, and PVT1 expression served as an independent predictor for gastric cancer patient's overall survival [34]. Moreover, high levels of PVT1 expression in hepatocellular carcinoma could serve as a biomarker for predicting tumor recurrence in hepatocellular carcinoma patients [29, 30].

PVT1 has been suggested to paly important role in regulating cell proliferation, metastasis, cell cycle, apoptosis, stemness, and drug resistance [20]. Zeng et al showed PVT1 knockdown by RNA interference led to suppression of the MYC protein level, and cell proliferation was inhibited in acute promyelocytic leukemia [28]. In gastric cancer, Zhang et al found that PVT1 was overexpressed in gastric cancer tissues of cisplatin-resistant patients and cisplatin-resistant cell lines, and cisplatin-resistant cell lines transfected with PVT-1 siRNA and treated with cisplatin exhibited significant lower survival rate and high percentage of apoptotic tumor cells [34]. Similar results have been reported by You et al in pancreatic cancer [36] and Liu et al in ovarian cancer [37]. In addition, Wang et al's study suggested that PVT1 promotes cell growth, cell cycling, and the acquisition of stem cell-like properties in hepatocellular carcinoma cells by stabilizing NOP2 protein [30]. In NSCLC, knockdown of PVT1 significantly suppressed

cellular proliferation, cell cycle progression, migration and invasion [21, 22]. Interestingly, our study showed decreased PVT1 expression has no effect on the growth of SCLC cells, but effectively suppressed SCLC cell migration and invasion in vitro. The discrepancy between our data in SCLC and Yang et al's and Cui et al's data in NSCLC [21, 22] would be most likely due to the heterogenicity of lung cancer.

In conclusion, PVT1 was overexpressed in SCLC tissues and cell lines, and correlated with malignant status and poor prognosis of SCLC patients. Knockdown of PVT1 expression suppressed SCLC cell migration and invasion in vitro. PVT1 could serve as a new biomarker and a potential therapeutic target for SCLC patients.

Ethics statement

This study was approved by the Research Ethics Committee of Shandong Cancer Hospital Affiliated to Shandong University, Shandong Academy of Medical Sciences. The informed written consents were collected from all eligible patients and the entire study was performed based on the Declaration of Helsinki.

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Disclosure of conflict of interest

None.

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