Long noncoding RNA PVT1 promotes cervical cancer progression through epigenetically silencing miR-200b

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Long noncoding RNA PVT1 has been reported to be dysregulated and play vital roles in a variety of cancers. However, the functions and molecular mechanisms of PVT1 in cervical cancer remain unclear. The objective of this study was to investigate the expression, clinical significance, biological roles, and underlying functional mechanisms of PVT1 in cervical cancer. Our results revealed that PVT1 is upregulated in cervical cancer tissues. Enhanced expression of PVT1 is associated with larger tumor size, advanced International Federation of Gynecology and Obstetrics stage, and poor prognosis of cervical cancer patients. Using gain-of-function and loss-of-function approaches, we demonstrated that overexpression of PVT1 promotes cervical cancer cells proliferation, cell cycle progression and migration, and depletion of PVT1 inhibits cervical cancer cell proliferation, cell cycle progression, and migration. Mechanistically, we verified that PVT1 binds to EZH2, recruits EZH2 to the miR-200b promoter, increases histone H3K27 trimethylation level on the miR-200b promoter, and inhibits miR-200b expression. Furthermore, the effects of PVT1 on cervical cell proliferation and migration depend upon silencing of miR-200b. Taken together, our findings confirmed that PVT1 functions as an oncogene in cervical cancer and indicated that PVT1 is not only an important prognostic marker, but also a potential therapy target for cervical cancer.

Key words: Long noncoding RNA; epigenetics; microRNA; cervical cancer; progression.

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Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death among females worldwide (1, 2). Although many diagnostic techniques and therapeutic strategies for cervical cancer have improved, the overall prognosis of cervical cancer patients remains poor (3, 4). Thus, a better understanding of the molecular mechanisms underlying cervical cancer development and progression is urgent for improving cervical cancer patients’ prognosis (5).

Increasing amount of evidence has shown that long noncoding RNAs (lncRNAs), which are more than 200 nucleotides in length with no protein coding potential, play critical roles in many cellular biological processes and are deregulated in various types of cancers (6–9). As to functional mechanisms, lncRNAs can modulate gene expression in transcriptional, post-transcriptional, translational, or post-translational levels (10–16). Particularly, numerous lncRNAs have been identified to associate with Polycomb Repressive Complex 2 (PRC2) to change chromatin formation in specific gene loci (17, 18). PRC2 contains enhancer of zeste homolog 2 (EZH2), SUZ12, and EED. EZH2 is a histone methyltransferase that induces histone H3 lysine 27 trimethylation (H3K27me3) in specific gene loci and then inhibits gene expression (19).

Long noncoding RNA PVT1 maps to chromosome 8q24, a region with frequent copy number amplification (20). Many reports have shown that PVT1 is overexpressed in many cancers, and is associated with reduced survival in hepatocellular carcinoma, breast cancer, ovarian cancer, gastric cancer, and colorectal cancer patients (21–25). Furthermore, PVT1 play critical roles in various biological processes, such as stem cell-like property, proliferation, cell cycle, apoptosis, and mobility (20, 21, 26, 27). However, the functions and molecular mechanisms of PVT1 in cervical cancer remain largely unknown.

MicroRNAs (miRNAs) are another class of non-coding RNAs with lengths of 17–25 nucleotides,
which regulate gene expression by inducing mRNA degradation and/or blocking mRNA translation (28, 29). miRNAs also play critical roles in diverse cellular biological processes and are deregulated in various types of cancers (30, 31). miRNAs have been implicated as oncogenes or tumor suppressors (32, 33). However, whether lncRNA could regulate miRNA transcription, and the interaction between lncRNA, histone methylation and miRNA remain to be investigated.

In this study, we evaluated the expression of PVT1 in cervical cancer tissues and adjacent non-cancerous tissues, and then analyzed the correlation between PVT1 expression and tumor size, clinical stage, patients' prognosis. Using gain-of-function and loss-of-function approaches, we investigated the effects of PVT1 on cervical cancer cells proliferation, cell cycle, and migration. Furthermore, we explored the molecular mechanisms underlying how PVT1 exerts its functional roles. Our results demonstrated that PVT1 directly binds to EZH2, recruits EZH2 to the miR-200b promoter, increases H3K27me3 level on the miR-200b promoter, and inhibits miR-200b expression.

MATERIALS AND METHODS

Patients and samples

A total of 90 cervical cancer tissues and adjacent non-cancerous tissues were obtained with informed consent from patients who were treated at Yantaishan Hospital, Yantai, Shandong Province, China. All patients underwent radical resections and were classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria (34). The clinical characteristics of the 90 cervical cancer patients are described in Table S1. The Ethics Review Committee of Yantaishan Hospital approved this study.

Cell culture

The human cervical cancer SiHa and HeLa cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were grown in DMEM medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) under a humidified atmosphere of 5% CO2 at 37 °C.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. First-strand cDNA was generated using the M-MLV Reverse Transcriptase (Invitrogen). Real-time PCR was carried out using the standard SYBR-Green PCR kit protocol on ABI-7300 (Applied Biosystems, Foster City, CA, USA). GAPDH was employed as an endogenous control for PVT1. The primer sequences used were as follows: for PVT1: 5'-ATA GATCCTGCCCCTGTGTC-3' (forward) and 5'-CATTTC CTGCGCCTTTTCT-3' (reverse); for GAPDH: 5'-GGA GCGAGATCCCTCAAAAT-3' (forward) and 5'-GGCT GTTTGCTATCTTCATGG-3' (reverse). For miR-200b expression analysis, real-time PCR was performed as above, using TaqMan microRNA assays according to the manufacturer’s protocols (Applied Biosystems). U6 was employed as an endogenous control for miR-200b. The annealing temperature for real-time PCR was 60 °C.

Vectors construction

The cDNA encoding PVT1 was PCR amplified by the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher, Waltham, MA, USA) and subcloned into the Kpn I and BamH I sites of pcDNA3.1 (Invitrogen), named pcDNA3.1-PVT1. The primers used were as follows: 5'-GGGGATCCCTCCGGGACACGCAGGCGTGG-3' (forward) and 5'-GGGGATCCCTCCGGGACACGCAGGCGTGG-3' (reverse). pcDNA3.1-PVT1 was double digested with Kpn I and BamH I, and the PVT1 fragment was subcloned into pSPT19 (Roche, Mannheim, Germany), named pSPT19-PVT1.

Small interfering RNA (siRNA) synthesis and transfection

siRNAs specifically targeting PVT1 and miR-200b mimics were purchased from Applied Biosystems. Transfection and co-transfection were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. The transfected cells were harvested at directed time after transfection.

Cell proliferation assay

Identical number of cervical cancer cells was plated in 96-well plates 24 h after transfection. Cell proliferation was detected using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. The cell proliferation curves were acquired using the absorbance at 450 nm at each time point. Ethynyl deoxyuridine (EdU) incorporation assays were performed with an EdU Kit (Roche) according to the manufacturer’s protocols.

Cell cycle analysis

Forty-eight hours after transfection, SiHa and HeLa cells were plated in medium without FBS in the upper chamber of a transwell insert (Millipore, Bedford, MA, USA). Medium containing 20% FBS was added...
to the lower chamber. After 24 h incubation, the cells that migrated into the outer surface of the transwell insert were stained with 0.5% crystal violet solution and manually counted for three independent experiments.

RNA pull-down assay

PVT1 were in vitro transcribed and biotin-labeled from vector pSPT19-PVT1, with SP6 RNA polymerase (Roche), and Biotin RNA Labeling Mix (Roche). One milligram of protein lysates from HeLa cells were mixed with 3 μg of purified biotinylated PVT1 for 1 h at 4 °C. Complexes were incubated with streptavidin agarose beads (Invitrogen) for 1 h and then washed three times with PBS at room temperature. The protein present in the pull-down material was detected by Western blot analysis.

Western blot

The retrieved proteins from RNA pull-down assay were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After incubation with antibodies specific for EZH2 (Millipore, Bedford, MA, USA) or β-actin (Cell Signaling Technology, Boston, USA), the blots were incubated with streptavidin–horseradish peroxidase conjugate and visualized with enhanced chemiluminescence.

RNA Immunoprecipitation (RIP)

RIP experiments were carried out using the EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) and EZH2 antibody (Millipore) according to the manufacturer's protocol. Retrieved RNAs were detected by RT-PCR. The primer sequences used were as follows: for PVT1: 5'-ATAGATCCTGCCTTTGGA-3' (forward) and 5'-CATTCCTGCTGGTTTTC-3' (reverse); and for β-actin: 5'-GGGAAATCGTGCGTGACAT-3' (forward) and 5'-TGTTGGACGTACAGGTC-3' (reverse).

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were carried out using the EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore), EZH2 antibody (Millipore), and H3K27me3 antibody (Millipore). ChIP-derived DNA was quantified using RT-PCR with SYBR-Green PCR kit (Applied Biosystems). The primers specific for the miR-200b promoter were: 5'-CTGCCTCACGACTCTGG-3' (forward) and 5'-AACAATCGCCCTCCTCTG-3' (reverse).

Statistical analysis

SPSS 18.0 software package was used for all statistical analyses. For comparisons, Wilcoxon signed-rank test, Log-rank test, Student’s t-test and Pearson correlation analysis were performed as indicated (16). p < 0.05 was defined as statistically significant differences.

RESULTS

PVT1 is overexpressed in cervical cancer and correlates with poor prognosis

To investigate the role of PVT1 in cervical cancer, we first examined PVT1 expression in 90 pairs of cervical cancer tissues and adjacent non-cancerous tissues using RT-PCR. The results showed that PVT1 were significantly upregulated in cervical cancer tissues compared with corresponding non-cancerous tissues (Fig. 1A). Next, we analyzed the correlation of PVT1 expression level with clinical characteristics of the 90 cervical cancer patients. The results showed a significant association between PVT1 upregulation and larger tumor size (Fig. 1B), advanced FIGO stage (Fig. 1C).

We further examined the relationship between PVT1 expression level and cervical cancer patients' prognosis. Kaplan–Meier survival analysis and log-rank test showed that patients in PVT1 high expression group had a significant poorer overall survival than those in PVT1 low expression group (Fig. 1D).

PVT1 promotes the proliferation and migration of cervical cancer cells

We next study the function of PVT1 through gain-of-function and loss-of-function approaches. PVT1 overexpression vector or PVT1-specific siRNA was transiently transfected into SiHa or HeLa cells. The overexpression or depletion of PVT1 was confirmed by measuring PVT1 expression (Fig. 2A,B). CCK-8 assays showed that upregulation of PVT1 significantly promoted cell proliferation (Fig. 2C); and while the depletion of PVT1 significantly inhibited cell proliferation (Fig. 2D). EdU incorporation assays were also performed to evaluate effect of PVT1 on cell proliferation. As shown in Fig. 2E,F, enhanced expression of PVT1 upregulated the percentage of EdU positive cells, and while PVT1 knockdown decreased the percentage of EdU positive cells. To evaluate whether the effect of PVT1 on cervical cancer cell proliferation is through affecting cell cycle progression, we measured the cell cycle distribution of cervical cancer cells after PVT1 overexpression or knockdown. The results showed that enhanced expression of PVT1 decreased the percentage of cells at G0/G1 phase, and increased the percentage of cells at G2/M phases (Fig. 2G). PVT1 knockdown increased the percentage of cells at G0/G1 phase, and decreased the percentage of cells at S and G2/M phases (Fig. 2H). These data demonstrated that PVT1 significantly promotes cervical cancer...
cell proliferation through promoting cell cycle progression.

To examine the effect of PVT1 on cervical cancer cell migration, we performed transwell migration assays. As shown in Fig. 2I,J, enhanced expression of PVT1 significantly increased the number of migrated cells, and while PVT1 knockdown significantly decreased the number of migrated cells. Collectively, these results confirm that PVT1 promotes cervical cancer cells proliferation and migration.

PVT1 physically associates with EZH2 and epigenetically silences miR-200b

Recent studies have shown that twenty percent of lncRNAs could physically associate with Polycomb Repressive Complex 2 (PRC2) and many lncRNAs could change PRC2 genomic location (17, 18). To evaluate whether PVT1 could also bind PRC2 and affect gene expression in such a manner, we used in vitro transcribed biotin-labeled PVT1 to pull down endogenous proteins associated with PVT1. Biotin-labeled PVT1 was incubated with HeLa cell lysates, followed by protein extraction and detection by Western blot with an antibody against EZH2 (an important subunit of the PRC2). The results showed a significant enrichment of EZH2 (but not β-actin) in the presence of PVT1 compared with antisense RNA (negative control) (Fig. 3A). In addition, we performed RIP assays to further confirm the association between PVT1 and EZH2 (Fig. 3B).

EZH2 has been shown to induce histone H3 lysine 27 trimethylation (H3K27me3) on the miR-200b promoter and silence miR-200b expression (35). miR-200b has been reported to have a critical role in many cancer cells proliferation and migration (36, 37). So we next determine the functional relevance of the association between PVT1 and EZH2 on miR-200b. ChIP assays showed that enhanced expression of PVT1 promoted the binding of EZH2 and increased H3K27me3 levels on the miR-200b promoter (Fig. 3C). PVT1 knockdown inhibited the binding of EZH2 and decreased H3K27me3 levels on the miR-200b promoter (Fig. 3D). Analysis with RT-PCR showed that overexpression of PVT1 inhibited miR-200b expression, and while depletion of PVT1 increased miR-200b expression (Fig. 3E,F). These data...
demonstrated that through associating with EZH2, PVT1 promoted the binding of EZH2 on the miR-200b promoter, upregulated H3K27me3 levels on the miR-200b promoter, and silenced miR-200b expression.

PVT1 expression is inversely correlated with miR-200b expression in human cervical cancer tissues

We next measured the miR-200b expression levels in the same set of 90 pairs of cervical cancer tissues.
Fig. 3. PVT1 binds EZH2 and epigenetically silences miR-200b. (A) RNA pull-down assays show a significant enrichment of EZH2 in the presence of PVT1. Biotin-labeled PVT1 was incubated with HeLa cell lysates, and the extracted protein was detected by Western blot using an EZH2 or β-actin antibody. (B) RIP experiments were performed in HeLa cells using an EZH2 antibody or non-specific IgG, and specific primers were used to detect PVT1 or β-actin. (C) ChIP analyses in SiHa cells 48 h after pcDNA3.1-PVT1 transient transfection were performed on the miR-200b promoter regions using anti-EZH2 and H3K27me3 antibodies. Enrichment was determined relative to input. (D) ChIP analyses in HeLa cells 48 h after PVT1 siRNA transient transfection were performed on the miR-200b promoter regions using anti-EZH2 and H3K27me3 antibodies. Enrichment was determined relative to input. (E) The expression of miR-200b in SiHa cells 48 h after pcDNA3.1-PVT1 transient transfection. (F) The expression of miR-200b in HeLa cells 48 h after PVT1 siRNA transient transfection. Data are shown as mean ± SD based on three independent biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001 by Student’s t-test.
and adjacent non-cancerous tissues shown in Fig. 1A. The results showed that miR-200b was significantly downregulated in cervical cancer tissues compared with corresponding non-cancerous tissues (Fig. 4A). We then investigated the correlation between PVT1 and miR-200b expression levels. A statistically significant inverse correlation was found between PVT1 and miR-200b expression levels in cervical cancer tissues (Fig. 4B).

The effect of PVT1 on cervical cancer cells proliferation and migration is dependent upon silencing miR-200b

To test whether miR-200b mediated the effect of PVT1 on cervical cancer cells proliferation and migration, we overexpressed miR-200b in PVT1 upregulated SiHa cells (Fig. 5A) and then measured SiHa cells proliferation, cell cycle, and cell migration. CCK-8 assays and EdU incorporation assays showed that overexpression of miR-200b abolished the effect of PVT1 on cervical cancer cells proliferation (Fig. 5B and C). Cell cycle analyses showed that overexpression of miR-200b abolished the effect of PVT1 on cell cycle progression (Fig. 5D). Transwell migration assays showed that overexpression of miR-200b abolished the effect of PVT1 on cervical cancer cells migration (Fig. 5E). These results demonstrated that PVT1 promotes cervical cancer cells proliferation and migration through silencing miR-200b.

DISCUSSION

In this study, we measured the expression, function and functional mechanisms of long noncoding RNA PVT1 in cervical cancer. Our results showed that PVT1 is upregulated in cervical cancer tissues in comparison with adjacent non-cancerous tissues and that enhanced PVT1 expression is associated with larger tumor size, advanced FIGO stage, and poor prognosis of cervical cancer patients. Using gain-of-function and loss-of-function approaches, we demonstrated that overexpression of PVT1 promotes cervical cancer cells proliferation, cell cycle progression and migration, and depletion of PVT1 inhibits cervical cancer cell proliferation, cell cycle progression and migration. Collectively, our results confirmed that PVT1 functions as an oncogene in cervical cancer.

Many reports have shown that lncRNAs may modulate gene expression through binding to proteins, mRNAs or miRNAs, and then changing their expressions, locations or functions (9, 13, 14, 38). Although PVT1 has been studied in hepatocellular carcinoma, breast cancer, gastric cancer, non-small cell lung cancer, colorectal cancer, and ovarian cancer (21–24, 39), its possible functions and molecular mechanisms in cervical cancer are still largely unknown. Previous study showed that PVT1 binds to c-Myc and protects c-Myc from phosphorylation and subsequent degradation in breast cancer (20). Another report showed that PVT1 binds to NOP2 and increases its protein stability in hepatocellular carcinoma (21). In gastric cancer, PVT1 was shown to bind to EZH2 and epigenetically regulating p15 and p16 (23). These studies suggest that in different cancers PVT1 may exert its function through different mechanisms. In this study, using RNA pull-down, RIP and ChIP assays, we found that PVT1 binds to EZH2, recruits EZH2 to the miR-200b promoter, increases H3K27me3 level on the miR-200b promoter, and inhibits miR-200b expression in cervical cancer. Furthermore, the effects of PVT1 on cervical cell proliferation and migration depend upon silencing of miR-200b.
Belonging to the miR-200 family, miR-200b has been reported to be deregulated in esophageal squamous cell carcinoma, gastric cancer, breast cancer, lung cancer, ovarian cancer, glioma, and hepatocellular carcinoma (40–46). In addition, miR-200b has been shown to inhibit cancer cell proliferation and migration (46, 47). However, the expression of miR-200b in cervical cancer and the factors contributing to miR-200b deregulation is still unknown. In this study, we have also shown that miR-200b is downregulated in cervical cancer, and PVT1 inhibits miR-200b expression through changing histone methylation. PVT1 and miR-200b expression levels are inversely correlated in cervical cancer tissues, which support the regulation of miR-200b by PVT1. Our study shows a new epigenetically modulation between lncRNA and miRNA.

In conclusion, our studies demonstrate that PVT1 is upregulated in cervical cancer and
indicates poor prognosis for cervical cancer patients. By physically associating with EZH2, PVT1 epigenetically silences miR-200b expression. By silencing miR-200b, PVT1 promotes the proliferation, cell cycle progression, and migration of cervical cancer cells. These findings suggest that PVT1 may not only be an important prognostic marker in cervical cancer, but also be a potential target for cervical cancer therapy.

**CONFLICTS OF INTEREST**

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found 
online in the supporting information tab for this 
article:
Table S1. The clinical characteristics of the 90 
cervical cancer patients.