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Long non-coding RNA PVT1 promotes cell proliferation in ERα+ breast cancer through the ERα-BTG2 axis

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ABSTRACT

Background: Plasmacytoma variant translocation 1 (PVT1) is recognized as an oncogenic long non-coding RNA (lncRNA) in multiple cancer types including breast cancer (BC). Estrogen receptor alpha-positive (ERα+) BC is the major BC subtype, accounting for about 70% of cases. However, the role of PVT1 in this subtype and the underlying mechanisms are not yet fully elucidated.
Objective: To investigate the clinical significance and biological function of PVT1 in ERα+ BC.
Methods: The expression level and prognosis significance of PVT1 in ERα+ BC was explored through *in silico* analysis based on public databases. The biological function of PVT1 in ERα+ BC was examined in MCF7, a typical ERα+ BC cell line. MTS, plate clone formation and EdU assay were used to detect the effect of PVT1 knockdown on BC cell proliferation. The impact of PVT1 knockdown on estrogen signaling was assessed by measuring the expression of ERα and its downstream molecules through quantitative real-time PCR (qRT-PCR) and/or western blot. The regulatory effect of PVT1 on ERα was further investigated using protein stability experiments. To confirm the role of ERα in PVT1-regulated cell proliferation, rescue assay was conducted by targeting its downstream molecule BTG2.
Results: PVT1 expression is elevated in BC tissues compared to adjacent non-cancerous tissues, particularly in the ERα+ subtype. PVT1 knockdown substantially inhibited the proliferation of MCF7 cells as well as the estrogen signaling, which was evidenced by the marked downregulation of ERα and its downstream molecules including TFF1 and GREB1. Mechanistically, PVT1 knockdown was proved to significantly accelerate the degradation of ERα protein. BTG2 is a downstream molecule negatively regulated by ERα, which was upregulated upon PVT1 knockdown. In the rescue assay, interference of BTG2 could largely attenuated the inhibitory effect of PVT1 knockdown on cell proliferation.
Conclusion: PVT1 knockdown could inhibit cell proliferation partly through disrupting the ERα-BTG2 axis in ERα+ breast cancer.

Introduction

Breast cancer (BC) has been the most commonly diagnosed cancer in the world since 2020 and is the leading cause of cancer mortality among women.[1] BC is a disease of high molecular heterogeneity and mainly classified according to the expression status of estrogen or progesterone receptors and human epidermal growth factor 2.[2] Approximately 70% of BC patients are estrogen

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receptor alpha-positive (ERα+), seriously threatening women's health.[3]

The existence of ERα and functional estrogen signaling plays a critical role in the initiation and progression of ERα+ BC. Canonically, upon binding with estrogen, ERα dimerizes and translocates to the nucleus where it's specifically anchored to the estrogen response element in DNA, further regulating the transcription of downstream genes related with multiple biological function, such as cell proliferation and differentiation.[4] Endocrine therapy focused on estrogen signaling pathway is the standard clinical treatment for ERα+ BC. Selective ERα modulators represented by tamoxifen have achieved great benefits in ERα+ BC patients.[5] However, after 5 years of adjuvant endocrine therapy, breast-cancer recurrences continue to occur within 5–20 years in 10–41% patients.[6] Multiple reasons might explain the resistance to endocrine therapy. For example, loss of ERα expression occurs in a minority (~10%) of endocrine-resistant BC.[7] Upregulation of ERα expression is another important reason. High abundance of ERα can still exert transcriptional activity and maintain cell proliferation under conditions of estrogen deprivation or tamoxifen treatment.[8] Thus, elucidation of the regulation mechanisms underlying ERα expression and activity is of great significance for understanding the pathogenesis of BC and further reversing the resistance to endocrine therapy.

Plasmacytoma variant translocation 1 (PVT1) is an oncogenic long noncoding RNA (lncRNA) highly expressed in multiple cancer types, including colorectal cancer,[9] gastric cancer,[10] hematological cancer,[11] hepatocellular cancer,[12] ovarian cancer[13] as well as BC.[14] PVT1 can promote the progression of cancer through a variety of mechanisms while it is best known for its relation with the oncogene MYC. Both PVT1 and MYC are located at the 8q24 region, of which the genomic amplification is found in all major cancers.[15] PVT1 can enhance the stability of MYC by blocking its phosphorylation at the threonine 58 residue.[16] Furthermore, MYC increases the transcription of PVT1 by binding to the enhancer E-boxes located in the PVT1 promoter region.[17] Thus, PVT1 and MYC may be involved in a positive feedback loop to synergistically promote tumorigenesis.

The role and underlying mechanism of PVT1 in BC are far from being clarified, which have so far only been reported in the triple-negative subtype or just regardless of subtypes. For example, PVT1 was proved to enhance the progression of triple-negative BC by stabilizing KLF5 and activating the beta-catenin signaling.[14] Liu et al.[18] found that the expression of PVT1 in plasma had no difference among subtypes, and it promoted BC proliferation and metastasis by binding miR-128–3p and UPF1 in both ERα+ and triple-negative BC cells. However, the special function of PVT1 in ERα+ BC has not been elucidated yet.

In this study, we found that PVT1 was significantly elevated in ERα+ BC patients compared to ERα-negative (ERα-) patients. Interference of PVT1 could inhibit ERα+ BC cell proliferation largely through inhibiting the ERα-BTG2 axis. Moreover, the high expression of PVT1 in ERα+ patients indicates a worse overall survival and relapse-free survival after endocrine therapy. Our study has revealed for the first time that there is a positive relationship between PVT1 and ERα and might provide a potential target for treating patients with ERα+ BC.

Materials and methods

Bioinformatical analysis of PVT1 expression and prognosis in BC

The expression level of PVT1 in BC was explored in UALCAN (http://ualcan.path.uab.edu/index.html), an effective online cancer data analysis and mining website mainly based on The Cancer Genome Atlas (TCGA) database.[19] In addition, the expression and clinical files of the TCGA BC cohort, as well as the expression data of GEO datasets (GSE29431 and GSE70947) were downloaded using R software (version 4.3.3) and normalized to obtain the expression of PVT1. The prognosis significance of PVT1 was evaluated either by R 4.3.3 or by the Kaplan-Meier Plotter tool (https://www.kmplot.com/analysis/), which is capable of assessing the correlation between gene expression and survival in 35k+ samples from 21 tumor types based on databases including GEO, EGA, TCGA, and Metabric.[20]

Cell lines and chemicals

The human ERα+ BC cell line MCF7 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml of penicillin and 1 mg/ml of streptomycin (Biological Industries, Cromwell, CT, USA). The cell was maintained in a humidified incubator with 5% CO_2 at 37 °C with the medium changed every 2 days. Cycloheximide, fulvestrant and actinomycin D were purchased from Selleck Chemicals (Houston, TX, USA). They were dissolved in DMSO and stored at −20 °C until use.

Transfection of small interfering RNA (siRNA)

The siRNAs and negative control (NC) were obtained from GenePharma (Shanghai, China). Briefly, after cells were seeded in plates and cultured for 24 h, they were transfected with 50 nM single siRNA, NC or combined siRNA mixture using RNAiMAX reagents (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The siRNA sequences were as follows: PVT1-si1, 5'-CCC AAC AGG AGG ACA GCU UTT-3'; PVT1-si2, 5'-GCU UGG AGG CUG AGG AGU UTT-3'; PVT1-si3, 5'-GGC ACA UUU CAG GAU ACU ATT-3'; ERα-si, 5'-GGA GAA UGU UGA AAC ACA ATT-3'; BTG2-si, 5'-CAA GAA CUA CGU GAU GGC AGU-3'.

Cell viability assay

Cells were seeded into 96-well plates at a density of 3000 cells per well, cultured for 24 h and then transfected with single siRNA, NC or combined siRNA mixture. After 48 or 72 h, cell viability was detected by the MTS assay with a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as instructed by the manufacturer. The absorbance values were detected at 490 nm using a microplate reader.

Plate clone formation assay

Cells were seeded into 12-well plates at a density of 500 cells per well, cultured for 24 h and then treated with single siRNA, NC or combined siRNA mixture. A week later, when most clones contained > 50 cells, they were fixed with 4% paraformaldehyde (Sigma-Aldrich, Louis, MO, USA) for 30 min and stained with 1% crystal violet for 30 min. After washing and air drying, the clone numbers were quantified by ImageJ software (Bethesda, MA, USA).

EdU assay

DNA replication activity was detected using the Cell-Light EdU Apollo567 In Vitro Kit (RiboBio, Guangzhou, China) following the manufacturer's instructions. In brief, cells were seeded into 96-well plates at a density of 3000 cells per well, cultured for 24 h, and then transfected with single siRNA, NC or combined siRNA mixture. After 48 h, cells were incubated with EdU (10 μM) for 2 h prior to fixation and staining. The ratio of EdU-incorporated cells was calculated to indicate the proliferation activity.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Takara Bio Inc., Dalian, China) and its concentration was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was reversely transcribed with the PrimeScriptTM RT reagent Kit (Takara Bio Inc.,

Table 1
qRT-PCR Primer Sequences.

Gene	Primer Sequence (5′→3′)
PVT1	Forward: TGAGAACTGTCCTTACGTGACC Reverse: AGAGCACCAAGACTGGCTCT
ERα	Forward: CCACCAACCAGTGCACCATT Reverse: GGTCTTTTCGTATCCCACCTTTC
BTG2	Forward: GCGAGCAGAGGCTTAAGGTCTTC Reverse: ATGCGAATGCAGCGGTAGC
TFF1	Forward: CATCGACGTCCCTCCAGAAGAG Reverse: CTCTGGGACTAATCACCGTGCTG
GREB1	Forward: GGCAGGACCAGCTTCTGA Reverse: CTGTTCCCACCACCTTGG
β-actin	Forward: TCAAGATCATTGCTCCTCCTGAG Reverse: ACATCTGCTGGAAGGTGGACA

Dalian, China) in a 20 μL reaction system. The reaction products were subsequently diluted to a final volume of 100 μL with distilled water. The quantitative real-time PCR reaction mixture comprised 2 μL of diluted reverse transcription product, 10 μL of 2 × SYBR® Premix DimerEraser (Takara Bio Inc., Kusatsu, Japan) and 0.6 μL of forward and reverse primers (0.3 μM). The reaction was conducted in a Light Cycler@ 480 II Sequence Detection System (Roche, Basel, Switzerland). The conditions were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin serving as an endogenous control. The primers employed were detailed in Table 1.

Western blot analysis

Total cellular proteins were extracted from the harvested cells with RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Cellular proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Blots were incubated for 1 h at room temperature in a blocking buffer made of non-fat dry milk dissolved in Tris-buffered saline with 0.5% Tween 20 (TBS-T) at a ratio of 5 g per 100 ml. After washing with TBS-T, the blots were incubated with a specific antibody against ERα (Abcam Inc., Cambridge, MA, USA), BTG2 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), or β-actin (Sigma Chemical Co., St. Louis, MO, USA) overnight at 4 °C. Then the blots were incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Finally, the signal was detected using an ECL Western Blotting reagent (Promega, Madison WI, USA) and visualized with the Bio-Rad ChemiDocTM MP system. Subsequent quantification of western blot bands in each lane was conducted using the Image Lab software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

GraphPad Prism 7.0 was used for plotting and statistical analysis. The experimental data were obtained from independent replicates and expressed as mean ± standard error (SEM). Differences among groups were compared using Mann-Whitney rank-sum test (two groups) or Kruskal-Wallis test (multiple groups). All statistical tests were two-tailed and $P < 0.05$ was considered to be statistically significant.

Results

PVT1 was abnormally overexpressed in ERα+ BC

Through online analysis in UALCAN, the expression level of PVT1 was found to be elevated in BC tissues (Fig. 1A), which is consistent with previous reports.[14,18] Similar findings were validated in two additional GEO datasets (GSE29431 and GSE70947) (Fig. 1B-C). When BC tissues were classified into major subclasses, PVT1 was found to be mostly overexpressed in the luminal subtype (Fig. 1D). Since a majority of luminal BC is ERα+ , it is suggested that there is a potential relationship between PVT1 and ERα. We then divided the

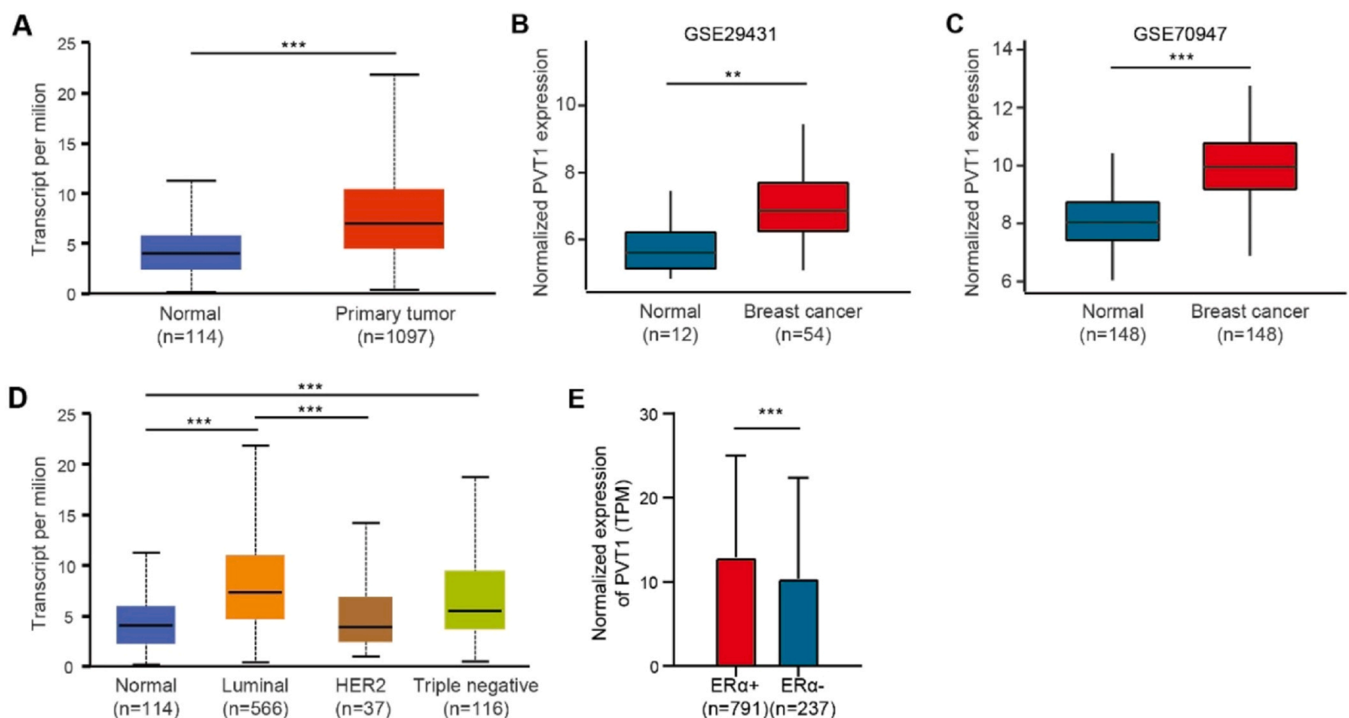

Fig. 1. Bioinformatical analysis of PVT1 expression in breast cancer. (A) Expression level of PVT1 in normal tissues and breast cancer tissues from UALCAN. (B-C) Expression level of PVT1 in normal tissues and breast cancer tissues based on GEO datasets GSE29431 and GSE70947. (D) Expression level of PVT1 in normal tissues and various breast cancer subclasses from UALCAN. (E) Expression level of PVT1 in ERα+ and ERα- breast cancer tissues based on TCGA expression data using R software. *$P < 0.05$; ***$P < 0.001$.

patients into ERα+ and ERα- groups according to the TCGA clinical profile and compared the expression level of PVT1 between them. Notably, PVT1 exhibits a higher expression in the ERα+ group (Fig. 1E).

Knockdown of PVT1 inhibited the proliferation of ERα+ BC cells

To verify the role of PVT1 in ERα+ BC, we chose a classical ERα+ cell line MCF7 as the *in vitro* model. Three siRNAs targeting PVT1 were synthesized and the one with the best knockdown efficiency was used for subsequent experiments (Fig. 2A). As shown in Fig. 2B-C, PVT1 knockdown significantly inhibited the cell viability and clone formation of MCF7 cells. In the EdU assay, the ratio of marked proliferating cells was severely decreased (Fig. 2D). These results all indicated that PVT1 mediated the proliferation activity of MCF7 cells.

Knockdown of PVT1 inhibited the activity of estrogen signaling through downregulating ERα

The proliferation of ERα+ BC cells can be regulated by various signaling pathways, while estrogen signaling is one of the best known. To examine whether PVT1 has an effect on estrogen signaling, TFF1 and GREB1 were chosen from the literature as two classical downstream genes.[21] As expected, when ERα was knocked down, the expression of TFF1 and GREB1 were markedly decreased (Fig. 3A). Furthermore, the expression of these genes was also significantly decreased after PVT1 was knocked down, indicating a probable inhibition of estrogen signaling (Fig. 3B). To examine how PVT1 affected the activity of estrogen signaling, the expression of ERα was then detected. It was found that only the protein level of ERα was decreased by PVT1 knockdown rather than mRNA, suggesting that ERα was post-transcriptionally regulated by PVT1 (Fig. 3C-D).

In order to find out the modulatory mechanism of ERα protein, a protein synthesis inhibitor cycloheximide was used to pretreat MCF7 cells before PVT1 knockdown. However, the expression of ERα was still decreased, indicating that PVT1 knockdown didn't have an effect on ERα synthesis (Fig. 3E). Then, we treated MCF7 cells with cycloheximide for indicated intervals after PVT1 had been successfully knocked down. It was found that ERα expression decreased more rapidly in the PVT1-knockdown group than in the control group (Fig. 3F). These results revealed that PVT1 participated in the stabilization of ERα. Therefore, knockdown of PVT1 could inhibit the activity of estrogen signaling via decreasing the expression of ERα due to the reduction of its stability.

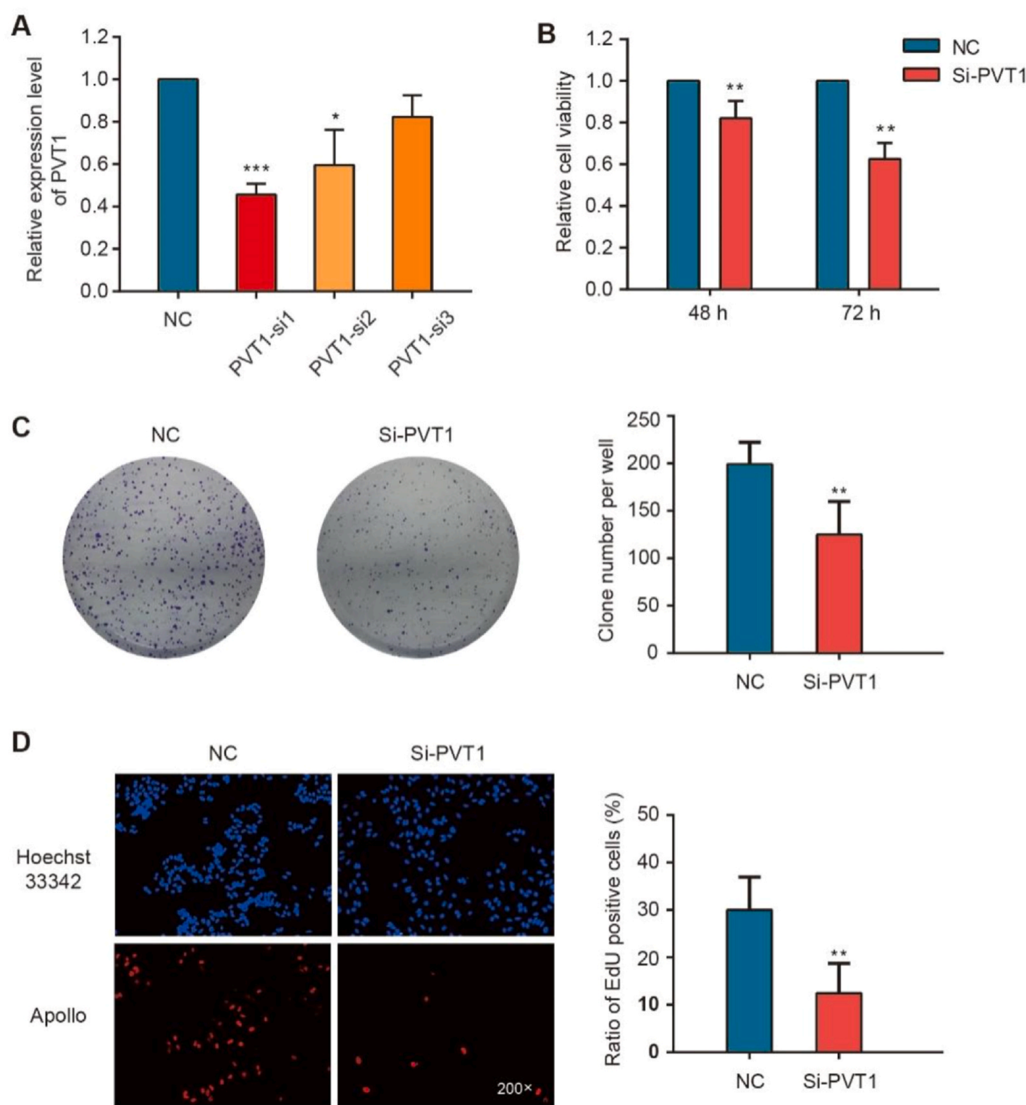

Fig. 2. Effect of PVT1 knockdown on proliferation in ERα+ MCF7 breast cancer cells. (A) Relative expression of PVT1 after knockdown using three siRNAs. (B) Relative cell viability of MCF7 cells after 48 and 72 h of PVT1 knockdown measured using the MTS assay. (C) Representative images of cell clones and the quantitative bar chart of the clone number of MCF7 cells after PVT1 knockdown in the plate clone formation assay. (D) Representative images of Hoechst 33342 and Apollo dyed cells and the quantitative bar chart of the ratio of EdU-positive cells in MCF7 cells after 48 h of PVT1 knockdown in the EdU assay. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

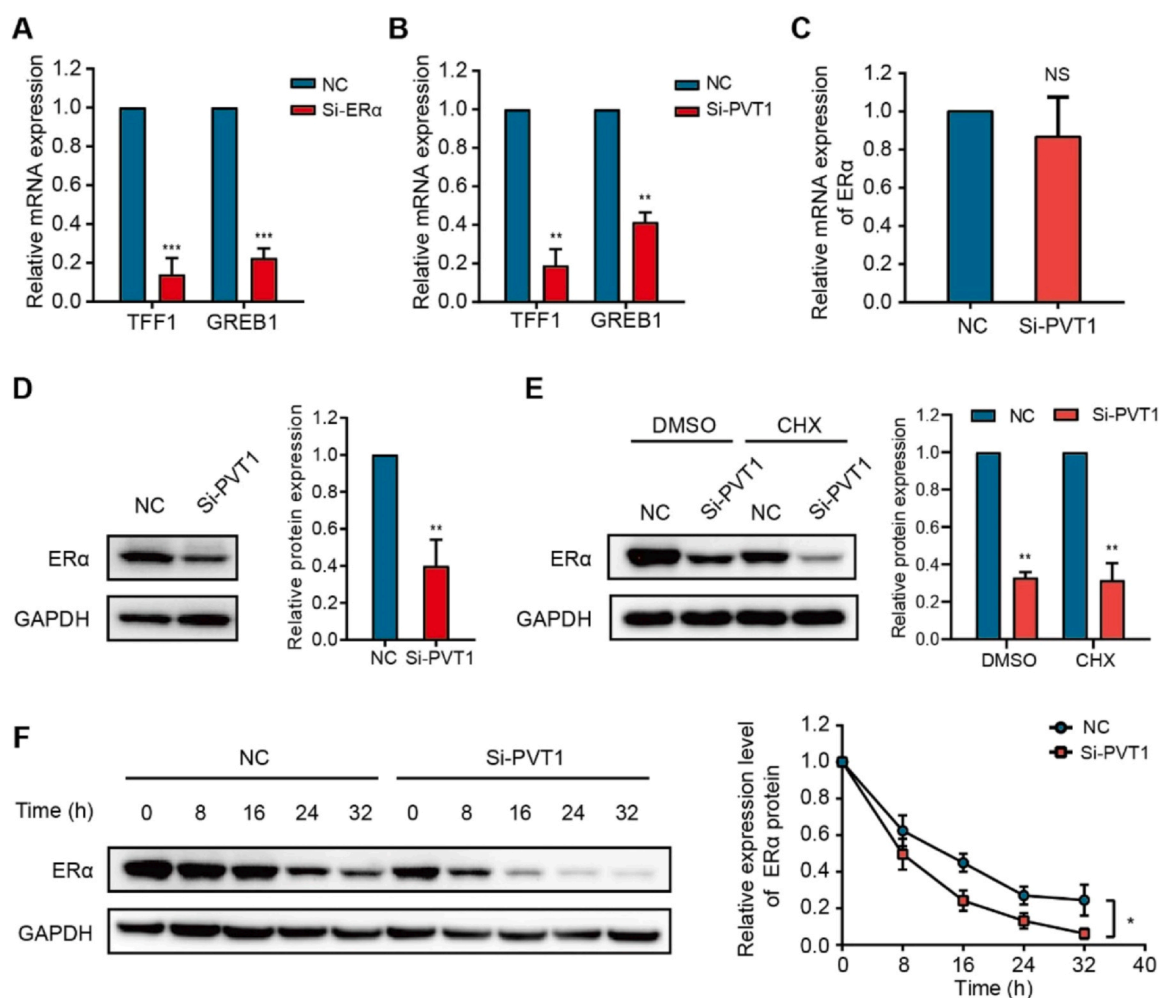

Fig. 3. Effect of PVT1 knockdown on estrogen signaling pathway in MCF7 breast cancer cells. (A) Relative expression of TFF1 and GREB1 after ERα knockdown. (B) Relative expression of TFF1 and GREB1 mRNA after PVT1 knockdown. (C) Relative expression of ERα mRNA after PVT1 knockdown. (D) Representative western blot images of ERα after PVT1 knockdown and the quantitative bar chart of relative ERα expression. (E) After pretreating MCF7 cells with 1‰ DMSO or 300 μM cycloheximide (CHX) for 1 h, PVT1 was knocked down and expression of ERα was detected using western blot analysis after 48 h. Representative western blot images of ERα and the quantitative bar chart of relative ERα expression were showed. (F) After PVT1 knockdown, MCF7 cells were incubated with 1‰ DMSO or 300 μM CHX for different time intervals, and the expression of ERα were detected using western blot analysis. Representative western blot images of ERα and the time-course line graph of relative ERα expression were showed. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Knockdown of PVT1 upregulated the expression of BTG2 which was mediated by ERα

BTG2 is a member of the BTG/TOB anti-proliferation gene family, which can inhibit cell proliferation in a variety of tumors.[22] Notably, it has been reported that BTG2 was inhibited by the estrogen signaling.[23,24] Since knockdown of PVT1 could decrease the expression of ERα, we doubted whether there would be any change in the expression of BTG2.

As was shown in Fig. 4A-B, the expression of BTG2 increased significantly after PVT1 knockdown in MCF7 cells, while it was not observed in the ERα- cell line BT549 (Fig. 4C). Furthermore, when MCF7 cells were treated with an ERα siRNA or an ERα antagonist fulvestrant, both of them elevated the mRNA expression of BTG2 (Fig. 4D-E). All these results confirmed an ERα-mediated negative regulation of PVT1 on BTG2.

Since ERα was a nuclear receptor transcription factor, we speculated that its regulation of BTG2 was directly related to its transcriptional function. To confirm this, we pretreated MCF7 cells with actinomycin D, an mRNA synthesis inhibitor, to inhibit the transcription of BTG2. We found that the upregulation of BTG2 mRNA induced by PVT1 knockdown could then be absolutely reversed (Fig. 4F). Moreover, when MCF7 cells were treated with actinomycin D for different intervals after PVT1 had been successfully knocked down, the degradation speed of BTG2 mRNA was comparable to that in the control group (Fig. 4G). The above results indicated that PVT1 knockdown-induced upregulation of BTG2 expression was not related to mRNA stability, but simply caused by increased transcription.

Knockdown of PVT1 inhibited the proliferation of MCF7 partly through ERα-BTG2 axis

To verify that PVT1 knockdown-induced inhibition of cell proliferation is caused by BTG2 upregulation, a series of rescue experiments were conducted. Concretely, when PVT1 was knocked down, we simultaneously used a siRNA targeting BTG2 to block its upregulation. As was shown in Fig. 5A-B, the impairment of both cell viability and clone formation was largely reversed by BTG2 interference. In addition, in the EdU assay, the ratio of proliferating cells also acquired a great recovery in the BTG2 upregulation-blocked group (Fig. 5C). These results all demonstrated that PVT1 knockdown-induced inhibition of cell proliferation was partly mediated by the ERα-BTG2 axis.

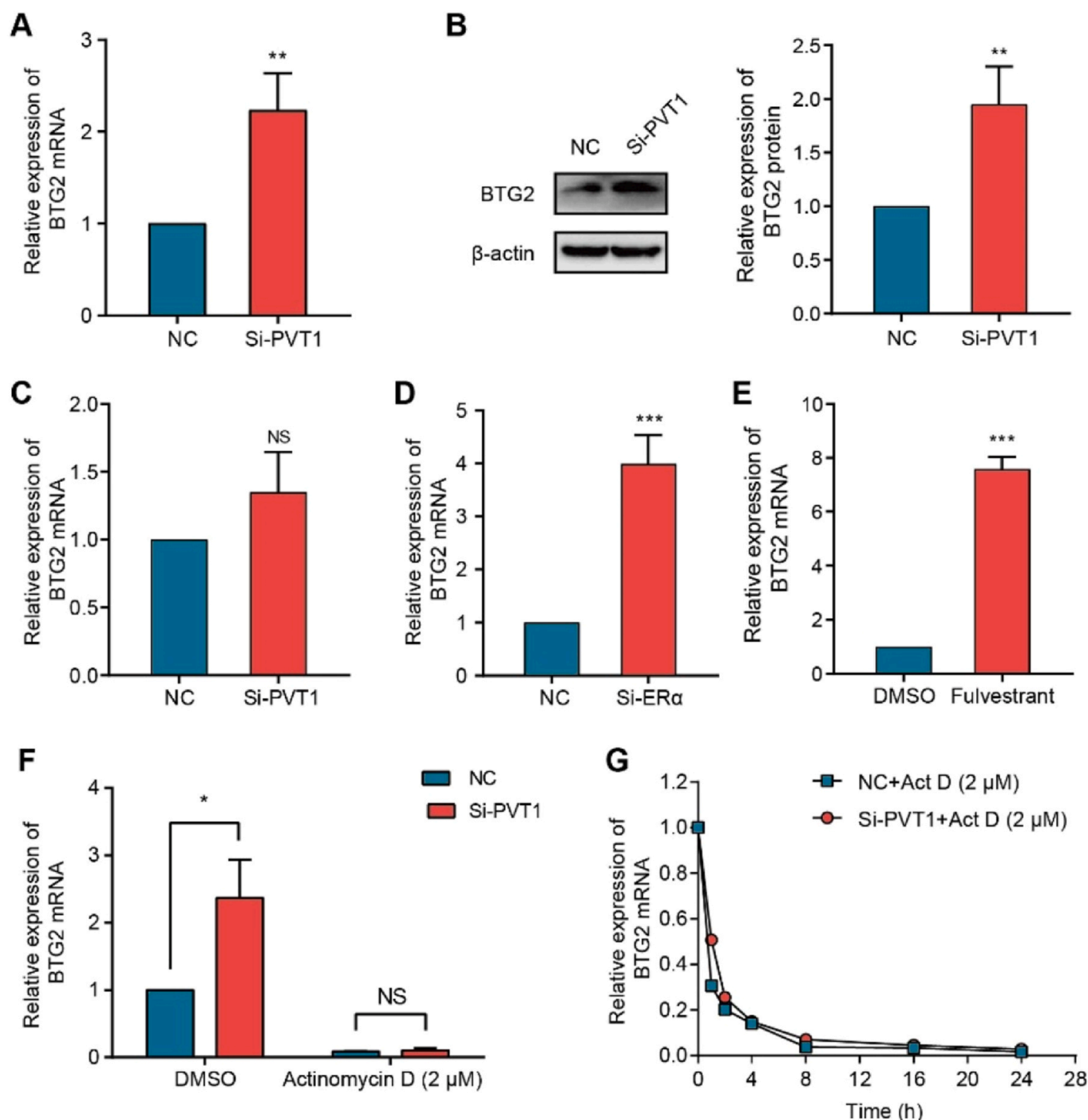

Fig. 4. PVT1 knockdown upregulates the expression of BTG2 through estrogen signaling pathway in MCF7 breast cancer cells. (A) Relative expression of BTG2 mRNA after PVT1 knockdown in MCF7 cells. (B) Representative western blot images of BTG2 protein after PVT1 knockdown in MCF7 cells and the quantitative bar chart of relative BTG2 expression. (C) Relative expression of BTG2 mRNA after PVT1 knockdown in an ERα- cell line BT549. (D-E) Relative expression of BTG2 mRNA after ERα knockdown or fulvestrant treatment (100 nM) in MCF7 cells. (F) After pretreating MCF7 cells with 1‰ DMSO or 2 μM actinomycin D (Act D) for 1 h, PVT1 was knocked down and relative expression of BTG2 mRNA was detected using qRT-PCR after 48 h. (G) After PVT1 knockdown, MCF7 cells were incubated with 1‰ DMSO or 2 μM actinomycin D for different time intervals, and the relative expression of BTG2 mRNA were detected using qRT-PCR analysis. NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

High PVT1 expression indicates poorer survival in ERα+ patients

To explore the clinical significance of PVT1 in ERα+ BC, we analyzed its impact on overall survival in luminal-subtype BC patients (predominantly ERα+) from the TCGA cohort. As shown in Fig. 6A, patients with higher PVT1 expression exhibited poorer overall survival (HR = 1.57). Considering that endocrine therapy targeting ERα is the standard treatment for ERα+ BC, PVT1 might also cause changes in patients' response to endocrine therapy by regulating ERα expression. Intriguingly, high PVT1 expression was consistently and significantly associated with worse relapse-free survival in three independent GEO datasets (Fig. 6B-D), suggesting that PVT1 may contribute to the development of endocrine therapy resistance.

Discussion

In this study, we found that PVT1 was abnormally elevated in BC, especially the luminal subtype, and knockdown of PVT1 could inhibit the proliferation of ERα+ BC cells through post-transcriptionally downregulating ERα and transcriptionally upregulating downstream BTG2. Our study suggested PVT1 as a new positive regulator of estrogen signaling. Its clinical role in the diagnosis and treatment of ERα+ BC was therefore worth further study.

BTG2 is the earliest identified member of the BTG/TOB anti-proliferation gene family, which is highly expressed in the pancreas, thymus, kidneys, prostate, lungs, and skeletal muscles.[22] BTG2 is involved in important life activities such as cell proliferation, apoptosis

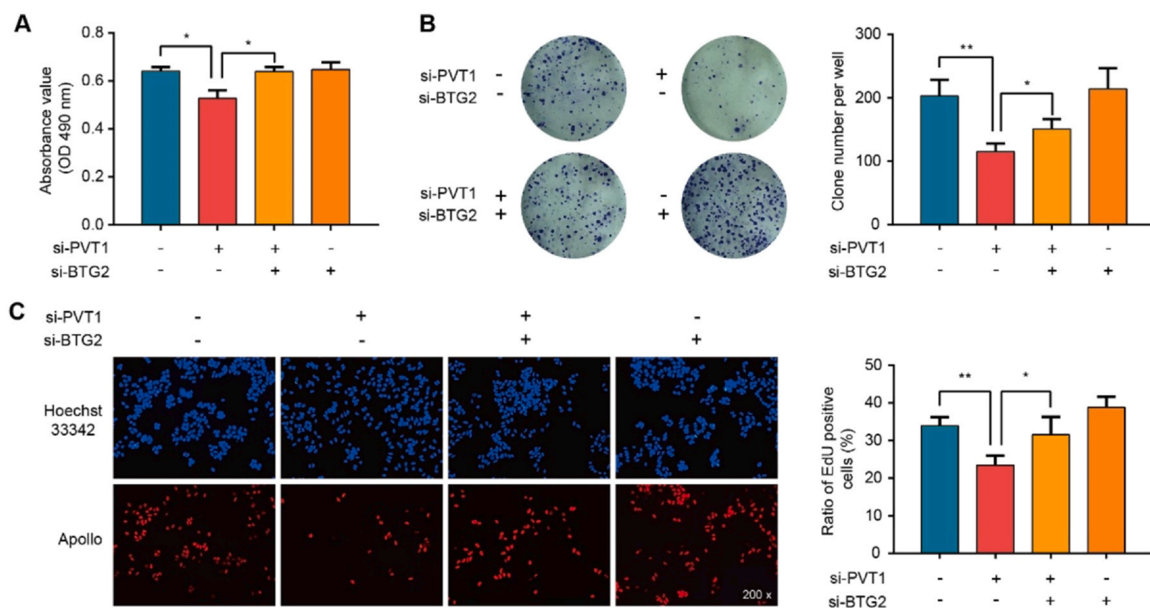

Fig. 5. PVT1 knockdown inhibits the proliferation of MCF7 breast cancer cells partly through upregulating the expression of BTG2. (A) Cell viability of MCF7 cells were detected using the MTS assay after knocking down PVT1 or BTG2 alone or simultaneously. (B) Representative images of cell clones and the quantitative bar chart of the clone number of MCF7 cells after knocking down PVT1 or BTG2 alone or simultaneously in the plate clone formation assay. (C) Representative images of Hoechst 33342 or Apollo dyed MCF7 cells and the quantitative bar chart of the ratio of EdU-positive cells after knocking down PVT1 or BTG2 alone or simultaneously. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

and DNA damage repair, and its dysregulation is closely related to cancer occurrence.[25] Loss of nuclear BTG2 expression was observed in 46% of BC samples and was associated with a higher pathological grade of ERα+ BC.[26] Overexpression of BTG2 in MCF7 cells could suppress cell growth.[27] Moreover, BTG2 is a significant factor in tamoxifen response through modification of AKT activation.[28] Besides, BTG2 downregulated Twist1 protein expression at a translational level, which made it a promising target to combat with metastatic BC.[29] In our study, the upregulation of BTG2 also proved to show an inhibitory effect on proliferation, which was consistent with its tumor suppressive role in the previous reports.

In estrogen signaling, the molecular regulation of ERα on downstream genes and underlying mechanisms are the focus of recent research. Although most studies have revealed the transcriptional activation of ERα on its target genes, it is important to note that for some genes ERα can also exert an inhibitory role.[30] For BTG2, it has been reported to be negatively regulated by ERα, which was also verified in our study. However, the molecular mechanism remained unclear. Karmakar et al.[23] showed that the transcriptional inhibition of BTG2 by ERα might be related to the histone acetylation activity in the promoter region of *BTG2*, while the transcriptional corepressor REA also played a certain role. Recently, Melone et al.[31] proposed that ERα needed the recruitment of PRC2 for EZH2-mediated H3K27Me3 and chromatin closure for E2 depending BTG2 transcriptional repression. To elucidate how BTG2 is transcriptionally regulated by ERα will undoubtedly help to deepen our understanding of the way estrogen signaling works.

The expression of ERα is regulated by various mechanisms, including transcriptional activity,[32] epigenetic modification,[33] mRNA stability[34] and post-translational modulation,[35] which may trigger dysregulation of ERα and inappropriate estrogen signaling in BC. It was revealed here that PVT1 might have an effect on protein stability of ERα, whereas the precise mechanism was barely known. As previously mentioned, Liu et al.[18] demonstrated that PVT1 promotes carcinogenesis in both ERα+ and triple-negative BC by functioning as a ceRNA of miR-128–3p and binding UPF1. However, their study did not explore the downstream consequences of this axis in the context of ERα protein stability. Since no significant change of ERα mRNA was observed in our study, it is reasonable to infer that PVT1 regulate ERα independently of sponging miRNAs as a ceRNA. Moreover, no direct relationship between UPF1 and ERα can be found from existed literature. Future work is needed to determine whether this downstream axis of PVT1 functions in parallel with or independently of the ERα-BTG2 pathway uncovered in our work.

The ubiquitin-proteasome system has been reported to be a key regulator of ERα stability and degradation.[36,37] ERα lysing residues, K302 and K303, were suggested as the lysine targets for ubiquitination.[35] And several E3 ubiquitin ligases and deubiquitinases, such as PSMD14,[38] USP36,[39] Hbo1[40] and USP15,[41] specifically mediate the covalent binding of ubiquitin to ERα residues. Additionally, the ubiquitination of ERα is also influenced by other modification forms. For example, ufmylation of ERα dramatically increases its stability by inhibiting the ubiquitination.[42] Whether PVT1 participates a certain kind of modification on ERα and further regulate its stability is an issue urgently needed to be addressed.

Limitations of the study should also be acknowledged. It should be noted that most of our conclusions were based on *in vitro* experiments and public database analyses. Although these results provide solid initial evidence, *in vivo* experiments are still needed to further validate the functional role of PVT1 and the ERα-BTG2 axis in tumor growth and progression. In addition, future studies involving specimens collected from our own clinical cohort will be crucial to confirm our findings and enhance their clinical and mechanistic significance.

Overall, our study established PVT1 as a new regulator of ERα expression, which might be involved in the progression of ERα+ BC by regulating cell proliferation. Since ERα is the core of endocrine therapy, and drug resistance happens frequently as for recent therapeutic regimens, our result helps to provide a potential target for treatment of ERα+ BC.

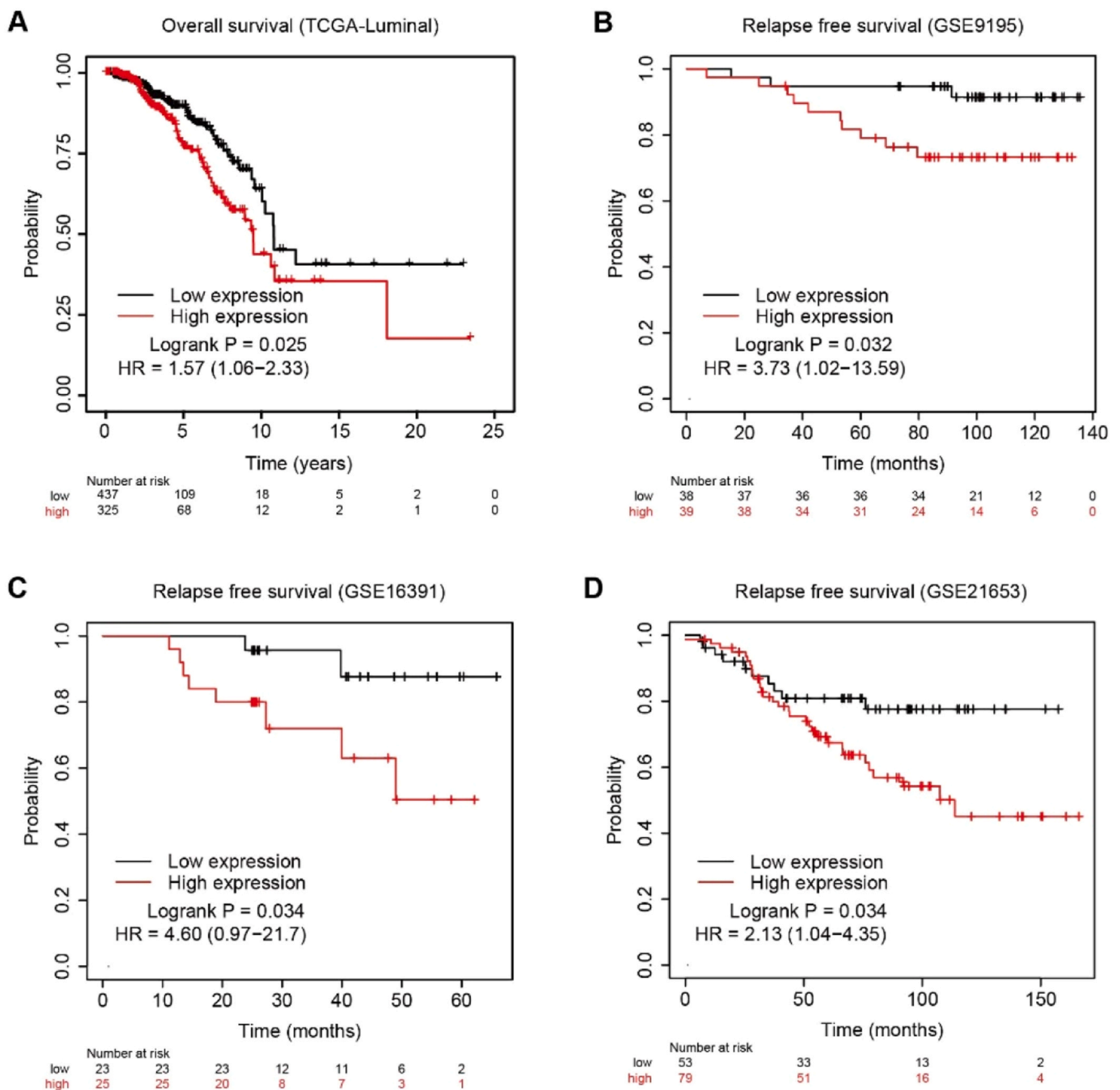

Fig. 6. The prognosis significance of PVT1 in ERα+ breast cancer patients (A) The impact of PVT1 expression level on overall survival prognosis in luminal-subtype breast cancer patients (predominantly ERα+) from TCGA; (B-D) The impact of PVT1 expression level on relapse free survival prognosis in ERα+ breast cancer patients receiving endocrine therapy in three independent GEO datasets (GSE9195 (B), GSE16391 (C), and GSE21653 (D)) in the Kaplan-Meier Plotter website.

Declarations

Not applicable.

CRediT authorship contribution statement

Haibo Zhang: Conceptualization, Methodology, Investigation. Haibo Zhang, Shuoguo Tan: Data curation, Formal analysis, Visualization. Ying Zeng, Junli Deng, Yarui Liu: Writing – original draft. Rui Liang, Li Nan, Xiaomei Zhou, Guoying Fang: Writing – review & editing. Guo Wang: Validation, Supervision.

Ethics approval and consent to participate

This article uses publicly available database data and does not involve ethical approval or informed consent.

Consent for publication

All authors have read and agreed to the published version of the manuscript and give their consent for publication in this journal.

Availability of data and materials

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Declarations of Competing Interest

The authors declare no competing interests.

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Authors' other information

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