lncRNA PVT1 promotes hepatitis B virus-positive liver cancer progression by disturbing histone methylation on the c-Myc promoter

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Abstract. Long noncoding RNA (LncRNA) PVT1 has recently been reported to be involved in the development of hepatocellular carcinoma (HCC). We aimed to elucidate the correlation of PVT1 with hepatitis B virus-positive HCC in the clinic, and the roles of PVT1 in liver cancer cell biology, as well as to investigate the underlying molecular mechanisms. qPCR analysis was performed to examine the expression of PVT1 in hepatitis B virus-positive HCC tissues and liver cancer cell lines. lncRNA PVT1 overexpression and knockdown were achieved by transfection of an overexpression vector or shRNA. Cell proliferation, colony formation, migration, apoptosis, and invasion capabilities were examined, accordingly. RNA pull-down assay was employed to examine the connection between PVT1 and the PRC2 complex. Chromatin immunoprecipitation was employed to test the combination with EZH2 protein and H3K27me3 level on the *MYC* promoter. The results revealed that upregulation of PVT1 was detected in hepatitis B virus-positive HCC tissues compared with that noted in the HBV-negative samples. lncRNA PVT1 enhanced cell proliferation, migration, and invasion in the hepatitis B virus-positive Hep3B cells rather than the hepatitis B virus-negative HepG2 cells. PVT1 was able to bind EZH2 and obstruct the recruitment of EZH2 to the promoter of MYC therefore promoting *MYC* expression by altering H3K37me3 status in Hep3B liver cancer cells, and EZH2 protein was negatively correlated with lncRNA-PVT1 expression. In conclusion, our results indicate that lncRNA PVT1 promotes hepatitis B virus-positive liver cancer progression by disturbing histone methylation on the *MYC* promoter, suggesting that lncRNA PVT1 may be a potential target for developing diagnostic and therapeutic strategies of hepatitis B virus-positive liver cancer at the early stages.

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Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor with high lethality, and infection with hepatitis B virus (HBV) is the leading cause of HCC (1). HBV can accelerate HCC via multiple mechanisms. First, HBV induces immune reactions that lead to repeated hepatic inflammation, fibrosis and a deficient immune microenvironment. Subsequently, HBV can modify host genes near the insertion point through DNA integration to cause host cell genome instability and to generate carcinogenic fusion proteins (2,3). However, the approaches for HCC screening in HBV-infected individuals, the risk factors of HCC, the possible mechanisms leading to HCC and the potential therapeutic approaches of HBV-related HCC have not been systematically reviewed. Thus, it is urgent to characterize the pathogenic mechanisms of HBV-related HCC in order to identify novel targets for its treatment.

The lncRNA plasmacytoma variant translocation 1 (PVT1) has 1,716 nucleotides and is located in the chr8q24.21 region, which is 57 kb downstream of the *MYC* gene on human chromosome 8q24 (4,5). PVT1 produces a wide variety of spliced non-coding RNAs as well as a cluster of six annotated microRNAs: miR-1205, miR-1204, miR-1206, miR-1207-5p, miR-1208, miR-1207-3p, as a P53-inducible target gene (6). Several researchers have observed that lncRNA PVT1 plays an important role in tumorigenesis in non-small cell lung cancer, ovarian and breast cancer, gastric cancer, and high expression of PVT1 predicts poor patient prognosis (7-10). As to liver cancer, lncRNA PVT1 has been reported to participate in tumor progression by promoting cell proliferation, invasion and metabolism of HCC cells (11,12), and these studies indicate the important roles of PVT1 in HCC carcinogenesis, yet the mechanisms remain unclear (13). lncRNA-PVT1 participates in many physiological and pathological processes by modulating gene expression at the transcriptional,

post-transcriptional and epigenetic levels (14,15). lncRNAs recruit the PRC2 complex to chromatin, such as HOTAIR, Kcnq 1ot1, and Braveheart, suggesting the specific association between PRC2 and lncRNAs (16). RNA immunoprecipitation with microarray analysis revealed that 20% of the lncRNAs in human cells are associated with the PRC2 complex (17). Polycomb repressive complex-2 (PRC2) is a highly conserved histone methyltransferase for H3K27 methylation, and contains the subunits Enhancer of Zeste 2 (EZH2), Embryonic Ectoderm Development (EED), and Suppressor of Zeste 12 (SUZ12) (18). EZH2 is detectable in various HCC cell lines, and was found to play a critical role in HCC tumorigenesis *in vivo* and may serve as a powerful diagnostic biomarker (19). Although the important roles of PVT1 in many cancer have been reported, the mechanism of PVT1 in the occurrence and development of hepatitis B virus-positive HCC remains unclear.

In the present study, it was demonstrated that lncRNA PVT1 is highly expressed in hepatitis B virus-positive HCC tissues and regulates cell proliferation, migration, and invasion in liver cancer cell lines. Importantly, we present evidence that lncRNA PVT1 interferes with the recruitment of EZH2 onto the *MYC* promoter, which contributes to the suppression of H3K37me3 modification and promotes c-Myc expression in hepatitis B virus-positive HCC cells. Moreover, EZH2 protein was found to be negatively correlated with lncRNA PVT1 expression. Taken together, our study may provide a new approach and facilitate lncRNA-PVT1-directed diagnostic and therapeutic strategies for hepatitis B virus-positive liver cancer at the early stages.

Materials and methods

Patient tissue samples. A total of 24 HCC tissues were obtained from patients undergoing routine hepatic resection at Tianjin Second People's Hospital. Fifteen of the patients presented with HBV-positive HCC. Patients included 14 males and 10 females, with ages ranging from 33 to 74 years, with a median age of 46 years. Pathological progression was classified as TNM I (n=5), TNM II (n=9), and TNM III (n=10) according to the International Union Against Cancer TNM classification system (20). These patients did not receive any form of chemotherapy or radiotherapy before surgery. The tissues were immediately snap-frozen and stored in liquid nitrogen after resection. Written informed consent was obtained from all patients enrolled in this study, and the present study protocol was approved by the Ethics Committee of Tianjin Second People's Hospital.

Isolation of primary human hepatocytes (PHHs). Primary hepatocyte isolation was prepared according to a previous report (21). Briefly, tissues obtained from resected human liver of patients with benign local liver diseases were digested with EDTA/collagenase perfusion technique, and the cell suspension was centrifuged at 50 x g for 5 min at 4˚C to separate the non-parenchymal cell (NPC) fraction, and the cell pellet was used to isolate the PHHs. The PHH fraction was subjected to a 25% Percoll density gradient centrifugation at 1,250 x g for 20 min at 4˚C without stopping to remove non-viable cells, and then the cells were washed with phosphate-buffered saline (PBS) and re-suspended and cultured in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell culture. The human liver cancer cell lines Hep3B and HepG2 were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin-streptomycin, and cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Quantitative real-time PCR. Total RNA was extracted from the frozen tissues or cell lines with TRIzol™ reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed with SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific, Inc.). The quantitative real-time PCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche) on QuantStudio 3 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification conditions consisted of 95˚C for 10 min; 95˚C for 15 sec, 60˚C for 60 sec; 40 cycles. The relative expression of *PVT1* was calculated using the $2^{-\Delta\Delta Cq}$ method (22). The gene-specific primers were as follows: *PVT1* forward, 5'-GGGAATAACGCTGGTGGAAC-3' and reverse, 5'-GATCTCAACCCTCTCAGCCA-3'; *GAPDH* forward, 5'-AATGGCAGCCGTTAGGAAA-3' and reverse, 5'-GCCCAATACGACCATCAGAG-3'.

MTS cell proliferation assay. Cells were seeded in 96-well microplates at a density of 3,000 cells per well. Cell viability was assessed using the CellTiter 96 AQueous One Solution Reagent (Promega) according to the manufacturer's instructions.

Colony formation assay. Single-cell suspension of cells at the concentration of $1x10^4$ cells in DMEM with 10% FBS were plated on the bottom layer containing 0.8% agarose in a 6-well plate. The cells were photographed on day 14 after plating (Motic AE2000; Xiamen).

Cell mobility analysis. Cell migration and invasion were examined by wound-healing and Transwell assay. For the wound-healing assay, Hep3B and HepG2 liver cancer cells were seeded with a defined cell number of $3x10^3$ cells/cm^2 in 24-well plates. A scratch was made using a pipette tip as a cross through the whole well after reaching a uniform confluence of 95%. The scratch area was monitored and measured with Fiji software (ImageJ V1.48; National Institutes of Health, Bethesda, MD, USA) at time-points 0, 6, 12 and 24 h.

A Transwell chamber (8-μm pore size; Millipore) with Matrigel (BD Biosciences) matrix was used to determine cell invasion. Two hundred microliters of the cell suspension ($1x10^4$ cells) in serum-free medium was plated in the top chamber, whereas the lower chamber contained 600 μl. The cells on the top surface of the membrane were removed by a cotton swab after 24 h. Cells on the bottom surface of the membrane were stained for 15 min at room tempreture with crystal violet solution and image were captured (Motic AE2000; Xiamen).

Cell cycle and apoptosis analysis. Hep3B and HepG2 cells were cultured as described in the above section. Subsequently, cells were trypsinized and fixed in 70% ethanol and incubated at 4˚C overnight followed by incubation with DNAse-free RNAse A (Sigma-Aldrich; Merck KGaA) containing propidium iodide (PI)/Triton X-100 staining solution for 20 min at room tempreture. The analysis was performed according to the manufacturer's instructions using a FACS Calibur instrument.

Cell apoptosis was detected with the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich; Merck KGaA) according to the instruction manual. Briefly, the cells were washed with PBS and incubated with Dead Cell Apoptosis Kit (Thermo Fisher Scientific, Inc.) with Annexin V FITC and PI, for flow cytometry at 4˚C for 30 min. The flow cytometry data were analyzed with CellQuest 3.0 software (BD Biosciences).

Construction of vectors and cell transfection. The lncRNA-PVT1 small hairpin RNA (shRNA) oligos and the non-targeting scramble (NC Ctrl) were synthesized by Shanghai Invitrogen Biotechnology (Shanghai, China), and the sequence tested with the highest efficacy was GGACATGAGAAGGACAGAATA. The lncRNA-PVT1 overexpression (OE) vector and the vector control (Vector) were constructed by GeneChem Co. (Shanghai, China). Vector transfections were all performed by using FuGENE® HD Transfection Reagent (Promega) following the manufacturer's instructions.

Western blot analysis. Total protein was extracted using RIPA protein extraction reagent containing protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics), and the concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). The protein lysates were analyzed with 10% SDS-PAGE and subsequently transferred to a PVDF membrane. Antibodies for EZH2 (cat. no. 5246), c-Myc (cat. no. 9402), SUZ12 (cat. no. 3737) and EED (cat. no. 51673) were purchased from Cell Signaling Technology (all at 1:1,000 dilution), and anti-GAPDH (cat. no. G9545) was purchased from Sigma-Aldrich/Merck KGaA (1:5,000 dilution). The HRP-conjugated secondary antibody (cat. no. A0545) was purchased from Sigma-Aldrich; Merck KGaA. The signal was detected with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) and exposed using the Gel Doc™ EZ System (Bio-Rad Laboratories).

RNA pull-down assay. RNA pull-down assays were performed using a Magnetic RNA Protein Pull-Down kit (Thermo Fisher Scientific, Inc.) according to the kit manual. Biotinylated PVT1 RNA was synthesized by RiboBio. For each assay, 50 pM biotinylated RNA was incubated with 50 μl prewashed streptavidin-agarose beads for 1 h at 4˚C. Then, RNA-bound beads were incubated with lysates from Hep3B cell cytosolic/nuclear extracts, and eluted proteins were detected by western blot analysis.

RNA immunoprecipitation assay. The EZ Magna RNA Immunoprecipitation Kit (Millipore) was used following the manufacturer's guidelines. Briefly, Hep3B cells were lysed in RIPA lysis buffer. Magnetic beads were pre-incubated with antibodies for 30 min at room temperature, and the cell lysates were immunoprecipitated with beads for 6 h at 4˚C. Then, RNA was purified and detected by RT-qPCR.

Chromatin-immunoprecipitation-qPCR (ChIP-qPCR). Cells ($4x10^7$) were washed in PBS and cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched by addition of glycine for 5 min. Chromatin was fragmented to 200-500 bp using 14 cycles using the Vibra-Cell Ultrasonic Liquid Processors (Sonics and Materials, Inc.). For each IP, chromatin was immunoprecipitated with 2 mg antibody, EZH2 (cat. no. 5246) and H3K27me3 (cat. no. 9733) (Cell Signaling Technology), in IP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) at 4˚C overnight. Chromatin was precleared for 2 h each with protein G agarose beads before immunoprecipitation. The samples were removed from the beads, reversed cross-linked overnight at 65˚C and DNA was isolated using QIAquick PCR Purification Kit (Qiagen). Precipitated DNA was analyzed by qPCR using upstream primer of the *c-Myc* gene, 5'-CCTACCCTCTCAACGACAGC-3' and downstream primer, 5'-CTTGTTCCTCCTCAGAGTCGC-3'.

Statistical analysis. Data are shown as mean ± SD for at least three independent experiments. Differences between groups were determined using paired two-tailed Student's t-test for matched data in two groups or one-way ANOVA for three or more groups, and the Least Significant Difference (LSD) was used for post hoc analysis. A P-value <0.05 was considered statistically significant.

Results

lncRNA PVT1 expression is upregulated in hepatitis B virus-positive HCC tissues and liver cancer cells. In order to assess the lncRNA-PVT1 expression pattern associated with HBV virus infection in HCC tissues, we analyzed lncRNA-PVT1 expression in 24 HCC tissues from HBV-positive and HBV-negative patients using real-time PCR assay. Our results showed that HBV-positive HCC tissues showed a significantly higher PVT1 expression than the HBV-negative HCC patient tissues (Fig. 1A). To investigate the difference between pathological stages, we further assessed samples of different stages (TNM I, II and III) by qPCR. The results showed that PVT1 expression was significantly upregulated in patients with stage TNM II and III HCC compared with stage TNM I (Fig. 1B). Furthermore, in comparison with the primary human hepatocytes (PHH) from healthy donors and the HBV-negative HepG2 liver cancer cell line, PVT1 was highly expressed in the HBV-positive liver cancer cell line, Hep3B (Fig. 1C). Our study revealed that PVT1 was upregulated in HBV-positive HCC tissues, suggesting that PVT1 may play a specific role in the progression of HBV-positive liver cancer.

PVT1 promotes HBV-positive liver cancer cell proliferation. To investigate the biological role of PVT1 in liver cancer cells, we transfected HepG2 and Hep3B cells with PVT1 shRNA to knock down PVT1 expression, or the cell lines were transfected with expression vectors to overexpress the *PVT1* gene, and the non-targeting scramble (NC) or vector control (Vector)

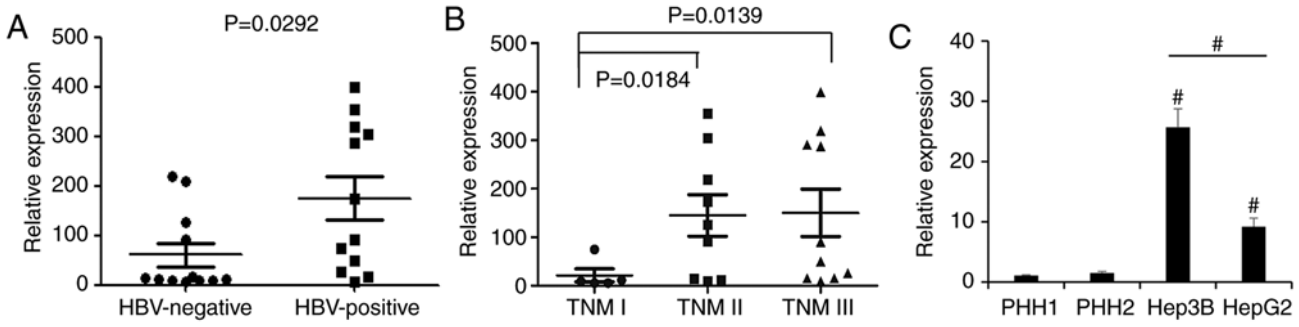

Figure 1. lncRNA PVT1 is upregulated in HBV-positive HCC tissues and liver cancer cells. (A) Total RNA was extracted from HCC patient tissues. HBV-induced HCC liver tissues and non-HBV-induced HCC liver tissues were subjected to real-time PCR assay to examine the expression of lncRNA PVT1. (B) HCC tissue samples from different disease stages, TNM I, II and III, were subjected to quantitative reverse transcription-PCR to examine the expression of lncRNA PVT1. (C) Cell extracts were analyzed by RT-qPCR to determine the profile of PVT1 expression. Primary human hepatocytes (PHH) isolated from two independent donors were used as controls. Each bar represents mean ± SD from three independent experiments. #P<0.01. lncRNA, long non-coding RNA; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

were used as mock controls, respectively. The transfection efficiency was examined by qPCR. PVT1 was successfully overexpressed in the Hep3B and HepG2 cells after transfection with synthesized PVT1 RNA compared with the vector control (Fig. 2A and B). The effect of PVT1 on cell proliferation was investigated by MTS assay and colony formation assay. As shown in Fig. 2C and D, the cell proliferation was significantly increased at 12, 24, 48 and 72 h following PVT1 overexpression in the Hep3B cell line but not in the HepG2 cell line. Compared with non-target scrambles (NC), the shRNA of PVT1 had a significant knockdown (KD) efficacy in the HepG2 and Hep3B cell lines (Fig. 2E and F). Following PVT1 KD, the proliferation of Hep3B cells was significantly suppressed at 24, 48 and 72 h when compared with the HepG2 cell line which showed no significant decrease (Fig. 2G and H). In addition, the colony formation capacity was significantly enhanced following overexpression of PVT1 in the Hep3B cells while the colony formation capacity in the HepG2 cells was only markedly increased without significance (Fig. 3A and B); in contrast, significantly fewer colonies were formed in the Hep3B cells rather than the HepG2 cells when PVT1 was KD by shRNA compared with the NC group (Fig. 3C and D).

PVT1 enhances HBV-positive liver cancer cell migration and invasion. To assess the effects of PVT1 on the migration and invasion of HBV-positive liver cancer cells, we performed a wound-healing assay and invasion assay, respectively. Our results showed that PVT1 overexpression (OE) significantly enhanced the wound healing capacity in Hep3B cells rather than HepG2 cells (Fig. 4A and B). Moreover, KD of PVT1 by shRNA significantly reduced migration capacity of the Hep3B cells rather than the HepG2 cells compared with the NC group (Fig. 4C and D). The invasion ability of HBV-positive Hep3B cells was significantly enhanced following PVT1 OE (Fig. 4E and F) and was impeded by PVT1 KD compared with the Vector and NC groups (Fig. 4G and H).

PVT1 causes changes in cell cycle distribution and induces apoptosis of HBV-positive HCC cells. Based on our results, alterations in PVT1 expression led to cell proliferation, migration and invasion changes. Therefore, we investigated whether PVT1 would disrupt the cell cycle profile of HBV-positive liver cancer cells. We found that in contrast to the Vector group, the percentage of cells in the S phase was increased following PVT1 OE in the Hep3B cells (Fig. 5A), and the percentage of cell in the S phase was decreased following PVT1 KD in the Hep3B cells (Fig. 5B). Furthermore, the cell apoptosis rate was significantly increased in the PVT1 KD Hep3B cells (Fig. 5C); on the other hand, following PVT1 OE, the apoptotic ratio of the Hep3B cells was significanly decreased (Fig. 5D).

PVT1 negatively regulates EZH2 expression and dysregulates recruitment of EZH2 to the MYC promoter. To further study the regulatory role of PVT1 in HBV-positive HCC cells, RNA immunoprecipitation assay was performed. As shown in Fig. 6A, we detected a strong interaction between PVT1 with polycomb repressive xomplex 2 (PRC2) subunits, such as EZH2, SUZ12 and EED (Fig. 6A). Next, to confirm this regulated interaction, we determined the protein levels of PRC2 complex members in PVT1 OE and KD Hep3B cells by western blot analysis. When the PVT1 expression was KD or OE, the c-Myc protein level was markedly downregulated or upregulated respectively, and EZH2 showed a negative correlation with the PVT1 expression, but no obvious changes in EED and SUZ12 were observed (Fig. 6B). Moreover, the c-Myc protein level was positively correlated with PVT1 expression level in the PHH cells and liver cancer cells, where in the HBV-positive Hep3B cells the c-Myc protein level and PVT1 mRNA level were obviously high than that in the HBV-negative HepG2 and PHH cells (Fig. 6C). To investigate a potential effect on the recruitment ability of PVT1, we designed another experiment which assessed the EZH2 protein and H3K27me3 level of the MYC promotor by ChIP-qPCR. Lower PVT1 expression in the Hep3B cell line induced EZH2 recruitment to the specific target gene, MYC that contributed to H3K37me3 alteration (Fig. 6D). PVT1 OE significantly inhibited EZH2 recruitment to the MYC promoter and decreased the H3K27me3 level of this region (Fig. 6E).

Discussion

The present study investigated the function of long non-coding RNA (lncRNA) PVT1 in HBV-positive liver cancer development and progression. We found that lncRNA PVT1 was

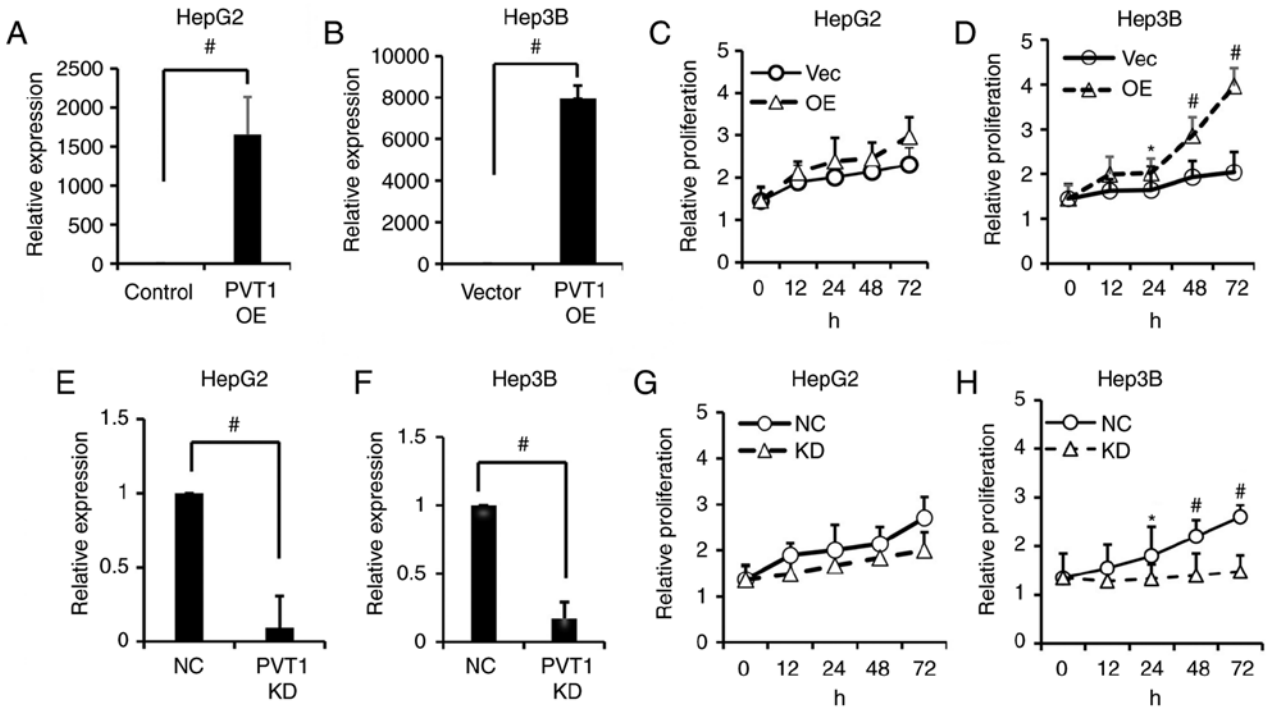

Figure 2. lncRNA PVT1 enhances cell proliferation. (A) HepG2 and (B) Hep3B cells were transfected with the PVT1 overexpression plasmid (PVT1 OE) or vector control (Vector) and transfection efficiency was confirmed by RT-qPCR. Cell proliferation of (C) HepG2 and (D) Hep3B cells transfected with PVT1 overexpression plasmid (PVT1 OE) or vector control (Vector) was detected with MTS assay at different time points. (E) HepG2 and (F) Hep3B cells were transfected with PVT1 shRNA (PVT1 KD) or non-targeting scrambles (NC) and transfection efficiency was confirmed by real-time PCR assays. Cell proliferation of the (G) HepG2 and (H) Hep3B cells transfected with the PVT1 shRNA (PVT1 KD) or non-targeting scramble (NC) was detected with MTS assay at different time points. Each bar represents mean ± SD from three independent experiments. All data represent results from at least three independent experiments. $^{*}P<0.05$, $^{\#}P<0.01$ compared to the NC or Vector. lncRNA, long non-coding RNA.

upregulation in HBV-positive HCC tissues and liver cancer cell lines. We present evidence that the cell proliferation, migration and invasion of the liver cancer cells was regulated by PVT1. Knockdown of PVT1 significantly arrested HBV-positive liver cancer cell cycle and induced apoptosis. More importantly, the protein expression and recruitment of the core subunit of PRC2, EZH2, was negatively regulated by PVT1. These results indicated that PVT1 expression could be an independent prognostic and diagnostic factor for HBV-positive HCC and is critical for the tumorigenesis and progression of HBV-positive HCC.

lncRNA PVT1 has been studied in various human cancers and has been identified to play an oncogenic role. Increased PVT1 expression was significantly associated with histological grade, lymph node metastasis, and poor overall survival in non-small cell lung cancer (NSCLC) (10). The PVT1 locus nearby the normal-MML-network encompassed tumor-related genes indicating a possible role of surveillance to tumorigenesis (23). PVT1 was found to increase FOXM1 post-translation by directly binding FOXM1 protein, and FOXM1 also could bind to the PVT1 promoter to activate its transcription in gastric cancer (24). PVT1 is also highly expressed in the tissues of cisplatin-resistant gastric patients and cisplatin-resistant cells, and upregulation of PVT1 was found to increase the expression of MDR1, and exhibit an anti-apoptotic effect (25). Our results showed that lncRNA PVT1 was upregulated in HBV-positive HCC tissues and liver cancer cells, and PVT1 was significantly associated with the proliferation, migration, and invasion of HBV-positive liver cancer cells rather than HBV-negative liver cancer cells.

Enhancer of zeste homolog 2 (EZH2), a member of the polycomb group (PcG) protein family, suppresses many tumor-suppressor genes, including miRNAs by modified transcription at the epigenetic level (26,27). PVT1 binding to EZH2 epigenetically regulates P15 and P16 causing G1 arrest in gastric cancer (28). Zhang *et al* demonstrated that PVT1 could combine with EZH2 and recruit EZH2 to the miR-200b promoter, thereby increasing histone H3K27 trimethylation level of the miR-200b promoter, and inhibiting miR-200b expression in cervical cancer progression (29). RNA immunoprecipitation and chromatin immunoprecipitation assays demonstrated that PVT1 recruits EZH2 to the promoter of large tumor suppressor kinase2 (LATS2) and represses LATS2 transcription in NSCLC (30). The location of PVT1 is recognized as a cancer risk locus that is shared with the well-known MYC oncogene. The gain of PVT1 expression was found to be required for MYC protein upregulation in human cancer cells and was co-increased in more than 98% of MYC-copy-increase cancers (31). Guan *et al* found that inhibition of PVT1 induced apoptosis and suppression of MYC expression contributing independently to ovarian and breast cancer (7). In this study, we provide evidence that PVT1 binds EZH2 and interferes with the recruitment of EZH2 onto *MYC* promoter in HBV-positive liver cancer cells by RNA immunoprecipitation and chromatin immunoprecipitation assays. The important role of PVT1 in many types of cancer has been studied, and the present study explored its functional role in HBV-positive HCC. Nevertheless, whether the upregulation of lncRNA PVT1 is a consequence of HBV

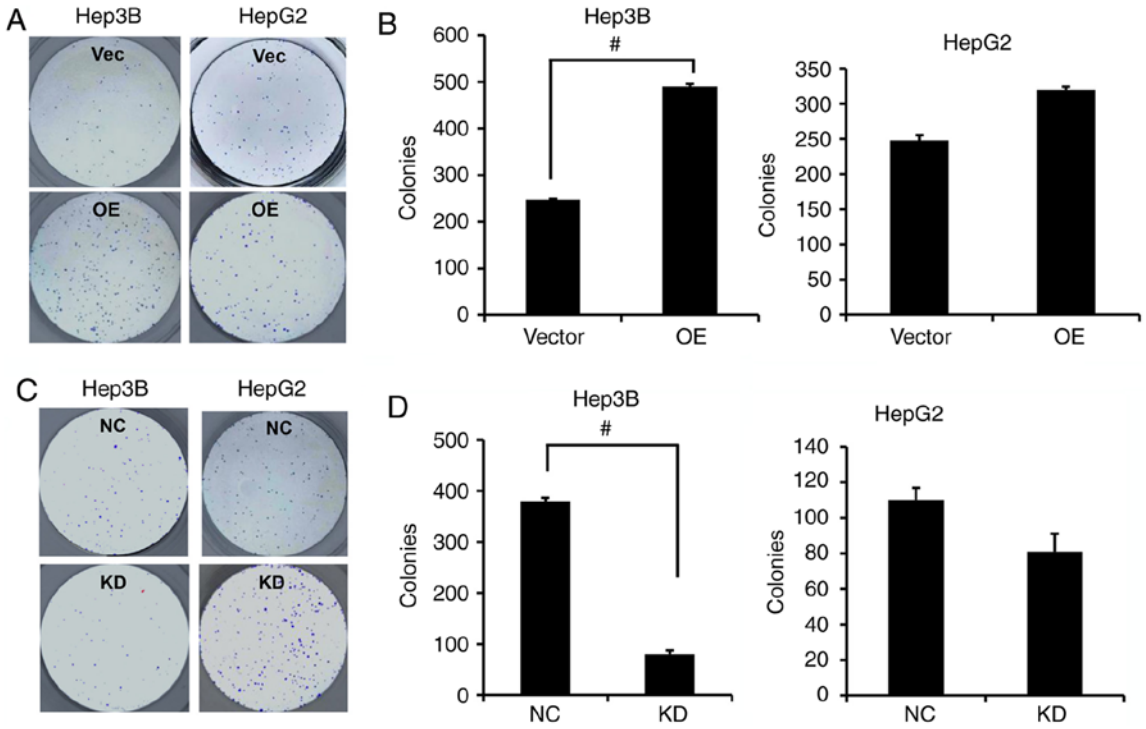

Figure 3. lncRNA PVT1 promotes cell colony forming ability. Representative images and quantification of colony formation of Hep3B and HepG2 cells after (A and B) PVT1 overexpression (PVT1 OE) and (C and D) PVT1 knockdown by shRNA (PVT1 KD), compared with the vector control (Vector) or non-targeting scramble (NC), respectively. Each bar represents mean ± SD from three independent experiments. All data represent results from at least three independent experiments. #P<0.01. lncRNA, long non-coding RNA.

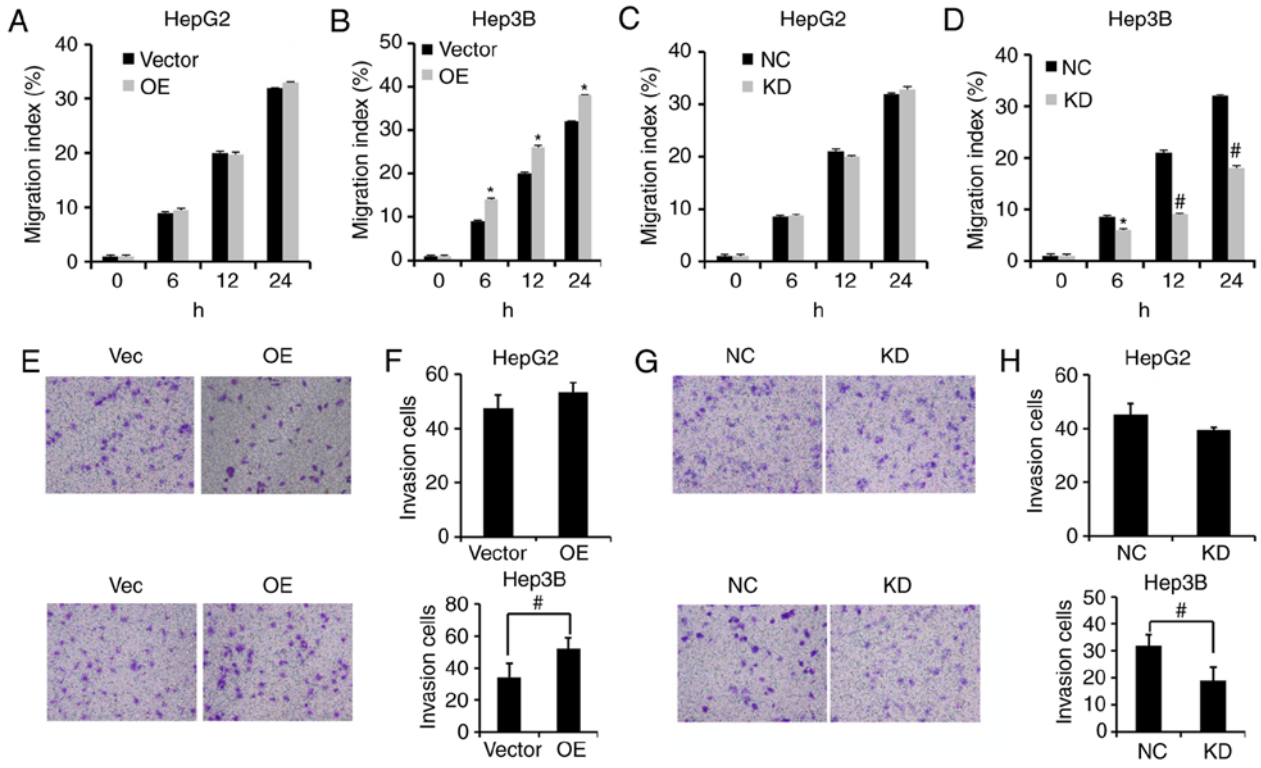

Figure 4. lncRNA PVT1 facilitates cell migration and invasion. The migration ability of (A) HepG2 and (B) Hep3B cells after PVT1 overexpression (OE) compared with the vector control (Vector). The migration ability of (C) HepG2 and (D) Hep3B cells after PVT1 knockdown by shRNA (KD) compared with the non-targeting scrambles (NC). (E) Representative Transwell assay showing the effect of PVT1 OE on HepG2 and Hep3B cells, and (F) the invasion cells number were counted after PVT1 OE compared with the Vector group. (G) Representative Transwell assay showing the effect of PVT1 knockdown (KD) on HepG2 and Hep3B cells, and (H) the invasion cell numbers were counted after PVT1 KD compared with the non-targeting scramble (NC). The upper chamber containing the invaded cells was fixed with 4% polyformaldehyde, washed, stained with Giemsa, and 10 visual fields under the microscope were selected for counting. Each bar represents the mean ± SD from three independent experiments. *P<0.05, #P<0.01 compared with the Vector or NC. lncRNA, long non-coding RNA.

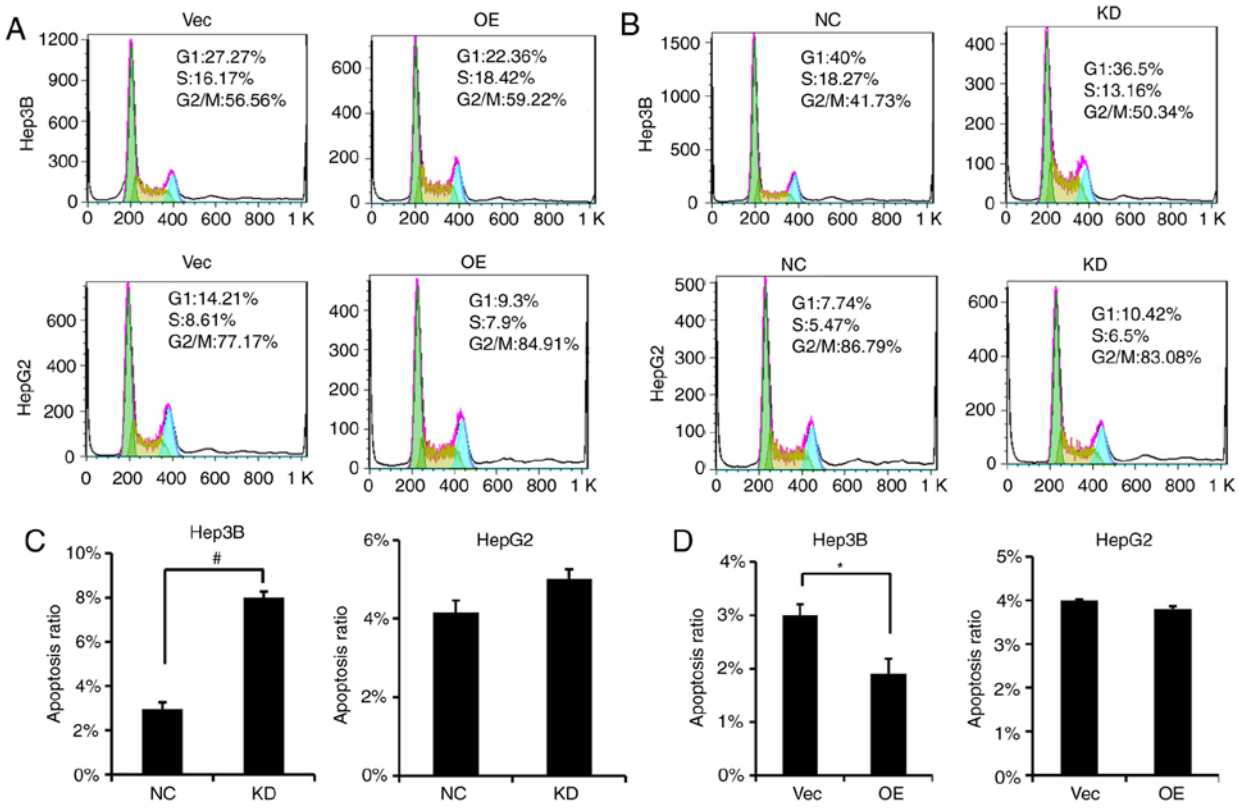

Figure 5. lncRNA PVT1 causes alterations in the cell cycle distribution and induces apoptosis of HBV-positive liver cancer cells. Cell cycle analysis of Hep3B and HepG2 cells after (A) PVT1 overexpression (OE) and (B) knockdown (KD) by shRNA compared with the vector (Vec) or non-targeting scramble (NC), respectively. Cell apoptosis rate analysis of Hep3B and HepG2 cell after (C) knockdown (KD) by shRNA and (D) overexpression (OE) as determined by Annexin V-FITC Apoptosis Detection Kit compared with the vector (Vec) or non-targeting scramble (NC), respectively. Each bar represents mean ± SD from three independent experiments. *P<0.05, #P<0.01. lncRNA, long non-coding RNA; HBV, hepatitis B virus.

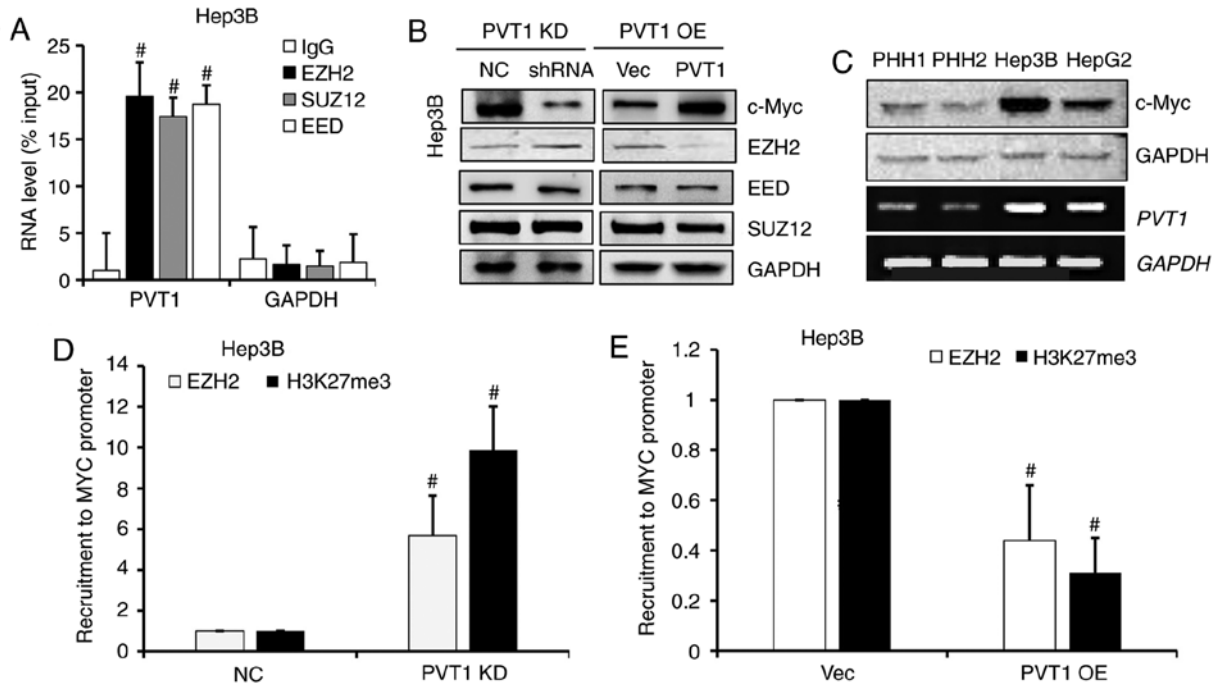

Figure 6. PVT1 dysregulates the recruitment of EZH2 to the MYC promoter. (A) Hep3B cells were lysed in RIPA buffer. RNA immunoprecipitation assay was used to detect the interaction of PVT1 and PRC2 subunits, EZH2, SUZ12, and EED in Hep3B cells. (B) Representative western blot analysis shows the EHZ2 protein expression level in PVT1 knockdown (PVT1 KD) and overexpression (PVT1 OE) cells compared with vector (Vec) or non-targeting scramble (NC), respectively. (C) Western blot analysis and RT-PCR to detect the protein level of c-Myc and RNA level of lncRNA PVT1 in primary human hepatocytes (PHH), HBV-positive Hep3B cells and HBV-negative HepG2 cells. The effect of lncRNA PVT1 on recruitment to MYC promoter after PVT1 KD (D) and PVT1 OE (E) was detected by CHIP-qPCR assay in Hep3B cells compared with vector (Vec) or non-targeting scramble (NC), respectively. All data represent results from at least three independent experiments. #P<0.01. lncRNA, long non-coding RNA; HBV, hepatitis B virus.

infection or tumorigenesis remains unelaborated in our study, and lack of comparison between normal liver tissue and HBV infected tissue is the main limitation of the present study. Our study clearly showed that expression of lncRNA PVT1 was higher in the HBV-positive HCC tissues when compared with the HBV-negative HCC tissues, and the expression was elevated gradually with the progression of HCC. Moreover, PVT1expression was higher in liver cancer cells compared with normal liver cells. These data indicate that, at least partially, high expression of PVT1 is associated with HBV infection and tumor development, but this conclusion should be tested in HBV-positive and HBV-negative normal liver tissues. Since virus infection is crucial for activation of gene transcription initiation and elongation (32), the lncRNA PVT1 is involved in HBV-related HCC development. Thus, the present study may provide a new approach and facilitate lncRNA PVT1-directed diagnostic and therapeutic strategies for HBV-positive HCC at the early stages.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BJ and BY contributed to the writing of the manuscript. BJ, QW, BY and XZ contributed to performing of the experiments and the statistical analyses. YG and XZ provided the patient samples and performed the clinical statistics. WL contributed to the design of the experiments. All authors have approved the final version of the publication and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from all patients enrolled in this study, and the present study protocol was approved by the Ethics Committee of Tianjin Second People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ringelhan M, Pfister D, O'Connor T, Pikarsky E and Heikenwalder M: The immunology of hepatocellular carcinoma. Nat Immunol 19: 222-232, 2018.
2. Liu J, Yang HI, Lee MH, Lu SN, Jen CL, Batrla-Utermann R, Wang LY, You SL, Hsiao CK, Chen PJ, *et al*: Spontaneous seroclearance of hepatitis B seromarkers and subsequent risk of hepatocellular carcinoma. Gut 63: 1648-1657, 2014.
3. Levrero M and Zucman-Rossi J: Mechanisms of HBV-induced hepatocellular carcinoma. J Hepatol 64 (1 Suppl): S84-S101, 2016.
4. Shtivelman E, Henglein B, Groitl P, Lipp M and Bishop JM: Identification of a human transcription unit affected by the variant chromosomal translocations 2;8 and 8;22 of Burkitt lymphoma. Proc Natl Acad Sci USA 86: 3257-3260, 1989.
5. Huppi K, Siwarski D, Skurla R, Klinman D and Mushinski JF: Pvt-1 transcripts are found in normal tissues and are altered by reciprocal(6;15) translocations in mouse plasmacytomas. Proc Natl Acad Sci USA 87: 6964-698, 1990.
6. Barsotti AM, Beckerman R, Laptenko O, Huppi K, Caplen NJ and Prives C: p53-Dependent induction of PVT1 and miR-1204. J Biol Chem 287: 2509-2519, 2012.
7. Guan Y, Kuo WL, Stilwell JL, Takano H, Lapuk AV, Fridlyand J, Mao JH, Yu M, Miller MA, Santos JL, *et al*: Amplification of PVT1 contributes to the pathophysiology of ovarian and breast cancer. Clin Cancer Res 13: 5745-5755, 2007.
8. Takahashi Y, Sawada G, Kurashige J, Uchi R, Matsumura T, Ueo H, Takano Y, Eguchi H, Sudo T, Sugimachi K, *et al*: Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers. Br J Cancer 110: 164-171, 2014.
9. Ding J, Li D, Gong M, Wang J, Huang X, Wu T and Wang C: Expression and clinical significance of the long non-coding RNA PVT1 in human gastric cancer. OncoTargets Ther 7: 1625-1630, 2014.
10. Yang YR, Zang SZ, Zhong CL, Li YX, Zhao SS and Feng XJ: Increased expression of the lncRNA PVT1 promotes tumorigenesis in non-small cell lung cancer. Int J Clin Exp Pathol 7: 6929-6935, 2014.
11. Xu Y, Luo X, He W, Chen G, Li Y, Li W, Wang X, Lai Y and Ye Y: Long non-coding RNA PVT1/miR-150/HIG2 axis regulates the proliferation, invasion and the balance of iron metabolism of hepatocellular carcinoma. Cell Physiol Biochem 49: 1403-1419, 2018.
12. Guo J, Hao C, Wang C and Li L: Long noncoding RNA PVT1 modulates hepatocellular carcinoma cell proliferation and apoptosis by recruiting EZH2. Cancer Cell Int 18: 98, 2018.
13. Derderian C, Orunmuyi AT, Olapade-Olaopa EO and Ogunwobi OO: PVT1 signaling is a mediator of cancer progression. Front Oncol 9: 502, 2019.
14. Cui M, You L, Ren X, Zhao W, Liao Q and Zhao Y: Long non-coding RNA PVT1 and cancer. Biochem Biophys Res Commun 471: 10-4, 2016.
15. Chen L, Sun L, Dong L, Cui P, Xia Z, Li C and Zhu Y: The role of long noncoding RNA-LET in cell proliferation and invasion of nasopharyngeal carcinoma and its mechanism. OncoTargets Ther 10: 2769-2778, 2017.
16. Davidovich C and Cech TR: The recruitment of chromatin modifiers by long noncoding RNAs: Lessons from PRC2. RNA 21: 2007-2022, 2015.
17. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, *et al*: Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 106: 11667-116672, 2009.
18. He A, Shen X, Ma Q, Cao J, von Gise A, Zhou P, Wang G, Marquez VE, Orkin SH and Pu WT: PRC2 directly methylates GATA4 and represses its transcriptional activity. Genes Dev 26: 37-42, 2012.
19. Chen Y, Lin MC, Wang H, Chan CY, Jiang L, Ngai SM, Yu J, He ML, Shaw PC, Yew DT, *et al*: Proteomic analysis of EZH2 downstream target proteins in hepatocellular carcinoma. Proteomics 7: 3097-3104, 2007.

20. Villanueva A: Hepatocellular Carcinoma. N Engl J Med 380: 1450-1462, 2019.
21. Pfeiffer E, Kegel V, Zeilinger K, Hengstler JG, Nüssler AK, Seehofer D and Damm G: Featured article: Isolation, characterization, and cultivation of human hepatocytes and non-parenchymal liver cells. Exp Biol Med (Maywood) 240: 645-656, 2015.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Colombo T, Farina L, Macino G and Paci P: PVT1: A rising star among oncogenic long noncoding RNAs. Biomed Res Int 2015: 304208, 2015.
24. Xu MD, Wang Y, Weng W, Wei P, Qi P, Zhang Q, Tan C, Ni SJ, Dong L, Yang Y, *et al*: A positive feedback loop of lncRNA-PVT1 and FOXM1 facilitates gastric cancer growth and invasion. Clin Cancer Res 23: 2071-2080, 2017.
25. Zhang XW, Bu P, Liu L, Zhang XZ and Li J: Overexpression of long non-coding RNA PVT1 in gastric cancer cells promotes the development of multidrug resistance. Biochem Biophys Res Commun 462: 227-232, 2015.
26. Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, Hannon G, Tarakhovsky A and Fuchs E: Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell 136: 1122-1135, 2009.
27. Gao SB, Zheng QF, Xu B, Pan CB, Li KL, Zhao Y, Zheng QL, Lin X, Xue LX and Jin GH: EZH2 represses target genes through H3K27-dependent and H3K27-independent mechanisms in hepatocellular carcinoma. Mol Cancer Res 12: 1388-1397, 2014.
28. Kong R, Zhang EB, Yin DD, You LH, Xu TP, Chen WM, Xia R, Wan L, Sun M, Wang ZX, *et al*: Long noncoding RNA PVT1 indicates a poor prognosis of gastric cancer and promotes cell proliferation through epigenetically regulating p15 and p16. Mol Cancer 14: 82, 2015.
29. Zhang S, Zhang G and Liu J: Long noncoding RNA PVT1 promotes cervical cancer progression through epigenetically silencing miR-200b. APMIS 124: 649-658, 2016.
30. Wan L, Sun M, Liu GJ, Wei CC, Zhang EB, Kong R, Xu TP, Huang MD and Wang ZX: Long noncoding RNA PVT1 promotes non-small cell lung cancer cell proliferation through epigenetically regulating LATS2 expression. Mol Cancer Ther 15: 1082-1094, 2016.
31. Tseng YY, Moriarity BS, Gong W, Akiyama R, Tiwari A, Kawakami H, Ronning P, Reuland B, Guenther K, Beadnell TC, *et al*: PVT1 dependence in cancer with MYC copy-number increase. Nature 512: 82-86, 2014.
32. Clark DN and Hu J: Hepatitis B virus reverse transcriptase-target of current antiviral therapy and future drug development. Antiviral Res 123: 132-137, 2015.