# Amplification and the clinical significance of circulating cell-free DNA of PVT1 in breast cancer

XUJING LI[1], WEIJUAN CHEN[2], HUI WANG[3], QUN WEI[2], XIAODI DING[1] and WENTONG LI[1]

[1]Department of Pathology, Weifang Medical University, Weifang, Shandong 261053; [2]Department of Pathology, and [3]The Second Department of Oncology, People's Hospital of Shouguang City, Shouguang, Shandong 262700, P.R. China

Received December 5, 2016; Accepted May 12, 2017

DOI: 10.3892/or.2017.5650

**Abstract.** Duplication in the chromosome 8q24 region is a frequent occurrence in carcinomas. The PVT1 oncogene (PVT1), a long non-coding RNA, is found in this locus. PVT1 amplification is a frequent event in cancers, such as in lymphomas, serous ovarian, colorectal and breast cancers. Ectopic PVT1 expression is related with reduced survival duration in cancer patients. In the present study, we proved that PVT1 is markedly augmented in breast cancer tissues compared with adjacent non-tumorous tissues. Thus, PVT1 is an independent prognostic factor for the survival duration of breast cancer patients. Furthermore, PVT1 is pivotal in regulating p21 expression. In addition, we detected PVT1 DNA in serum and found that circulating PVT1 DNA significantly increased in the serum of breast cancer patients. Compared with PVT1 RNA, DNA is the main form of the PVT1-derived segment. These relevant findings collectively demonstrate that PVT1 plays a pivotal role in breast cancer and is a possible target for novel breast cancer therapies. The detection of circulating PVT1 DNA fragments may be a convenient means to predict the prognosis of breast cancer patients.

## Introduction

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts with a base length of over 200 nucleotides; lncRNAs often lack a functional open reading frame (1). lncRNAs modulate the expression of protein-coding or non-protein-coding genes by adjusting *cis*-acting elements and *trans*-acting factors (2). lncRNAs also participate in the regulation of biological behavior, such as cell differentiation, development and carcinogenesis (3,4). Therefore, lncRNAs are potential biomarkers and therapeutic targets for malignant tumors, providing a comprehensive approach for exploring cancer pathogenesis and presenting an alternative therapeutic method for cancers (5).

Some lncRNAs have been recently identified as multi-functional gene regulating factors (6). PVT1 is located in the 8q24 region and likely functions as a highly conserved non-coding RNA in humans (7). The amplification of the 8q24 region is a common event in cancers; moreover, previous studies have related the ectopic expressions of local genes in this region with reduced survival period (8,9). Myc, an established oncogene, is mapped to 8q24. Myc amplification likely leads to the pathogenesis of cancers. PVT1 amplification in 8q24 is another frequent event in cancer pathophysiology and has been observed in colorectal cancers (10), lymphomas (11), serous ovarian and breast cancers (12). Moreover, PVT1 overexpression is related with the decreased survival duration of cancer patients.

Ectopic PVT1 expression is associated with various diseases, particularly malignant tumors, however, the definite function and intermolecular signal mechanism of PVT1 in breast cancer remains obscure. Furthermore, the detection of circulating lncRNA DNA provides valuable information for the treatment and prognosis of breast cancer patients. Therefore, utilizing circulating PVT1 DNA as a marker for breast cancer requires further study.

## Materials and methods

*Tissue samples and clinical data collection*. Fresh tumor tissue samples, normal adjacent tissue samples, blood sera and clinical data were gathered from 84 patients with invasive ductal breast cancer aged 30-79 (average age, 48.3 years) enrolled at the Affiliated Hospital of Weifang Medical University from July 2009 to July 2011. Negative control samples were collected from individuals with no breast cancer. Written informed consent was given by breast cancer patients and healthy individuals. The present study was approved by the Research and Ethics Committee of Weifang Medical University.

*Cell culture and transient transfection*. The human breast cancer cell lines MDA-MB-231, MDA-MB-468, SK-BR-3, MDA-MB-435, T47D, MCF-7 and immortalized normal human mammary epithelial cells MCF-10A were purchased from the Type Culture Collection of Chinese Academy

*Correspondence to:* Dr Wentong Li, Department of Pathology, Weifang Medical University, 7166# Baotong West Street, Weifang City, Shandong 261053, P.R. China
E-mail: liwentong11@163.com

*Key words:* lncRNA, PVT1, p21, proliferation, breast cancer

of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA). All cell lines were cultured at 37˚C and 5% $CO_2$.

MCF-7 and MDA-MB-231 cells were transfected with siRNA plasmids using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. Negative control siRNA and PVT-1-specific siRNAs were purchased from Cosmo Bio, Co., Ltd. (Tokyo, Japan), the siRNA target sequence for PVT1 are si-PVT1: sense, 5'-CCCAACAGGVAGGACAGCUUTT-3' and antisense, 5'-AAGCUGUCVCUCCUGUUGGGTT-3' si-PVT2: sense, 5'-GCUUGGAGGVCUGAGGAGUUTT-3' and antisense, 5'-AACUCCUCAGVCCUCCAAGCTT-3'. Plasmids were tranfected into breast cancer cells at a concentration of 50 nM. Cells were harvested at 48 h after transfection for further research.

*Detection of nucleic acid segments by qRT-PCR*. Total RNA was isolated from serum of breast cancer patients or breast cancer cells using TRIzol according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA), then reverse transcription was performed using TransScript reverse transcriptase (TransGen Biotech, Inc., Beijing, China) and random hexamer primers, then the supernatant was collected after instantaneous centrifugation. TIANamp Blood DNA kit (Tiangen Biotech, Co., Ltd., Beijing, China) was used to extracted DNA was from sera according to the manufacturer's instructions. Quantitative PCR was then implemented using SYBR-Green (Beyotime Institute of Biotechnology, Haimen, China) to measure the profiles of PVT1. Forward and reverse primers for PVT1 RNA were 5'-CCGACTCTTCCTG GTGAAGC-3' and 5'-GTATGGTCAGCTCAAGCCCA-3'; 5'-CCGACTCTTCCTGGTGAAGC-3' and 5'-CCACATCAT GGCTCCAAATCTG-3' for PVT1 DNA; 5'-TGAGCCGCGA CTGTGATG-3' and 5'-GTCTCGGTGACAAAGTCGAAG TT-3' for p21; 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GA AGATGGTGATGGGATTTC-3' for GAPDH; 5'-GTAAC CCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGT AGCG-3' for 18S rRNA.

*Cell proliferation assay and colony formation assay*. Breast cancer cells were plated at $5x10^3$/well in 96-well plates and cultured for 48 h, cell density was measured by the Cell Counting kit-8 (CCK-8; Beijing Solarbio Science and Technology, Co., Ltd., Beijing, China). The cell growth curves were drawn according to the absorbance at 595 nm. For colony formation assay, 200 cells were seeded into 10 cm plates and cultured in DMEM contain 10% FBS. Seven days later, the colonies were fixed and stained by 0.1% crystal violet. The number of colonies >50 cells was counted. All experiments were performed in triplicate.

*Immunohistochemistry*. Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissue sections and incubated with a primary antibody against p21 (Fuzhou Maixin Biotech, Co., Ltd., Fuzhou, China), followed by a horseradish peroxidase-conjugated secondary antibody (1:500; Fuzhou Maixin Biotech) and the proteins to be tested were shown with 3,3-diaminobenzidine. Each sample is graded on a standard of 0-9 according to the staining densities (13). All slides were evaluated by two pathologists blinded to the information of the clinicopathological characteristics.

*Western blot analysis*. Total cell proteins were extracted in RIPA buffer; cell lysates were cleared by centrifugation and prepared in sodium dodecyl sulfate buffer. A total of 50 $\mu$l of cell lysates were separated by SDS-PAGE. The proteins were then transferred onto PVDF membranes. After incubation with primary antibodies for p21 or GAPDH (Fuzhou Maixin Biotech), the blots in horseradish peroxidase-conjugated goat anti-rabbit IgG for 60 min and enhanced chemiluminescence detection were used to quantify densitometry.

*Statistical analysis*. SPSS software version 17.0 was used to perform data analysis. One-way ANOVA was performed to detect the relationship between PVT1 and clinicopathological characteristics. Survival analysis was used for Kaplan-Meier examination. Receiver operating characteristic (ROC) curves were used to assess the diagnostic value of PVT1.

## Results

*The expression of PVT1 in breast cancer samples and cell lines*. Direct reverse transcription followed by qPCR was performed to determine PVT1 in breast cancer tissues and cell lines normalizing to 18S rRNA. The results showed that the profiles of PVT1 were remarkably increased in breast cancer tissues ($P<0.01$; Fig. 1A). Moreover, we also detect the profiles of PVT1 in breast cancer lines and mammary epithelial cell line MCF-10A, ectopic expression of PVT1 was detected in the six breast cancer cell lines compared with MCF-10A cells (Fig. 1B).

*Association of PVT1 expression with clinicopathological features*. To make clear the clinical relevance of PVT1 expression in breast cancer, according to the median expression of PVT1 in the breast cancer samples, we divided the 84 breast cancer patients into PVT1 high-expression group and PVT1 low-expression group in accordance with the median value of PVT1 expression. As shown in Table I and Fig. 2A and B, the PVT1 level was associated with cancer histological grade, expression of Ki-67, tumor size and lymph node metastasis. Whereas, no significant correlation was found between the expression of PVT1 and other clinicopathological characteristics of patients, for example, age, expression of ER, PR and Her-2 in the tissue.

*Prognostic values of PVT1 for breast cancer patients*. To explore the correlation between PVT1 expression and prognosis of breast cancer patients, the Kaplan-Meier test and log-rank test were carried out. The results demonstrated that breast cancer patients with high PVT1 expression showed significant shorter 5-year overall survival (OS) compared with patients with low PVT1 expression (Fig. 2C). Moreover, the 5-year disease-free survival (DFS) for high PVT1 group was 7.1%, while was 23.8% for low PVT1 group (Fig. 2D). A multivariate Cox proportional hazards regression analysis was

Table I. Clinical relevance of PVT1 in breast cancer.

| Features | PVT1 | | P-value |
|---|---|---|---|
| | Low | High | |
| Age (years) | | | 0.617 |
| ≤45 | 33 | 37 | |
| >45 | 44 | 42 | |
| Tumor size (cm) | | | **0.010** |
| ≤2 | 42 | 26 | |
| >2 | 37 | 53 | |
| Histological grade | | | **0.013** |
| I | 28 | 17 | |
| II, III | 51 | 62 | |
| Clinical stage | | | 0.061 |
| I, II | 57 | 47 | |
| III, IV | 22 | 34 | |
| Lymph nodes metastasis | | | 0.117 |
| Positive | 45 | 30 | |
| Negative | 34 | 49 | |
| ER | | | 0.143 |
| Positive | 63 | 55 | |
| Negative | 16 | 24 | |
| PR | | | 0.365 |
| Positive | 53 | 51 | |
| Negative | 26 | 18 | |
| HER-2 | | | 0.093 |
| Positive | 14 | 31 | |
| Negative | 65 | 48 | |
| Ki-67 | | | **0.022** |
| Positive | 14 | 31 | |
| Negative | 65 | 48 | |

P-value in bold indicates the statistical significance at P<0.05.

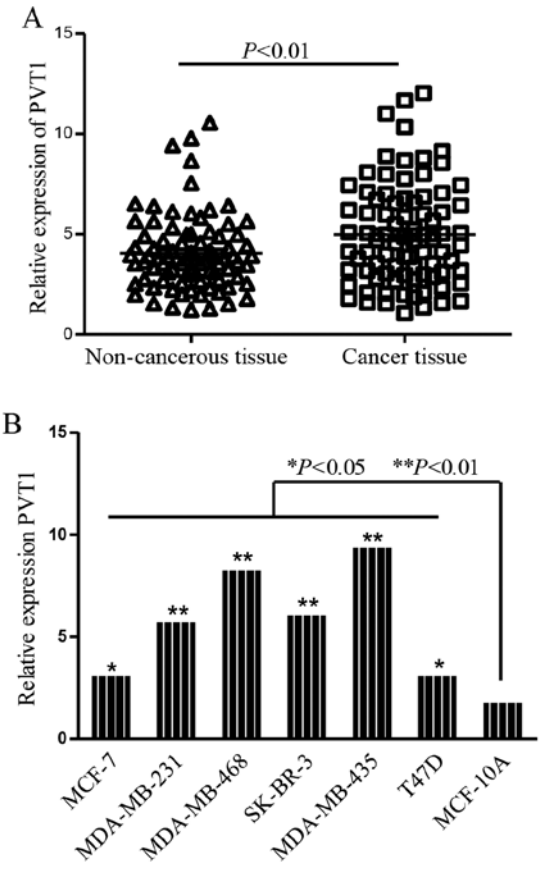


Figure 1. (A) PVT1 expression in breast cancer tissues was higher than that in the adjacent non-tumorous tissues. Wilcoxon signed-rank test was performed to analyzed statistical difference between two groups (n=84, P<0.01). (B) Expression of PVT1 in six breast cancer cell lines and human normal breast epithelial cell line MCF-7 10A. *P<0.05; **P<0.01.

performed to identify independent predictors of survival, the results showed that PVT1 expression was significantly correlated with DFS and OS (Table II).

Receiver operating characteristic (ROC) curve methodology was used to assess the diagnostic utility of PVT1 for breast cancers. As Fig. 2E showed, the best cut-off value for PVT1 in breast cancer was 4.97 with the sensitivity and specificity at 51.2 and 75.0%, respectively. The proportion under the ROC curve (AUC) was 0.63 (95% CI, 0.545-0.715, P=0.004; Fig. 2E), the Youden index was 0.425.

*Association of circulating PVT1 DNA with clinical stages of breast cancer.* To detect DNA profiles of PVT1 in serum, qPCR without reverse transcription using serum from the patients and healthy individuals were performed. The relative contents of PVT1 DNA were higher in the breast cancer group than that in healthy individuals, corresponding to a fold-change of 2.01 (Fig. 3A). A significant positive association is showed between the PVT1 in breast cancer tissues and the PVT1 DNA expression levels in the patient serum ($r^2$=-0.422, P=0.004; Fig. 3B). Next, the correlation of PVT1 DNA profiles with clinical features of breast cancer patients were examined further. As shown in Fig. 3C and D, copy number of PVT1 DNA was also associated with tumor size (P<0.05) and high histological grade (P<0.01).

We then assessed the diagnostic performance circulating DNA level of PVT1 by ROC analysis in breast cancer patients and healthy control individuals, the AUC was 0.66, indicating that circulating PVT1 is valuable in discriminating breast cancer patients from normal individuals (Fig. 3E).

*Downregulation of PVT1 inhibits proliferation and is inversely correlated with p21 in breast cancer.* To achieve cognition into the biological functions of PVT1 on breast cancer, PVT1 expression was downregulated by transfecting PVT1 siRNA into the MCF-7 and MDA-MB-231 cells, employing the scramble siRNA as a negative control. Results of cell proliferation showed that downregulation of PVT1 expression inhibited proliferation ability of MCF-7 and MDA-MB-231 cells significantly (Fig. 4A). Moreover, colony formation assays exhibited that the clonogenic survival was signifi-

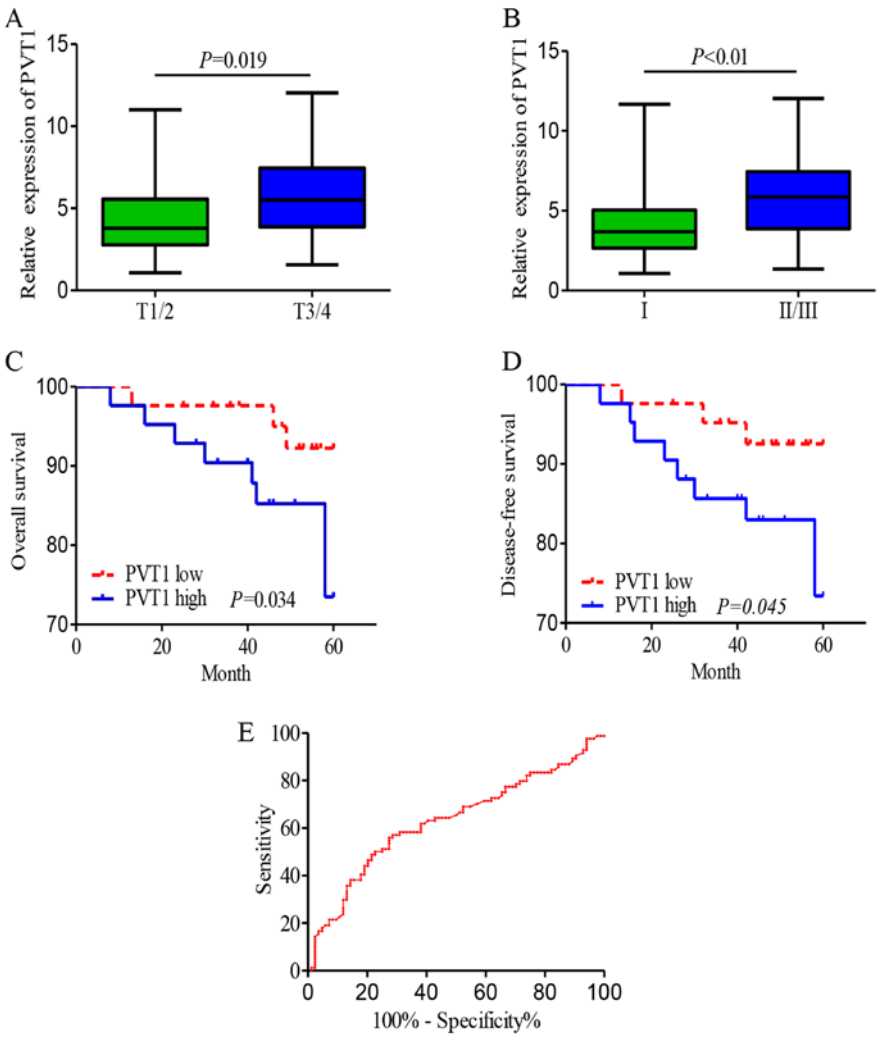


Figure 2. (A) The profiles of PVT1 were markedly higher in breast cancer patients with tumor size >2 cm than in patients with tumor size ≤2 cm. (B) Difference of PVT1 expression between high histological grade group and low histological grade group. (C) Correlations between the PVT1 expression and disease-free survival of breast cancer patients were analyzed by Kaplan-Meier analyses. (D) Kaplan-Meier analyses of correlations between the PVT1 expression level and overall survival of breast cancer patients. The median expression level was used as the cut-off. (E) ROC curve of PVT1 for differentiating breast cancer patients from healthy individuals. The area under the ROC curve is 0.63 (95% CI, 0.545-0.715, P=0.004).

Table II. Univariate and multivariate analysis of clinicopathological parameters influencing prognosis.

| | Overall survival | | | Disease-free survival | | |
|---|---|---|---|---|---|---|
| Features | HR | CI 95% | P-value | HR | CI 95% | P-value |
| Tumor size | 1.461 | 0.534-2.462 | 0.023 | 1.944 | 0.624-2.261 | 0.018 |
| Histologic grading | 1.279 | 0.915-3.517 | 0.008 | 2.279 | 0.815-2.481 | 0.021 |
| Lymphatic metastasis | 2.012 | 0.643-1.413 | 0.075 | 1.012 | 0.735-1.214 | 0.258 |
| Ki-67 expression | 1.121 | 0.921-3.276 | 0.041 | 0.926 | 1.845-3.321 | 0.035 |
| Her-2 expression | 0.625 | 1.213-2.016 | 0.152 | 1.142 | 0.663-1.435 | 0.105 |
| PVT1 expression | 2.167 | 1.108-4.265 | 0.015 | 3.167 | 1.416-3.187 | 0.009 |

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

cantly declined following inhibition of PVT1 in MCF-7 and MDA-MB-231 cells compared with control group (Fig. 4B). Above all, our results indicate that PVT1 plays a pivotal role in proliferation of breast cancer.

To verify the function role of PVT1 in breast cancer, IHC was used to determine the expression of p21 protein in breast cancer and corresponding non-tumorous tissues. Most of the non tumorous tissues exhibited strongly positive immuno-

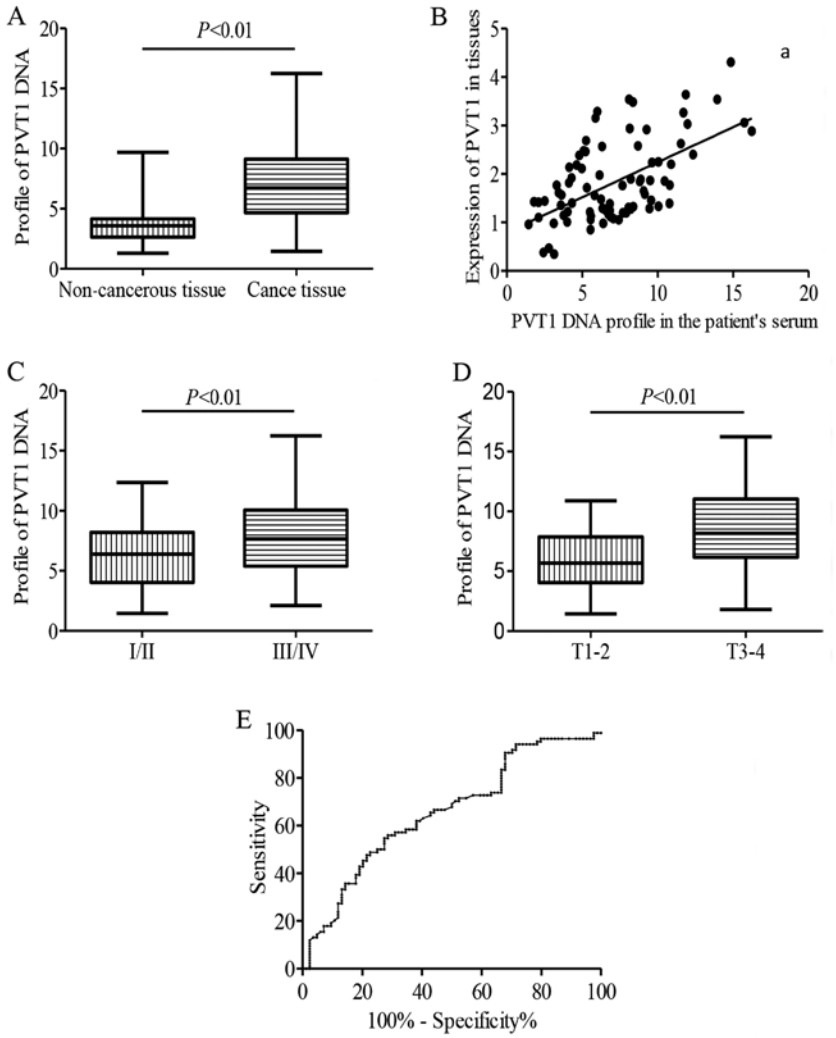


Figure 3. (A) Different expressions of PVT1 between breast cancer tissues and adjacent non-tumorous tissues. (B) The correlation between PVT1 transcript level and PVT1 DNA level in serum was measured. A significant positive correlation between the PVT1 expression in breast cancer tissues and the PVT1 DNA expression levels in the patient's serum. (C) The PVT1 expression was significantly higher in patients with higher pathological stage than in those with lower pathological stage. (D) The PVT1 expression was significantly higher in patients with high histological grade in patients with low histological grade. (E) ROC analysis of PVT1 could distinguish breast cancer patients from healthy individuals, the AUC was 0.66.

staining of p21 protein. In contrast, the corresponding breast cancer tissues showed negative or weakly positive immunostaining of p21 (Fig. 4C). Further analysis indicated that the expression of PVT1 was inversely correlated with p21 in breast tissues (Fig. 4D). To further understand the controlling relationship between PVT1 and p21, protein levels of p21 were examined in breast cancer cells subject to si-PVT1 transfection. Downregulation of PVT1 augmented p21 mRNA and protein expression (Fig. 4E and F).

## Discussion

PVT1 oncogene encodes a long non-coding RNA which locates to the region 8q24 (7). Amplification of PVT1 is a common event in various malignant tumors, such as breast, serous ovarian (12) and colorectal cancer (10). Amplification and upregulation of PVT1 have been associated with reduced survival duration in patients.

In the present study, our results illustrated that PVT1 expression was significantly upregulated in breast cancer tissues compared with adjacent non-tumorous tissues. Moreover, higher profiles of PVT1 in breast cancer patients were associated with lymph node metastasis and tumor size. Additionally, breast cancer patient with higher PVT1 expression seemed to have a shorter survival. These results demonstrated that the increase in copy number contributed to PVT-1 upregulation in breast cancer and showed that PVT1 might closely connect with the development of breast cancer, and might be applicable as a new biomarker for evaluating prognosis of breast cancer patients.

A recent screening for liver oncofetal lncRNAs in a mouse model for HCC proved the function of PVT1 in regulating proliferation and the same phenotype was confirmed in human HCC cell lines (14). Ectopic expression of PVT1 can contribute to cell proliferation, accelerate cell cycle, and lead to the achievement of stem cell-like characteristics by NOP2 (14). Significantly increased PVT1 blocked the cell cycle in a G1 phase by regulating expression of p15 and p16 epigenetically via binding to EZH2 in gastric cancer tissues (15).

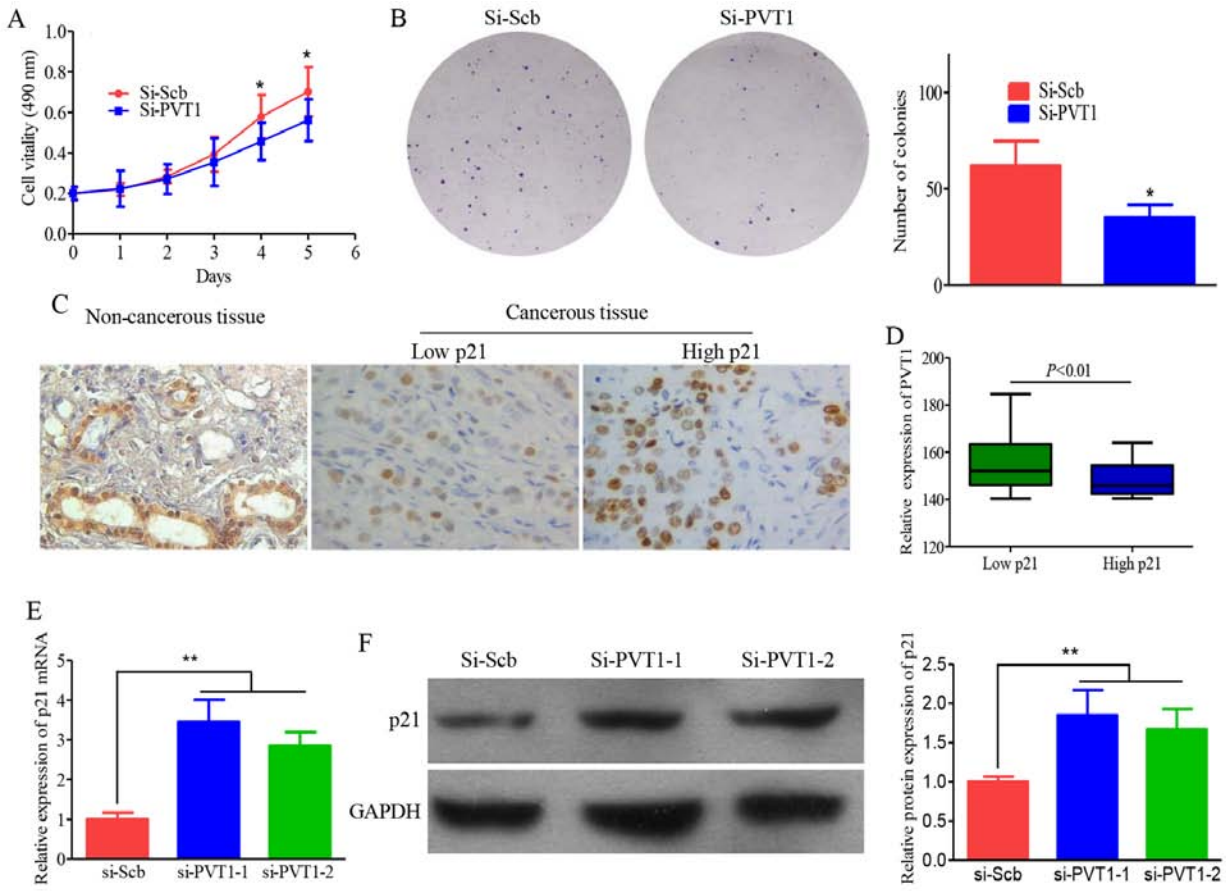


Figure 4. (A) Forty-eight hours after transfection, results of cell proliferation assay show downregulation of PVT1 retards the proliferation of MCF-7 cells. (B) Colony formation assay was performed to detect the effect of PVT1 on tumorigenesis of breast cancer cells; PVT1 knockdown significantly reduced the cell proliferation of MCF-7 cells. (C) Immunohistochemistry was performed to determine the protein levels of p21 in breast cancer tissues. (D) The expression of PVT1 was reversely correlated with the expression of p21. (E) Knockdown of PVT1 led to the increase of p21 transcripts. (F) Knockdown of PVT1 upregulated the expression of p21 protein. $^{*}$P<0.05, $^{**}$P<0.01.

p21 is a cell cycle control element and a CDK inhibitor. p21 could modulate G1 restriction point and G1/S checkpoint via binding with cyclin-CDK complexes and inhibiting cyclin-CDK complex kinase activity (16). Expression of p21 is closely regulated by p53, through this mechanism p21 is involved in the p53-dependent G1 cell cycle check point resulting from multiple stress stimuli (17,18). Here, we demonstrated that the expression of PVT1 was negatively associated with the expression of p15 and p21 in breast cancer tissues. We also inhibited PVT1 in breast cancer cells, likewise, the suppression of PVT1 downregulated the expression of p21. These results indicate that the tumor promoting activity of PVT1 is partially dependent on the negatively regulation on p21. Considering the combination of PVT1 with EZH2 epigenetically regulated p15 and p16 in Trans (15), and further studies are ongoing to define the detail regulatory mechanism of PVT1 on the expression of p21.

During the past decade, cell-free circulating nucleic acids in plasma, serum and other body fluids have a potential to change the way we make a diagnosis. Circulating DNA, miRNA and mRNA in blood may be useful for the detection of various human diseases (19,20). Circulating DNA in blood may be a very promising biomarker for precise diagnosis and treatment for breast cancer (20-22). Because the amplification of chromosomal 8q24 transcribing PVT1 has been identified in a number of cancers, including breast cancer, thus, we hypothesized that the circulating PVT1 DNA might be a useful marker for filtering breast cancer patients from healthy individuals.

In summary, in the present research, PVT1 was showed markedly unregulated in breast cancer tissues compared with adjacent non-tumorous tissues and upregulation of PVT1 might act as an independent prognostic factor for the survival of breast cancer patients. Furthermore, PVT1 played a pivotal part on the regulation of p21 expression. In addition, we detected profiles of PVT1 DNA in serum; compared with the RNA, DNA is the main form of PVT1-derived nucleic acid segment in serum, our results showed that circulating PVT1 DNA was apparently upregulated in breast cancer patients. Taken together, our results reveal that PVT1 plays a very important part in the development of breast cancer and might be potentially used for targeted therapies in breast cancer.

## Acknowledgements

The present study was supported by grants from the National Nature Scientific Foundation of China (81573717) and the Natural Science Foundation of Shandong Province (ZR2015HL064).

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