The long non-coding RNA PVT1 represses ANGPTL4 transcription through binding with EZH2 in trophoblast cell

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Abstract

Despite progress in diagnostics and treatment for preeclampsia, it remains the foremost cause of maternal and foetal perinatal morbidity and mortality worldwide. Over recent years, various lines of evidence have emphasized long non-coding RNAs (lncRNAs) which function as an innovative regulator of biological behaviour, as exemplified by proliferation, apoptosis and metastasis. However, the role of lncRNAs has not been well described in preeclampsia. Here, we identified a lncRNA, PVT1, whose expression was down-regulated in qRT-PCR analyses in severe preeclampsia. The effects of PVT1 on development were studied after suppression and overexpression of PVT1 in HTR-8/SVneo and JEG3 cells. PVT1 knockdown notably inhibited cell proliferation and stimulated cell cycle accumulation and apoptosis. Exogenous PVT1 significantly increased cell proliferation. Based on analysis of RNAseq data, we found that PVT1 could affect the expression of numerous genes, and then investigated the function and regulatory mechanism of PVT1 in trophoblast cells. Further mechanistic analyses implied that the action of PVT1 is moderately attributable to its repression of ANGPTL4 via association with the epigenetic repressor Ezh2. Altogether, our study suggests that PVT1 could play an essential role in preeclampsia progression and probably acts as a latent therapeutic marker; thus, it might be a useful prognostic marker when evaluating new therapies for patients with preeclampsia.

Keywords: preeclampsia – PVT1 – PRC2 – proliferation

Background

Preeclampsia (PE) is a multisystem syndrome of human gestation that is characterized by hypertension, proteinuria and oedema after 20 weeks of pregnancy. New guidelines from the American College of Obstetrics and Gynecology (ACOG) state that PE can also be diagnosed in the non-existence of proteinuria in hypertensive women complicated with pulmonary oedema, impaired liver function, thrombocytopenia and associated renal insufficiency, and visual disorders [1]. PE is the main reason for maternal and foetal perinatal morbidity and mortality worldwide [2, 3], affecting 3–5% of pregnancies and causing approximately 60,000 maternal deaths annually [4]. Delivery is the only cure for PE [5], and effective prevention strategies have not yet been developed.

PE originates from the placenta, and its sequelae also affect multiple organ systems. Many factors are associated with the etiopathogenesis of PE, including endothelial dysfunction [6], inflammatory cytokines [7–9], oxidative stress [10, 11], imbalances between pro-angiogenic and antiangiogenic factors [12, 13], and dietary and genetic factors [14]. Among these factors, endothelial dysfunction is the common trigger of most medical symptoms and could lead to hypertension, oedema, proteinuria and other symptoms [15]. During the early development of the placenta, extracellular trophoblasts (EVTs) originating from the foetus can migrate and invade the maternal spiral arteries of the myometrium and decidua, providing a substitute for the endothelial layers of the uterine spiral arteries. This process, known as maternal spiral artery remodelling, is vital for reducing blood flow resistance and/or aggregating uteroplacental perfusion [16]. In PE, this process is impaired [17, 18]. Owing to increased apoptosis [19, 20], reduced EVT proliferation [21], and impaired EVT migration and invasion capacity [22], EVTs are not able to effectively invade the spiral arteries in the myometrium in patients with PE. However, despite extensive research, the specific mechanisms related to the progression of cell proliferation, apoptosis, invasion and migration in preeclampsia remain uncertain.

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Recent studies have elucidated the genetic events that play essential roles in the progression and/or improvement of Preeclampsia. Non-coding RNAs have been revealed to have several principal regulatory functions in transcription and post-transcriptional processes [23]. In particular, abnormal regulation of IncRNAs, which are longer than 200 bop and do not encode proteins, is associated with numerous human disorders [24], including cardiovascular disease [25] and neurodegenerative diseases [26]. Moreover, IncRNAs play fundamental roles in both normal development and disease [26], and IncRNAs are correlated with diverse cellular processes, including stem cell pluripotency, apoptosis, proliferation, migration and invasion [27, 28]. We have identified two IncRNAs that participate in the occurrence and progress of PE in our previous study [29, 30]. However, most IncRNAs have not been shown to regulate the associated functions and mechanisms of trophoblasts or to be involved in PE. Accordingly, identifying PE-associated IncRNAs and related molecular mechanisms is essential for understanding the progress of preeclampsia and establishing improved treatment strategies.

Accordingly, in this study, we assessed the relative expression and role of the IncRNA PVT1 in placental tissues from women with normal pregnancies and PE. In addition, we further explored the effects of PVT1 on trophoblast cell proliferation, apoptosis, migration and invasion in vitro. Our results provide important insights into the role of PVT1 in the aberrant characteristics of trophoblasts in PE and may act as a potential biomarker for preeclampsia diagnosis and therapy.

Materials and methods

Tissue samples and patients

We obtained 52 paired placental tissues from normal pregnancies and preeclamptic women, who underwent caesarean deliveries in Jiangsu Province Hospital from 2015 to 2016, then all obtained tissue samples were instantaneously stored at −80 °C before RNA and protein extraction. And the clinicopathological characteristic of the normal pregnancies and pre-eclamptic women has been recapitulated in Table 1. And this research was authorized by the Ethnics Board of the First Affiliated Hospital of Nanjing Medical University, China. Relative written informed agreements were gotten from patients which meet the criteria were included in this study.

Cell culture

Trophoblast Cells HTR/SVneo, which derived from primary extra villous trophoblast cell, was kindly offered by Dr. Charles Graham, Queen’s University, Canada. And HTR/SVneo was cultured in RPMI 1640 (GIBCO, Nanjing, China), which added with 5% FBS (GIBCO, Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) in humidified air at 37°C with 5% CO2. Other cell lines, as exemplified by JEG-3, BeWo, Wish and HUVEC-C, were all purchased from the Chinese Academy of Sciences Committee (Shanghai, China).

Transfection of cell lines

Generally, HTR/SVneo and JEG3 were cultivated at six-well plates until confluent and then transfected with corresponding siRNAs (10 ul) or scrambled negative control siRNA (10 ul) or plasmid vectors (4 ug) by exploiting Lipofectamine 2000 (Invitrogen). Plasmid vectors (pcDNA3.1-PVT1, and pcDNA) were distilled by DNA Midiprep kit (Qiagen, Hilden, Germany). The nucleotide sequences of siRNAs for PVT1, EZH2 and scramble negative control (si-NC) purchased from Invitrogen and have been summarized in Table S2. To ectopically express the PVT1, the full-length complementary DNA of PVT1 was synthesized by Realgene (Nanjing, China) and subcloned into the pcDNA3.1(+) vector (Invitrogen), on the basis of the manufacturer’s instructions. At 36-hr post-transfection, cells would be harvested for further studies, such as qRT-PCR and Western blot analysis.

RNA preparation and qRT-PCR assays

Actually, the total RNAs were isolated from clinic sample tissues or cultured cells using TRIzol reagent (Invitrogen), following the manual. This experiment was executed in accordance with the relevant guidelines. 1 ug RNA was reverse transcribed to cDNA in a final volume of 20 ul with PrimerScript RT Master Mix (Takara, Dalian, China). Then we applied the
SYBR Premix Ex Taq (Takara, Dalian, China) to determine PVT1 levels, on the basis of the manufacturer’s protocol. The resulting data were normalized to the expression of GAPDH. The relevant primers were listed in Table S1. qRT-PCR assays were implemented on an ABI 7500, and our results were calculated and expressed relative to threshold cycle (shown as ΔCt) values, then converted to fold changes. This experiment was performed in accordance with the relevant guidelines and regulations.

**Subcellular fractionation location**

The isolation of the nuclear and cytosolic fractions was implemented by exploiting the PARIS Kit (Life Technologies, Carlsbad, CA, USA), following the manual.

**Cell proliferation assays**

Cell viability was supervised with the MTT (Cell Proliferation Reagent Kit I) Penzberg, Germany). The HTR/SVneo and JEG-3 were treated with si-PVT1 or pcDNA-PVT1(3500 cells/well) and were cultivated in 96-well plates with five duplicate. Cell viability was seasoned every 24 hrs according to the manual. The absorbance was detected at 490 nm with an ELx-800 University Microplate Reader (BioTek, Winooski, VT, USA).

**Ethynyl deoxyuridine analysis (Edu)**

As we know, the Edu assay was implemented as a complementary method to authenticate the proliferation level more accurately than MTT and colony formation assays. Usually, we exploit the 5-ethynyl-2-deoxyuridine labelling/detection kit (Ribobio, Guangzhou, China) to evaluate cell proliferation, following the manufacturer’s recommendation.

**Flow cytometric analysis of cell cycle and apoptosis**

These trophoblast cell lines, treated with si-PVT1-1# and si-PVT1-2#, were harvested 36 hrs after transfection, respectively and washed twice with cold PBS. Then, we exploit FITC-Annexin V and Propidium iodide (PI) using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences Franklin Lakes, NJ, USA) following the manufacturer’s manual. Cell cycle analysis was stained with propidium oxide by the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences) according to the protocol and analysed by FACSscan. The ratio of the cells in G0/G1, S and G2/M phase was calculated and compared.

**Cell migration and invasion assays**

The ability of cell migration and invasion were analysed by transwell assays as previously reported in Zuo et al. [31].

**Western blot assay**

WB assays were implemented to detected the relative level of protein as formerly reported in Zuo et al. [31], and incubated with specific antibodies (GAPDH, EZH2, ANGPTL4 purchased from CST or Protein-tech) at 1:1000 concentration. The intensity of autoradiogram protein bands was quantified by the Equipment of Quantity One software (Bio-Rad Hercules, CA, USA).

### RNA-seq bioinformatic analysis

The RNA-Seq experiments were implemented by Beijing Genomics Institute (Beijing, China). mRNA-seq library was arranged for exploiting standard Illumina manuals. Briefly, RNAs were obtained from si-NC, or si-PVT1-1# transfected HTR-8/SVneo cells, were isolated utilizing TRIzol reagent (Invitrogen). mRNA extraction was executed using Dynabeads-soligo (Invitrogen Dynal). To stabilize the mRNA-seq library, the cDNAs were next fragmented via nebulization and the standard Illumina protocol followed.

**RNA immunoprecipitation assays (RIP)**

RIP experiments were performed as formerly reported in Zuo et al. [31], following the manufacturer’s instructions. EZH2 and SUZ12 antibodies used for IP were from Millipore (Billerica, MA, USA). At the end of RIP experiment, the obtained RNA was subjected to qRT-PCR analysis to demonstrate the presence of PVT1 and IgG using specific primers which have been listed in Table S2.

### Statistical analysis

Student’s t-test was utilized to analyse data in vitro using SPSS 17.0 statistical software (IBM, Chicago, IL, USA). Furthermore, P-values of less than 0.05 were contemplated statistically significant. These resulting data are recounted as the mean ± S.D. *P* < 0.05(*) or **P** < 0.01 (**). At least three times for each experiment which was repeated independently.

### Result

**PVT1 expression in placental tissues from women with normal pregnancy and severe PE**

To ascertain whether PVT1 was differentially expressed in placental samples from severe preeclampsia women, we quantified PVT1 expression by qRT-PCR in 52 paired clinic placental samples from women with normal pregnancies and severe PE. The results showed
Fig. 1 Relative PVT1 expression in preeclampsia. (A and B) LncRNA PVT1 levels are lessened in PE placentas. The expression of PVT1 was significantly lower in PE samples \((n = 52)\) than that in normal placentas (log2). (C) The PVT1 expression in BeWo, WISH, JEG-3 and HTR-8/SVneo was normalized to that in HUVEC-C. At least three times of biological replicates have been performed and presented. \(* P < 0.05, ** P < 0.01.\)

Fig. 2 PVT1 promotes Cells proliferation in vitro. (A and B) MTT assays were implemented to detect the viability of si-PVT1-1#-treated or pcDNA3.1-PVT1-treated Trophoblast Cells. The cell viability after transfected with si-PVT1 was significantly lower than that treated with the control. (C and D). Proliferating Trophoblast Cells were labelled using Edu. The Click-it reaction shown Edu staining (red). Cell nuclei were stained with DAPI (blue). \(* P < 0.05, ** P < 0.01.\)
that PVT1 expression was lower in pregnant women with PE than that in women with normal pregnancies (Fig. 1A and B). Table 1 illustrates the clinical characteristics of these patients.

**PVT1** promote cell proliferation and apoptosis in trophoblast cells

As human lncRNAs play indispensable roles in numerous biological behaviour, we detected the expression of PVT1 in several trophoblast cell lines, including HTR-8/SVneo, BeWo, and JEG-3, WISH and HUVEC-C. As shown in Figure 1C, we discovered that the relative PVT1 level in this two cell lines was higher compared to WISH, HUVEC-C and BeWo. Then we examined whether PVT1 was functionally involved in PE using two different PVT1-specific siRNAs to down-regulate endogenous PVT1 expression in these two cell lines. qPCR analysis indicated that PVT1 expression was sufficiently silenced after treated with specific siRNAs (Fig. S1A); therefore, si-PVT1-1# and/or si-PVT1-2# were used for subsequent experiments. Conversely, ectopic overexpression of PVT1 was successfully induced by transfecting these two cells with a pcDNA 3.1-PVT1 expression vector (Fig. S1A).

MTT assays showed that knocking down PVT1 levels significantly inhibited the growth of HTR/SVneo and JEG-3 cells. Additionally, PVT1 overexpression promoted cell proliferation in trophoblast cells (Fig. 2A and B). Similarly, Edu/DAPI immunostaining further confirmed these results (Fig. 2C and D). Taken together, these findings supported the positive role of PVT1 in HTR/SVneo and JEG-3 cell proliferation, a crucial process in the progression of PE.

Next, we used flow cytometry analysis to explore the mechanisms through which PVT1 affected the proliferation of trophoblasts cells. The results revealed that knockdown of PVT1 noticeably induced apoptosis in HTR/SVneo and JEG-3 trophoblasts compared with that in control cells (Fig. 3A). Moreover, cell cycle progression was meaningfully accumulated at G0/G1 phase in trophoblasts cells transfected with si-PVT1-1# compared with that in cells treated with scramble.

![Fig. 3](image-url) Effect of PVT1 on apoptosis and cycle in Trophoblast cells. Trophoblast Cells were treated with siRNAs or pcDNA3.1-PVT1. (A) The apoptotic rates of cells were measured by Flow cytometry. LL, dead cells; UL, viable cells; LR, early apoptotic cells; UR, terminal apoptotic cells. (B) Cell cycle analyses by Flow cytometry in vitro. *P < 0.05, **P < 0.01.
Conversely, overexpression of PVT1 reduced cell cycle accumulation in G0/G1 phase (Fig. 3B).

**Effects of PVT1 on the migration and invasion of HTR/SVneo and JEG-3 trophoblasts**

The migration and invasion of trophoblast cells are critical for diseases progression; therefore, we next evaluated the effects of PVT1 on HTR-8/SVneo and JEG-3 cell migration and invasion using transwell assays. The results showed that decreased PVT1 expression blocked cell migration and invasion compared with that in the control cells (Fig. 4A and B). These results implied that knockdown of PVT1 repressed the migration and invasion of trophoblast cells.

**Gene expression profiling**

To elucidate PVT1-associated changes in gene expression, we implemented RNA transcriptome sequencing using control and PVT1-depleted HTR/SVneo cells. A total of 59 mRNAs presented at least a twofold increase in abundance, whereas 74 mRNAs exhibited a twofold or greater reduction in abundance in HTR/SVneo cells after depletion of PVT1 (Fig. 5A; Table S1). Evaluation of pathways activated by PVT1 using GO and KEGG databases showed that cell growth was altered in PVT1-depleted cells (Fig. 5B). Additionally, using qRT-PCR, we verified changes in the expression of representative genes involved in cell growth in HTR-8/SVneo and JEG-3 cells. Among transcripts that were related to cell growth, apoptosis, and migration; Encoding angiopeptin-like 4 (ANGPTL4) was significantly up-regulated/after PVT1 knockdown (Fig. 5C). On the contrary, it was down-regulated after overexpression of PVT1. Western blotting was also performed and further demonstrated that the ANGPTL4 proteins were significantly increased/decreased in PVT1-depleted/PVT1-overexpression cells (Fig. 5D).

**PVT1 epigenetically silenced ANGPTL4 transcription by binding to EZH2**

The subcellular location of a molecule may provide insights into its molecular mechanism. Therefore, we evaluated the localization of PVT1 in nuclear and cytoplasmic fractions in HTR/SVneo and JEG-3 cells using U1 (RNU1) and GAPDH as markers of the nucleus and cytoplasm, respectively (Fig. 6A). Notably, PVT1 RNA was predominantly located in the nucleus in HTR/SVneo and JEG-3 cells. Accordingly, PVT1 may have functions in transcriptional regulation.

Previous studies have shown that have covered that nearly 20% of human lncRNAs can bind to PRC2 which contained EZH2, SUZ12 and embryonic ectoderm development [EED] in various cells and exert their regulatory functions through interacting with RNA-binding proteins (RBPs), particularly PRC2 [32, 33]. Thus, we hypothesized that PVT1 might modulate ANGPTL4 expression by recruiting PRC2 in HTR/SVneo and JEG-3 cells. Indeed, RNA immunoprecipitation

**Fig. 4** Effect of PVT1 on Trophoblast cell migration and invasion in vitro. (A and B) Transwell assays were performed to detect the role of PVT1 on trophoblast cell migration and invasion in vitro. The migration and invasion viability of the trophoblast cell transfected with si-PVT1 were meaningfully lower than that of the control. The cells on the lower chamber were stained by crystal violet and displayed, and cell number in random five perspectives was analysed. *P < 0.05; **P < 0.01.
(RIP) showed sufficient enrichment of PVT1 by anti-EZH2 antibodies compared with that of the nonspecific IgG control (Fig. 6B).

We further explored the functional relevance of the relationship between PVT1 and EZH2. Repression of EZH2 expression by siRNAs showed that ANGPTL4 was up-regulated (Fig. 6C). Additionally, because PRC2 is known to regulate transcription through histone modification, H3K27me3 in the promoter region of target genes [34, 35] we speculated that PVT1 may modulate ANGPTL4 through epigenetic mechanisms in trophoblast cells. Using ChIP assays with antibodies against EZH2 and H3K27me3, we analysed that Ez2 and H3K27me3 were enriched in the promoter region of ANGPTL4 genes. Importantly, after PVT1 silencing using a siRNA, EZH2 enrichment and H3K27me3 levels were significantly decreased in the promoter regions of ANGPTL4 (Fig. 6D).
ANGPTL4 expression levels are elevated in PE placental tissues

To establish the clinical significance of ANGPTL4, we first tested the expression levels of ANGPTL4 mRNAs in placental tissues from women with severe PE and normal pregnancies. The results indicated that ANGPTL4 mRNAs were up-regulated in placental tissues from women with severe PE (Fig. 7A). In addition, immunohistochemistry showed that ANGPTL4 protein levels were increased in placental tissues from women with severe PE (Fig. 7B). Taken together, our findings confirmed that ANGPTL4 expression levels were inversely related to PVT1 levels in placental tissues from patients with severe PE.

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Discussion

Gene expression profiling technologies and genome-wide sequencing have recently been utilized to accelerate the identification of human genome transcripts. Approximately 90% of transcriptional products do not encode proteins [36]; these transcripts are known as nonprotein-coding RNAs (ncRNAs) and include microRNAs (miRNAs) and IncRNAs. Many previous studies have shown that IncRNAs are involved in various human disorders and cellular development [27, 37]. Moreover, imbalance in IncRNAs affects essential cell biological processes, including cell proliferation, apoptosis [38–40], migration [40] and metastasis, via various molecular mechanisms [41, 42]. We observed roles of maternally expressed 3 (MEG3) and sprouty RTK [40] and metastasis, processes, including cell proliferation, apoptosis [38–40], migration [40] and metastasis, via various molecular mechanisms [41, 42]. We observed roles of maternally expressed 3 (MEG3) and sprouty RTK signalling antagonist 4-intronic transcript 1 (SPRY4-IT1) in our previous studies evaluating the progression of PE [29, 30]. Consequently, identification of PE-associated IncRNAs and exploration of their biological functions and clinical significance may provide insights into the improvement of lncRNA-based diagnosis and prognosis in PE. However, the biological behaviour and molecular mechanisms of these IncRNAs in the pathogenesis of PE are still unclear.

PVT1 has been explored in numerous pathological processes, including colorectal cancer [43], diabetic nephropathy [44] and gastric cancer [33]. In the present study, we found that the relative PVT1 levels were dramatically reduced in placental samples from women with PE compared with that in the control. Moreover, knockdown of PVT1 inhibited HTR/SVneo cell proliferation and promoted apoptosis in vitro; conversely, overexpression of PVT1 promoted proliferation. Silencing of PVT1 expression stimulated G0/G1 phase accumulation and S phase reduction; however, PVT1 overexpression promoted the progression of the trophoblast cell cycle. Together, our findings imply that PVT1 might play an indispensable role in the proliferation and apoptosis of trophoblasts in PE and that PVT1 might be a promising marker for foreseeing PE.

In this study, we found that PVT1 was predominantly localized in the nucleus, indicating that PVT1 may affect transcriptional regulation. LncRNAs, which serve as molecular bait, can indirectly exert biological functions by binding to or titrating away RNAs or proteins, such as growth arrest and DNA-damage inducible, alpha [45] and WD repeat domain 5 [26]. Among these RBPs, approximately 20% of all human IncRNAs bind to PRC2 to regulate downstream targets; [32] the PRC2 complex can mediate gene silencing by catalysing the trimethylation of H3K27. Our results showed that endogenous PVT1 can interact with EZH2 protein in HTR/SVneo and JEG-3 cells. Thus, PVT1 may modulate PRC2-mediated epigenetic regulation to affect the function of trophoblast cells and then may contribute to the occurrence and progression of PE.

Through a combination of RNA transcriptome sequencing and qRT-PCR, we found that angiopoietin-like 4 (ANGPTL4), which has been reported to be related to cell cycle and apoptosis [46] was markedly up-regulated after PVT1 knockdown. ANGPTL4, a protein-coding RNA gene located on chromosome 19p13.2, was identified to encode an adipokine involved in multiple biological processes such as cell progression and metastasis. However, the function of ANGPTL4 in preeclampsia has not been studied. Here, we showed that EZH2 silenced ANGPTL4 expression by epigenetic regulation. Thus, we propose that PVT1 may exert its functions in part by binding to PRC2 and inhibiting ANGPTL4 expression in trophoblasts in the context of PE.

In summary, we showed that PVT1 was down-regulated in clinical samples from patients with PE and that down-regulation of this IncRNA may be associated with the pathogenesis of PE. Moreover, our study provides insights into the role of PVT1 as a participant of the PRC2-mediated epigenetic regulatory pathway in PE and as a novel indication for early diagnosis and treatment of PE. Further studies are required to illuminate other potential mechanisms through which PVT1 participates in the biological functions of trophoblasts in PE.

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Y.X., Y.L., N.Y. designed and performed the research and wrote the manuscript together. Y.X. analysed the data, and Y.X., Y.L., O.Z. helped to design the research. The others (S.H., Y.C., D.W., L.S.) contributed to the placental specimen collection.
Conflicts of interest

No conflicts of interest were stated by authors.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

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