Long Non-coding RNA PVT1 Promotes Cell Proliferation and Migration by Silencing ANGPTL4 Expression in Cholangiocarcinoma

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Cholangiocarcinoma (CCA) is the most common biliary tract malignancy, with a low survival rate and limited treatment options. Long non-coding RNAs (lncRNAs) have been verified to have significant regulatory functions in many kinds of human cancers. It was discovered in this study that the lncRNA PVT1, whose expression is significantly elevated in CCA, could be a molecular marker of CCA. Experiments indicated that PVT1 knockdown greatly inhibited cell migration and proliferation in vitro and in vivo. According to RNA sequencing (RNA-seq) analysis, PVT1 knockdown dramatically influenced target genes associated with cell angiogenesis, cell proliferation, and the apoptotic process. RNA immunoprecipitation (RIP) analysis demonstrated that, by binding to epigenetic modification complexes (PRC2), PVT1 could adjust the histone methylation of the promoter of ANGPTL4 (angiopoietin-like 4) and, thus, promote cell growth, migration, and apoptosis progression. The data verified the significant functions of PVT1 in CCA oncogenesis, and they suggested that PVT1 could be a target for CCA intervention.

INTRODUCTION
Cholangiocarcinoma (CCA), the most common biliary tract cancer, represents 3% of all gastrointestinal malignancies, and it is described as a malignancy originating from the ductal epithelial cells lining the intrahepatic and extrahepatic biliary ducts.1 According to its anatomic origin, CCA is classified as intrahepatic (ICC), perihilar (pCCA), or distal (dCCA) CCA.2 CCA is a destructive malignancy with an extremely poor general 5-year survival rate of less than 10%,3 and patients have a median survival of 24 months after diagnosis, indicating that CCA has a poor prognosis.3 Surgical resection and liver transplantation are possibly curative treatments for early stage CCA patients, and the median 5-year survival after R0 resection is approximately 30%;4 however, because of the destructive malignancy of CCA, most patients already have advanced disease at diagnosis.3 Furthermore, patients with CCA have insensitivity to conventional chemotherapy or radiotherapy.6 Therefore, there are no potentially curative clinical therapeutic interventions for CCA, and no targeted molecular therapies have been adopted for use in CCA.

Long non-coding RNAs (lncRNAs) represent enormous RNA families that are defined by a length of over 200 nt. lncRNAs have limited protein-coding potential and lack detectable open reading frames (ORFs), which are necessary for protein-coding potential.7–11 Recently, lncRNAs have been demonstrated to play pivotal roles in many biological processes, such as cellular proliferation, development, and differentiation. lncRNAs can adjust gene expression in diverse biological functions by binding with transcription factors,12 chromatin-modifying factors,13,14 and heterogeneous nuclear ribonucleoproteins (hnRNPs);15 lncRNAs can also act as regulators targeting the splicing, translation, or stability of host mRNAs through post-transcriptional mechanisms, or they can act as endogenous microRNA sponges to modulate microRNA targets.16–18 It is noteworthy that the abnormal expression of IncRNAs has been proven in many cancers, such as CCA.19–26

Plasmacytoma variant translocation 1 (PVT1) localizes downstream of the MYC gene and maps to chromosome 8q24. PVT1
specifically facilitates the invasive pathophysiology of ovarian cancer, breast cancer, and esophageal squamous cell carcinoma, and overexpression of PVT1 is an effective predictor of oncogenesis and overall survival in patients with multifarious cancers, including colorectal cancer and gastric cancer. However, the association between the abnormal expression and biological functions of PVT1 in CCA and the underlying mechanisms remains undiscovered.

We discovered a CCA-specific upregulated lncRNA, PVT1, that displayed markedly elevated expression levels in CCA compared to normal tissues. The functional association between the underlying molecular mechanism and the effect of its overexpression in CCA was not determined.

RESULTS

PVT1 Is Upregulated in Human CCA Tissues

PVT1, situated at the chromosomal locus 8q24.21, encodes a transcript of 1,957 bp. To conduct a comprehensive characterization of aberrantly expressed lncRNAs in CCA, an analysis of GSE CCA and normal tissue microarray data was conducted; the results showed that PVT1 expression is higher in tumor tissues than in ordinary tissues in the GEO: GSE61850 and GSE63420 datasets (Figures 1A and 1B). To verify this finding, PVT1 expression in a cohort of 17 paired CCA tumors and ordinary tissues was detected with qRT-PCR, and the results confirmed that PVT1 was markedly upregulated in carcinoma tissues (Figure 1C). However, the functional association and underlying molecular mechanism of PVT1 and the effectors involved in its overexpression were not determined.

Knockdown of PVT1 Inhibits CCA Cell Proliferation and Migration In Vitro

First, qRT-PCR was conducted to study the biological functional effects of PVT1 dysregulation in CCA. As shown in Figure 2A, the qRT-PCR results showed that the expression of PVT1 in the small interfering RNA (siRNA)-mediated knockdown group was significantly lower than that in the scrambled negative control siRNA (si-NC) group for the HuCCT1 and RBE cell lines. Colony formation was greatly decreased with knockdown of PVT1 (Figure 2B). Additionally, CCK-8 assays revealed that knockdown of PVT1 expression significantly reduced cell viability in both the HuCCT1 and RBE cell lines compared with that in the control cells (Figure 2C). Transwell assays showed that knockdown of PVT1 dramatically repressed the migration of cells (Figure 2D).

Knockdown of PVT1 Causes Apoptosis by Promoting Cell-Cycle Arrest In Vitro

To further study whether PVT1 could affect apoptosis in CCA cell lines, flow cytometry was performed. The findings revealed that the HuCCT1 and RBE cell lines transfected with PVT1 siRNA had higher apoptotic rates than did the control group (Figure 3A). Next, to determine whether the impacts of PVT1 on CCA cell proliferation and migration were due to PVT1-mediated alterations in the progression of the cell cycle, we performed flow cytometry assays in both the HuCCT1 and RBE cell lines. The flow cytometry assays revealed that PVT1 knockdown increased the proportion of cells in the G0/G1 phase and reduced the proportion of cells in the S and G2/M phases compared to the proportions in the control cells (Figure 3B). All the data suggested that PVT1 could accelerate cell proliferation and migration by
influencing cell cycle progression and inhibiting apoptosis in CCA cell lines.

**Knockdown of PVT1 Inhibits CCA Cell Tumorigenesis In Vivo**

To confirm whether PVT1 influences CCA tumorigenesis in vivo, HuCCT1 cells transfected with sh-PVT1 or a control vector were injected into nude mice. At 16 days post-injection, the tumors established in the sh-PVT1 group were dramatically smaller than those in the control group (Figures 4A and 4B). Correspondingly, the average tumor volumes and weights in the final experiment were markedly lower in the sh-PVT1 group than in the control vector group (Figures 4C and 4D). These findings indicated that silencing PVT1 could repress CCA tumor growth in vivo.

**Related Target Genes of PVT1 in CCA**

To define the target mRNAs that could be regulated by PVT1 in CCA, RNA transcriptome sequencing was performed after transfection with control siRNA or siRNAs against PVT1. Upon PVT1 silencing, a set of 540 common mRNAs showed ≥1.5-fold increases in abundance, while 755 genes showed decreased abundance (≤1.5-fold). To prioritize the genes most related to PVT1, attention was paid to the genes that were the most highly expressed upon knockdown of PVT1. Interestingly, many well-known genes related to proliferation and migration (e.g., ETV5, EREG, ENCI, ANGPTL4, sprouty RTK signaling antagonist 4 [SPRY4], GDF15, and others) were included (Figure 5A; Table S2). Moreover, gene ontology analysis indicated that the most markedly overrepresented biological processes were...
pathways involved in angiogenesis, cell proliferation, and apoptosis progression (Figure 5B). Some of these genes were verified by qRT-PCR after knockdown (Figures 5C and 5D) and overexpression (Figure 5E) of PVT1 in HuCCT1 and RBE cells, respectively.

**PVT1 Binds with EZH2 to Coregulate Target Genes, Especially ANGPTL4**

According to recent literature, many lncRNAs have been verified to cooperate with chromatin-modifying enzymes to accelerate epigenetic activation and, thus, silence target gene expression. In particular, PRC2, a classic methyltransferase consisting of enhancer of zeste homolog 2 (EZH2), EED, and SUZ12 subunits, can serve as a catalyst not only in the dimethylation but also in the trimethylation of lysine residue 27 of histone 3 (H3K27me3), thus epigenetically repressing the expression of target genes.

In our study, to investigate the mechanism of PVT1-mediated regulation, subcellular fractionation assays were first performed, which verified the localization of PVT1 mainly in the nucleus (Figure 6A). Similarly, an RNA fluorescence in situ hybridization (FISH) assay showed that PVT1 localized mainly in the nuclei of RBE cells (Figure 6B). In addition, the probability of interaction of EZH2 and PVT1 was determined with an online tool (http://pridb.gdb.iastate.edu/RPISeq/index.html), and the result showed that EZH2 could bind with PVT1 well (random forest [RF] = 0.75, support vector machine [SVM] = 0.83) (Figure 6C). As revealed in Figure 6D, relative endogenous PVT1 expression was amplified in the anti-EZH2 RNA immunoprecipitation (RIP) fraction compared to the immunoglobulin G (IgG) fraction in the HuCCT1 and RBE cell lines. Furthermore, simultaneous RNA FISH to detect PVT1 and immunofluorescence for EZH2 showed nuclear co-localization mainly in HuCCT1 cells.
A tumor suppressor gene in CCA. PVT1 and EZH2, angiopoietin-like 4 (ANGPTL4) (Figure 6J). In summary, we elucidated one of the genes coregulated by PVT1 was downregulated when knocked down (Figure 6I). In contrast, the protein level of ANGPTL4 (Figure 6F) in the HuCCT1 and RBE cell lines. In contrast, decreased PVT1’sion of protein level of ANGPTL4 was upregulated when knockdown or EZH2 expression and PVT1-reduced EZH2 binding and H3K27me3 levels throughout the promoters of ANGPTL4 (Figure 7G). In contrast, EZH2 binding and H3K27me3 levels increased throughout the promoters of ANGPTL4 when PVT1 was overexpressed (Figure 7H). These results confirmed that EZH2 could directly bind to the promoter region of ANGPTL4 and then repress ANGPTL4 expression directly by mediating H3K27me3 demethylation (Figure 7I).

To further determine whether PVT1 suppressed the expression of ANGPTL4 by interacting with EZH2, chromatin immunoprecipitation (ChIP) analysis was carried out. ChIP assays revealed that knockdown of PVT1 reduced EZH2 binding and H3K27me3 levels throughout the promoters of ANGPTL4 (Figure 7G). In contrast, EZH2 binding and H3K27me3 levels increased throughout the promoters of ANGPTL4 when PVT1 was overexpressed (Figure 7H). These results confirmed that EZH2 could directly bind to the promoter region of ANGPTL4 and then repress ANGPTL4 expression directly by mediating H3K27me3 demethylation (Figure 7I).

Our results revealed that knockdown of PVT1 reduced EZH2 binding and H3K27 trimethylation levels throughout the promoters of ANGPTL4, confirming that ANGPTL4 is a target gene of PVT1. Our results demonstrated that PVT1 promotes CCA malignancy by binding with EZH2 and then epigenetically repressing the expression of ANGPTL4 in the nucleus.

**DISCUSSION**

Thus far, the newly identified lncRNAs have proven to be critical players in diverse human diseases, particularly in human cancers. The current study demonstrated that the expression of PVT1 in CCA tissues was much higher than that in matched non-tumor

**Figure 4. PVT1 Regulates CCA Cell Proliferation In Vivo**

(A) Tumors established with the mice in the scrambled control or sh-PVT1 group. (B) The size of tumors in the scrambled control or sh-PVT1 group. (C) After cell injection, the tumor volumes were calculated every 4 days. (D) The tumor weights are presented as the means ± SD. The error bars indicate the means ± SD. ***p < 0.001.

First, to assess ANGPTL4 expression in CCA tissues, we performed an analysis of the dataset GEO: GSE26566 (104 cancer tissues and 59 normal tissue samples), and we found that one of the candidate genes, ANGPTL4, was expressed at lower levels in CCA tissues than in normal tissues (Figure 7A). Subsequently, ANGPTL4 expression was found to be lower in CCA tumor tissues than in neighboring tissues in an analysis of a cohort of 17 pairs of CCA tumor tissues and adjacent tissues using qRT-PCR (Figure 7B). Furthermore, overexpression of ANGPTL4 could significantly suppress HuCCT1 and RBE proliferation and migration, and overexpression of ANGPTL4 could partly reverse PVT1-induced growth and migration (Figures 7C–7F).

**PVT1 Binds with EZH2 in the Nucleus and Epigenetically Silences ANGPTL4, Inhibiting Cell Proliferation and Migration in CCA Cell Lines**

ANGPTL4, which is located on chromosome 19p13.2 and encodes a glycosylated, secreted protein with a C-terminal fibrinogen domain, can not only regulate glucose and lipid metabolism and insulin sensitivity but also serve as an apoptosis survival element for vascular endothelial cells, helping these cells avoid metastasis by preventing vascular development and tumor cell invasion. In addition, it has been proven that hypermethylation of the ANGPTL4 promoter region facilitates ANGPTL4 transscriptional inactivation.35

Subsequently, to assess the correlation between EZH2 expression and the candidate target gene signature in CCA tissues, we analyzed the dataset GEO: GSE26566 (104 cancer tissue samples and 59 normal tissue samples) from the Molecular Signature Database, and correlation analysis revealed that ANGPTL4 had a significantly negative correlation with EZH2 in the GEO: GSE26566 dataset (Figure 6H). The protein level of ANGPTL4 was upregulated when PVT1 or EZH2 was knocked down (Figure 6I). In contrast, the protein level of ANGPTL4 was downregulated when PVT1 or EZH2 was overexpressed (Figure 6J). In summary, we elucidated one of the genes coregulated by PVT1 and EZH2, angiopoietin-like 4 (ANGPTL4), which might be a tumor suppressor gene in CCA.

**Figure 5. PVT1 Regulates CCA Cell Migration In Vivo**

(A) mRNA levels of PVT1 in CCA cell lines. (B) mRNA levels of EZH2 in CCA cell lines. (C) mRNA levels of ANGPTL4 in CCA cell lines. (D) Knockdown of PVT1 reduced the migration of HuCCT1 and RBE cell lines. **p < 0.01. ***p < 0.001.
This finding elucidated the fact that PVT1 might play a significant role in CCA malignancy. PVT1 is upregulated in pancreatic cancer,36 esophageal squamous cell carcinoma,29 breast cancer,37 hepatocellular carcinoma,38 and gastric cancer,39 and it has been found to promote the proliferation and invasion of cancer cells in many previous studies.

We discovered that silencing PVT1 could inhibit CCA cell proliferation and migration in vitro and in vivo. Although PVT1 has been claimed to have oncogenic functions in diverse kinds of cancer, the genes regulated by PVT1 have not yet been discovered. Gene ontology analysis of the RNA sequencing (RNA-seq) results after PVT1 knockdown revealed that the altered genes were especially related to proliferation and migration, which was consistent with the prevention of proliferation and migration in the CCA cell line after PVT1 knockdown. Recently, many lncRNAs have been verified to cooperate with chromatin-modifying enzymes to stimulate epigenetic activation and, thus, silence the expression of target genes.32 For instance, the lncRNA PVT1 can interact with EZH2, which is necessary for the repression of p15 and p16 in gastric cancer.31 Moreover, lncRNAs can serve as scaffolds for protein complexes.40–42 For example, the lncRNA CCAT1 can act as a scaffold for double epigenetic modification complexes (the 5′ domain of CCAT1 binds to PRC2 and the 3′ domain binds to SUV39H1), and then it can mediate histone methylation at the promoter locus of SPRY4 in esophageal squamous cell carcinoma.34 Our results showed that PVT1 could bind with EZH2 to form a kind of histone methylation modification complex in the nucleus. Thus, a series of gene expression patterns can be regulated in CCA.

Previous studies have shown that ANGPTL4 can act as a tumor suppressor gene in many types of cancers.43 Nevertheless, the function of ANGPTL4 in the tumorigenesis of CCA remains unknown. In addition, previous studies have verified that DNA methylation of the ANGPTL4 promoter region leads to the inactivation of ANGPTL4 transcription.44 Our data revealed that histone methylation (H3K27me3) modulated by PVT1 could facilitate the reduction in ANGPTL4 expression in CCA cell lines. Taken together, our results demonstrated that PVT1 binds with EZH2 and epigenetically silences ANGPTL4 in the nucleus.

In summary, our study demonstrated the regulatory mechanism of PVT1 in tumorigenesis. Through this mechanism, PVT1 can promote the malignancy of CCA by epigenetically regulating the transcription
of ANGPTL4 in the nucleus, thus facilitating cell survival and the metastasis of CCA. Our results suggest that PVT1 could be a potential novel biomarker and a therapeutic target for CCA patients (Figure 7).

**MATERIALS AND METHODS**

**Tissue Gathering and Ethics Statement**

Altogether, 17 patients were analyzed in this research, all of whom underwent CCA resection at the Second Affiliated Hospital, Nanjing Medical University. All the specimens were instantly frozen in tubes with RNAlater preservation liquid after being removed, and they were kept in liquid nitrogen until the extraction of RNA. Our research was permitted by the Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, PRC), and written informed consent was obtained from every patient.

**Figure 6. PVT1 Binds with EZH2 to Coregulate Target Genes, Especially ANGPTL4**

(A) After nuclear and cytosolic separation, RNA expression levels were measured by qRT-PCR. GAPDH was used as a cytosolic marker and U1 was used as a nuclear marker. (B) Fluorescent images of RBE cells treated with anti-PVT1 (red), anti-18S (red), and anti-U6 (red) RNA probes. DAPI staining is shown in blue. (C) The probability of interaction of EZH2 and PVT1 was determined with an online tool (http://pridb.gdb.iastate.edu/RPISeq/index.html). Predictions with probabilities >0.5 were considered positive. RPI-seq predictions are based on random forest (RF) or support vector machine (SVM). (D) An RIP experiment for EZH2 was performed, and the coprecipitated RNA was subjected to qRT-PCR for PVT1. (E) Expression and co-localization of EZH2 and PVT1 in HuCCT1 cells. Representative fluorescent images show HuCCT1 cells treated with fluorescently labeled anti-EZH2 antibody (green) and anti-PVT1 RNA (red). DAPI staining indicates the cell nuclei (blue). (F) Methylation-related genes were detected by qRT-PCR in the HuCCT1 and RBE cell lines after knockdown of EZH2. (G) Methylation-related genes were detected by qRT-PCR in the HuCCT1 and RBE cell lines after overexpression of EZH2. (H) The correlation between EZH2 and ANGPTL4 expression was detected by analyzing GEO: GSE26566 data. (I) The altered protein levels of ANGPTL4 were selectively confirmed by western blot analysis in cells with knockdown of PVT1 or EZH2. (J) The altered protein levels of ANGPTL4 were selectively confirmed by western blotting in cells overexpressing PVT1 or EZH2. The error bars indicate the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

**RNA Extraction and qRT-PCR Analyses**

All RNA was obtained from cultured cells or specimens with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, for qRT-PCR, 1 μg RNA was reverse transcribed into cDNA with a reverse transcription kit (Takara, Tokyo, Japan). Real-time PCR analyses were carried out with SYBR Green (Takara, Tokyo, Japan). The findings were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences are shown in Table S1.

**Cell Culture**

The CCA cell lines HuCCT1 and RBE were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in DMEM (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (ScienCell, Carlsbad, CA, USA), 100 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen, Shanghai, China), in humidified air at 37°C with 5% CO₂.
Transfection of Cell Lines
Usually, CCA cells were seeded in six-well plates and transfectioned with a particular siRNA (100 nM) or si-NC (100 nM) on the next day using Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol (Invitrogen). The si-NC was purchased from Invitrogen (Invitrogen, CA, USA). sh-PVT1 was cloned into the pENTR/U6 vector, and the sequences of the siRNAs and small hairpin RNA (shRNA) are summarized in Table S1.

Cell Proliferation Analysis
Cell viability was monitored with a CCK-8 (Houston, TX, USA) following the manufacturer’s suggestions. HuCCT1 and RBE cells transfected with siRNA or si-negative control (NC) (3,000 cells/well) were cultivated in five 96-well plates with six replicate wells. For the colony formation assay, 500 transfected cells were combined in a six-well plate and kept in medium with 10% FBS for 2 weeks, with the replacement of medium every 4 days. Then, the colonies were confirmed with methanol and dyed with 0.1% crystal violet (Sigma-Aldrich) for 15 min. Colony formation was determined as the quantity of visibly stained colonies. Wells were measured in triplicate for the different treatment groups.

Assays of Cell Invasion
For the migration assays, after 24 hr of transfection, 3.5 × 10^4 cells in medium with 1% FBS were added to the upper chamber of a transwell insert (Millipore, Billerica, MA, USA), while medium with 10% FBS was added to the lower chamber. After 24 hr of incubation, the remaining cells on the upper level of the membrane were cleaned, while those cells that had migrated through the membrane were dyed with methanol and 0.1% crystal violet and then imaged with an IX71 inverted microscope (Olympus, Tokyo, Japan). The experiment was performed in triplicate.

Flow Cytometric Analysis
Flow cytometry assays were performed as previously reported by Xu et al. After the cells were transfected with siRNAs for 48 hr, we harvested the cells and performed fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) staining using an FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer’s instructions. The cell cycle phase distribution was analyzed by staining with propidium oxide with a CycleTest Plus DNA Kit (BD Biosciences), following the manufacturer’s instructions and evaluating the staining with a FACScan system. The percentage of cells in each phase was assessed.

Western Blot Assay and Antibodies
Cells’ protein lysates were separated by 10% SDS-PAGE, transferred to 0.22-μm NC membranes (Sigma-Aldrich), and cultivated with specific antibodies. Densitometry (Quantity One software; Bio-Rad) was used to quantify the protein band density. A GAPDH antibody was employed as a control. Anti-EZH2 was obtained from Proteintech (Wuhan, China), and anti-ANGPTL4 was obtained from R&D Systems (Minneapolis, MN, USA).

In Vivo Tumor Formation Assay
The 4-week-old athymic mice, bought from the Animal Centre of Nanjing University (Nanjing, China), were kept under specific pathogen-free conditions. HuCCT1 cells were stably transfected with shRNA or empty vector, harvested from cell culture plates, cleaned with PBS, and resuspended at a density of 2 × 10^7 cells/mL. Then, the cells were xenografted into BALB/c male nude mice. The sizes of the tumors were calculated every 4 days with the following equation: length × width^2 × 0.5. At 16 days post-injection, the mice were asphyxiated with CO2, and the tumors were weighed and examined. The study was performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of the NIH. The protocol was licensed by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

Deep Sequencing of the Whole Transcriptome
Total RNA from the HuCCT1 cells with PVT1 knockdown and the control HuCCT1 cells was extracted and quantified. The concentration of RNA in each sample was measured with a NanoDrop 2000 (Thermo Scientific, USA), and the amount was evaluated with an Agilent 2200 system (Agilent, USA). The sequencing library of each RNA sample was prepared with an Ion Proton Total RNA-Seq Kit version (v.1), following the protocol recommended by the manufacturer (Life Technologies, USA). The data are provided in Table S2.

Subcellular Fractionation Assay
Nuclear and cytosolic fractions were separated with a PARIS Kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer’s instructions.

RNA FISH
A FISH kit designed for PVT1 was purchased from RiboBio (Guangzhou, China). Briefly, cells were rinsed in PBS and then fixed in 4% formaldehyde for 10 min. Then, the RBE cells were incubated in PBS containing 0.5% Triton X-100 at 4°C for 5 min, washed with PBS three times for 5 min, and prehybridized at 37°C for 30 min.

Figure 7. PVT1 Binds with EZH2 in the Nucleus and Epigenetically Silences ANGPTL4, Inhibiting Cell Proliferation and Migration in CCA Cell Lines
(A) Expression level of ANGPTL4 in cholangiocarcinoma based on the analysis of GEO: GSE26566 data. (B) ANGPTL4 expression was determined in 17 pairs of CCA tissues by qRT-PCR. (C-F) HuCCT1 and RBE cells transfected with vector/ANGPTL4/pcDNA-PVT1 and cells transfected with PVT1 followed by transfection with ANGPTL4. After transfection, the cells were analyzed by CCK-8 assays (C and D) and transwell assays (E and F). (G and H) ChIP of EZH2 and H3K27me3 of the promoter region of the ANGPTL4 locus after siRNA treatment with si-NC and si-PVT1 2# (G) or overexpression of PVT1 (H) in HuCCT1 cells. qPCR was performed to quantify the ChIP assay products. Enrichment was quantified relative to the input controls. Antibodies directed against IgG were used as a negative control. (I) Proposed model by which PVT1 regulates ANGPTL4 expression to promote CCA tumor growth. The error bars indicate the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

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For hybridization, anti-PVT1, anti-U6, and anti-18S oligodeoxynucleotide probes were used in hybridization solution at 37°C overnight in the dark. Subsequently, the cells were counterstained with DAPI and imaged with a confocal laser-scanning microscope (Carl Zeiss, Germany).

RIP Assays
A RIP assay was carried out to study whether PVT1 could interact with EZH2 with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA), following the manufacturer’s instructions. The EZH2 antibody used for the RIP assays was obtained from Millipore (Billerica, MA, USA).

Double Fluorescence Detection of EZH2 Protein and PVT1 mRNA in HuCCT1 Cells
HuCCT1 cells were fixed with 4% paraformaldehyde overnight, washed with PBS, and then dehydrated. After rehydration, in situ hybridization was performed with a fluorescein-labeled PVT1 probe (RiboBio, Guangzhou, China), followed by reaction with a goat anti-EZH2 antibody (Proteintech, Wuhan, China) at 4°C overnight. Then, the samples were counterstained with DAPI. The fluorescent signals were detected using a Zeiss LSM 700 laser-scanning microscope system (Carl Zeiss) and analyzed with ZEN lite 2.3 software (Carl Zeiss).

ChIP Assays
ChIP assays were carried out with an EZ-ChIP Kit following the manufacturer’s recommendations (Millipore, USA). Antibodies against EZH2 were purchased from Millipore (Billerica, MA, USA), and antibodies against H3 trimethyl Lys27 (H3K27me3) were purchased from Abcam (Cambridge, UK). The sequences of the ChIP primers are shown in Table S1. The equation by which we calculated the ChIP signals as percentages of the input DNA was as follows: \[ \text{Input Ct} - \text{Target Ct} \times 0.1 \times 100 \].

Statistical Analysis
Statistical analyses were carried out with GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). The statistical significance of the differences between various groups was calculated with Student’s t tests or chi-square tests, as appropriate. All data are presented as the means ± SD. A two-sided alpha level of 0.05 was used to determine statistical significance.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.10.001.

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