

Long noncoding RNA PVT1 promotes hepatoblastoma cell proliferation through activating STAT3

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Background: Hepatoblastoma is the most common liver malignancy in children. The long noncoding RNA (lncRNA) PVT1 plays oncogenic roles in human cancers; however, its regulation and function in hepatoblastoma remain poorly understood.

Purpose: This study was designed to investigate the regulation and function of PVT1 in hepatoblastoma.

Methods: PVT1 expression was compared between human hepatoblastoma tissues and adjacent non-tumor tissues, and then analyzed using Kaplan-Meier method. The proliferation of hepatoblastoma cells was determined by BrdU incorporation assay. The tumor xenograft model was used to assess tumor proliferation in vivo. The gene expression level was measured by qRT-pCR, Western blot and immunohistochemistry analyses.

Results: Compared with normal counterparts, PVT1 is upregulated in human hepatoblastoma tissues as well as in hepatoblastoma cell lines. Additionally, PVT1 promotes the proliferation of hepatoblastoma cells in vitro and accelerates tumor growth in xenograft model in vivo. Mechanistically, PVT1 promotes the activation of the signal transducer and activator of transcription 3 (STAT3), which leads to the transcriptional activation of downstream targets involved in cell cycle progression, and moreover, STAT3 inhibition with the selective inhibitor stattic abolishes PVT1 pro-proliferative role in hepatoblastoma cells.

Conclusion: PVT1 promotes hepatoblastoma cell proliferation through activating STAT3-induced cell cycle progression, which may implicate PVT1 as a potential therapeutic target for hepatoblastoma treatment.

Keywords: long noncoding RNA, PVT1, hepatoblastoma, proliferation, STAT3

Introduction

Hepatoblastoma, the most common liver tumor in infants and toddlers, accounts for over 65% of pediatric liver malignancies.¹ In general, hepatoblastoma is a malignant transformation of immature liver precursor cells and can be classified into epithelial or mixed epithelial/mesenchymal tissue based on histological structure.^{2,3} The complete resection is a curative treatment for primary hepatoblastoma, but 60–80% of cases are unresectable at diagnosis.⁴ Currently, the neoadjuvant chemotherapy, such as the platinum-based regimen,¹ is a standard treatment for unresectable hepatoblastoma, which makes tumors to be resectable in most patients. For those without active metastasis but remain unresectable after chemotherapy, liver transplantation is the choice.⁵ However, prognosis is still very poor for patients with unresectable metastatic hepatoblastoma and the event-free survival (EFS) is only

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20–50%.⁶ To develop reliable diagnostic biomarkers and enhance the therapeutic efficiency for hepatoblastoma patients, a deeper understanding of the mechanisms that lead to hepatoblastoma progression is imperative.

The long noncoding RNAs (lncRNAs) are a heterogeneous class of noncoding transcripts (>200 nucleotides in length) which play a wide range of activities in cancer, including both oncogenic and tumor-suppressive functions.^{7,8} In recent years, studies have shown that some lncRNAs are dysregulated in hepatoblastoma tissues and play important roles in regulating hepatoblastoma progression. For instance, in a genome-wide analysis, a total of 2736 lncRNAs were found differentially expressed in hepatoblastoma tissues compared with normal tissues.⁹ In addition, Dong et al have reported that TUG1 and CRNDE are upregulated in hepatoblastoma tissues and their targeting inhibits tumor growth and angiogenesis in hepatoblastoma.^{10,11} Moreover, LUCAT1 is highly expressed in hepatoblastoma tissues and promotes the proliferation, migration and invasion of hepatoblastoma.¹² However, despite these reports, how other lncRNAs are functionally involved in hepatoblastoma is unknown.

lncRNA PVT1 is encoded by the human PVT1 gene, and its oncogenic roles have been revealed in a variety of cancer types, such as gastric cancer, non-small cell lung cancer, pancreatic ductal adenocarcinoma and hepatocellular carcinoma.^{13–16} Yet, whether PVT1 is associated with hepatoblastoma and the underlying mechanism have not been investigated. In this study, we report the pro-proliferative role of PVT1 in hepatoblastoma cells and further relate it to the modulation of the activation of signal transducer and activator of transcription 3 (STAT3).

Materials and methods

Human tissue specimens

Human hepatoblastoma tissues and adjacent nontumor tissues were obtained from 43 patients who underwent hepatoblastoma resection in The Third Xiangya Hospital of Central South University. All tissues were snap-frozen immediately in liquid nitrogen after surgical resection and then stored at -80°C for further analyses. The written informed consent was obtained from each patient. This study was approved by the Ethics Committee of The Third Xiangya Hospital of Central South University. All the clinical specimens were collected in accordance with the Declaration of Helsinki.

Cell culture

Human hepatoblastoma cell lines, HepG2, HuH-6 and HepT1, and a nonmalignant liver cell line QSG-7701 were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and maintained at 37°C in a humidified incubator with 5% CO_2 .

Lentiviral infection and treatment

The short hairpin RNA sequence targeting human PVT1 was inserted into pLKO.1-puro lentiviral vector (Sigma-Aldrich) (LV-shPVT1). Human PVT1 coding sequence was cloned into the lentiviral expressing vector pLV-puro (Addgene) (LV-PVT1). The scrambled control shRNA (LV-shCtrl) and lentiviral expressing empty vector (LV-vec) were used as controls, respectively. Lentiviral particles were produced according to the standard protocols. HuH-6 and HepG2 cells were infected with LV-shCtrl, LV-shPVT1, LV-vec or LV-PVT1 at a multiplicity of infection of 200 PFU per cell. The infected cells were selected by the treatment of $1\ \mu\text{g/mL}$ puromycin (Invitrogen) for 4 weeks. For the inhibition of STAT3 activity, HuH-6 and HepG2 cells, stably expressing LV-vec or LV-PVT1, were treated with $10\ \mu\text{M}$ statin (Sigma-Aldrich, S7947) for 2 days.

Real-time quantitative PCR

Trizol reagent (Invitrogen) was utilized for isolating the total RNA of HuH-6 and HepG2 cells, and $2\ \mu\text{g}$ of total RNA was reversely transcribed using RevertAidHMinus First Strand cDNA synthesis kit (Fermentas) following manufacturer's instructions. The real-time qRT-PCR analysis was performed using AceQ qPCR SYBR GreenMaster Mix (Vazyme, Nanjing, China) and a PCR iQ5 platform (Bio-Rad Laboratories, Hercules, CA, USA). The expression level of PVT1 was normalized to internal control GAPDH. Gene expression was quantified by using $2^{-\Delta\Delta\text{Ct}}$ method.¹⁷ The following primers were used in this study: PVT1 sense: 5'-CAGCACTCTGGACGGAC-3' and antisense: 5'-CAACAGGAGAAGCAAACA-3'. GAPDH sense: 5'-ACCACAGTCCATGCCATCAC-3' and antisense: 5'-TCACCACCCTGTTGCTGTA-3'.

Western blot analysis

HepG2 and HuH-6 cells were harvested and washed with PBS for 2 times. Cells were homogenized on ice for 20 mins

using RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with EDTA-free protease inhibitor cocktail (Roche). After centrifugation at 12,000 rpm for 20 mins at 4°C, supernatants were collected and protein concentration was determined using BCA method. Protein samples were denatured and separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 2 hrs with 5% nonfat dry milk diluted in tris-buffered saline with Tween-20 (TBST) at room temperature (RT), followed by the incubation with primary antibodies overnight at 4°C. After washing with TBST, membranes were incubated with corresponding horseradish peroxidase (HRP)-linked secondary antibodies for 1 hr at RT. Protein blots were developed by the reaction with ECL chemiluminescence reagents (GE Healthcare, Pittsburgh, PA, USA). The quantification of blots was processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The primary antibodies against p-STAT3 (Tyr705) (mouse monoclonal, NBP2-24463) and STAT3 (mouse monoclonal, MAB1779) were purchased from Novus Biologicals. The secondary HRP-conjugated antibodies (sc-2005) were purchased from Santa Cruz.

Cell proliferation assay

HepG2 and HuH-6 cells were seeded at 3000 cells per well in 96-well plates. After 2 days of culture, proliferation was measured using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche, 11647229001) according to manufacturer's instructions. In brief, BrdU-labeling solution (100 μ M) was added into each well and incubated for 4 hrs at 37°C. Absorbance was measured at 370 nm (reference wavelength 492 nm) by a microplate reader (Infinite 200, Tecan, Salzburg, Austria).

Cell cycle analysis

HepG2 and HuH-6 cells were seeded in 6-well plates. After 2 days of culture, cells were trypsinized and resuspended in PBS. Cells were then fixed overnight with 70% ice-cold ethanol at 4°C. After washing with PBS for 3 times, cells were incubated with 20 μ g/mL RNase A and 50 μ g/mL PI for 30 mins at 37°C in the dark. Cells were analyzed using FACSCalibur flow cytometer, and cell cycle distribution was analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA).

In vivo tumor xenograft model

Female nude mice (BALB/c, nu/nu) were purchased from the animal center of Central South University, and then

housed in a specific pathogen-free condition and allowed to access to animal chow and water ad libitum. HepG2 cells were trypsinized and resuspended in sterile saline solution. Equal numbers of HepG2 cells (2×10^5) were implanted into the flanks of 8-week-old female nude mice ($n=9$). Tumor volume was measured every week until the maximum volume was 1000 mm³. Tumor volume was calculated with the following formula: length \times width² \times $\pi/6$.¹⁸ The tumors were removed for further analyses after mouse sacrifice. All animal studies were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals approved by the review board of The Third Xiangya Hospital of Central South University.

Immunohistochemistry

The xenografted tumors were removed from nude mice when sacrificed, and then paraffin-embedded sections with 4- μ m thickness were processed. Prior to immunohistochemical staining, sections were deparaffinized, rehydrated in distilled water and equilibrated in 10 mM citric acid for 10 mins, followed by antigen retrieval for 5 mins at 100°C. After repeated washing with PBS, sections were blocked for 10 mins in 2% H₂O₂ diluted in methanol and then incubated in 5% horse serum for 30 mins at RT. After mild rinse with PBS, sections were probed with anti-Ki-67 primary antibody (1:200 dilution, Novus Biologicals, NB500-170SS) overnight at 4°C in a humidified box. After repeated rinse with PBS, sections were incubated with HRP-labeled anti-rabbit IgG secondary antibody (1:2000 dilution, Novus Biologicals, HAF008) for 1 hr at RT. After repeated rinse with PBS, sections were incubated with DAB (diaminobenzidine) reagent (Roche, 11718096001) for 2 mins and counterstained with hematoxylin.

Statistical analysis

All results were presented as mean \pm SD. Statistical analysis was performed using two-tailed Student's *t*-test or one-way ANOVA test with the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The comparisons were considered statistically significant when $p < 0.05$.

Results

PVT1 is upregulated in hepatoblastoma and predicts poor prognosis

PVT1 is a potential oncogene in numerous human cancers.¹³ To investigate whether PVT1 has a possible connection with hepatoblastoma, we initially compared its expression levels

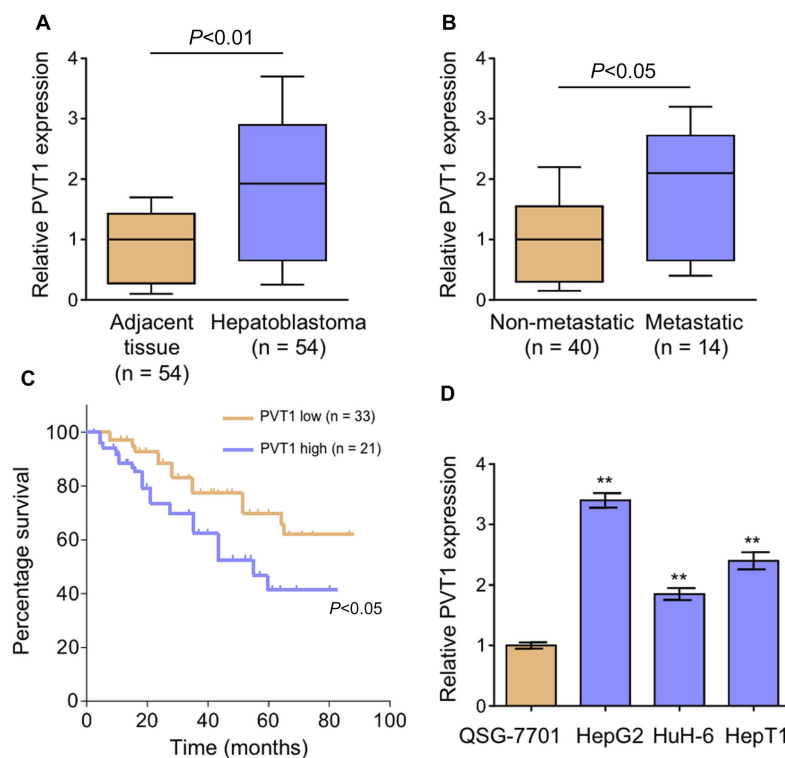


Figure 1 PVT1 is upregulated in hepatoblastoma tissues and cell lines and positively associated with the unfavorable prognosis of hepatoblastoma patients. **(A)** qRT-PCR analysis of PVT1 expression in hepatoblastoma tissues and the paired adjacent nontumor tissues (n=54). **(B)** Hepatoblastoma patients were divided into nonmetastatic group (n=40) and metastatic group (n=14). PVT1 expression was determined by qRT-PCR analysis. **(C)** Hepatoblastoma patients were divided using the mean value of PVT1 expression as a threshold. The percentage survival of hepatoblastoma patients with low (n=33) versus high (n=21) expression of PVT1 expression was determined by Kaplan–Meier analysis. **(D)** qRT-PCR analysis of PVT1 expression in hepatoblastoma cell lines, HepG2, HuH-6 and HepT1, and a nonmalignant liver cell line QSG-7701. Data are mean \pm SD (n=3). **P<0.01 versus QSG-7701. PVT1 expression was normalized to GAPDH and relative results were presented. **Abbreviations:** qRT-PCR, real-time quantitative PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

in hepatoblastoma tissues and adjacent noncancerous tissues. As analyzed by real-time qRT-PCR assay, a significantly higher PVT1 expression was observed in hepatoblastoma tissues than their matched noncancerous tissues (Figure 1A, n=54, $P<0.01$). In addition, hepatoblastoma tissues were further divided into nonmetastatic group (n=40) and metastatic group (n=14), and we found that PVT1 expression was even higher in the metastatic group compared with those in the nonmetastatic group (Figure 1B, $P<0.05$). Moreover, hepatoblastoma tissues were further divided into two groups with low-PVT1 (n=33) or high-PVT1 (n=21) expression using the mean value of PVT1 expression as a threshold. Kaplan–Meier analysis revealed that patients in the high-PVT1 group had shorter overall survival time than those patients in the low-PVT1 group (Figure 1C, $P<0.05$). These analyses suggest that PVT1 upregulation is associated with advanced progression and poor prognosis in hepatoblastoma. Furthermore, we also compared the PVT1 expression of three hepatoblastoma cell lines (HepG2, HuH-6 and HepT1) with that of a nonmalignant liver cell line QSG-

7701. As shown, PVT1 expression was markedly upregulated to varying degrees in these hepatoblastoma cell lines than that of QSG-7701 (Figure 1D). Thus, these lines of evidence indicate that PVT1 may be involved in hepatoblastoma progression.

PVT1 promotes hepatoblastoma cell proliferation

Lately, PVT1 has been shown to regulate the proliferation of multiple cancers, such as non-small cell lung cancer,¹⁹ gastric cancer²⁰ and pancreatic cancer.²¹ We wonder whether PVT1 plays a role in hepatoblastoma cell proliferation. To address this, an enforced ectopic expression of PVT1 was established in HepG2 and HuH-6 cells via lentiviral infection (Figure 2A). Next, BrdU incorporation assay showed that HepG2 (Figure 2B) and HuH-6 (Figure 2C) cells with PVT1 overexpression had a higher proliferation rate compared with those cells expressing vector control, suggesting that PVT1 promotes hepatoblastoma cell proliferation. To confirm this role of PVT1, we knocked it down in HepG2 and HuH-6 cells

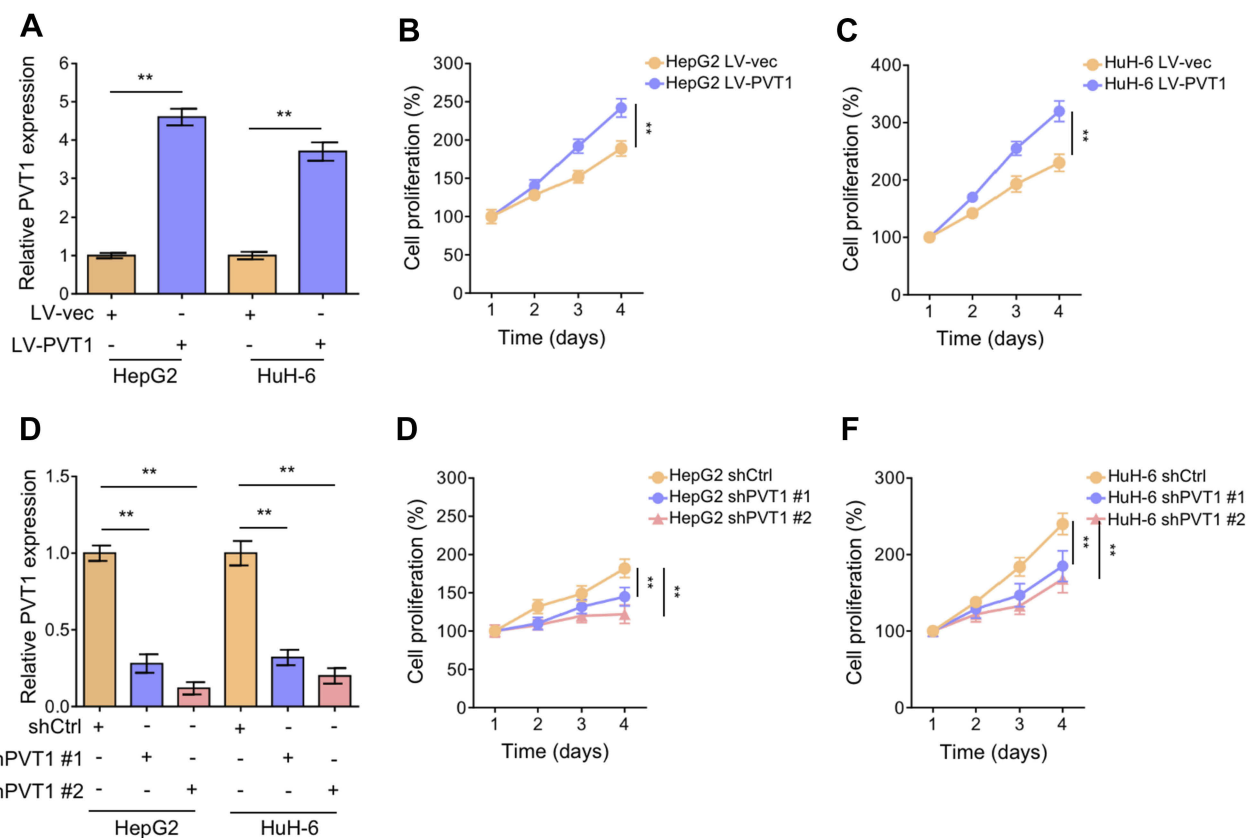


Figure 2 PVT1 promotes hepatoblastoma cell proliferation in vitro. **(A-C)** HepG2 and HuH-6 cells stably overexpressing empty vector control (LV-vec) or PVT1 (LV-PVT1) were established via lentiviral infection. **(A)** PVT1 expression was determined by qRT-PCR analysis. **(B-C)** The cell proliferation of HepG2 **(B)** and HuH-6 **(C)** was monitored by Cell Counting Kit-8 (CCK-8) assay during the course of continuous cell culture. **(D-F)** HepG2 and HuH-6 cells stably expressing control shRNA (shCtrl) or two different sets of PVT1 shRNA (shPVT1 #1 and shPVT1 #2) were established via lentiviral infection. **(D)** PVT1 expression was determined by qRT-PCR analysis. **(E-F)** The cell proliferation of HepG2 **(E)** and HuH-6 **(F)** was monitored by CCK-8 assay during the course of continuous cell culture. Data are mean \pm SD (n=5). **P<0.01. **Abbreviation:** shRNA, short hairpin RNA.

using two different sets of small hairpin RNA (shRNA #1, shRNA #2) (Figure 2D). In accordance with above-mentioned results, we found that the proliferation of HepG2 (Figure 2E) and HuH-6 (Figure 2F) cells expressing PVT1 shRNA #1 and PVT1 shRNA #2 was remarkably suppressed compared with that of cells expressing control shRNA. Together, these results indicate that PVT1 functions to promote hepatoblastoma cell proliferation, at least in vitro.

PVT1 promotes hepatoblastoma cell growth in a mouse xenograft model

To examine whether PVT1 promotes hepatoblastoma cell growth in vivo, HuH-6 cells with or without PVT1 overexpression were inoculated in BALB/c nude mice. The curve of tumor growth depicted that HuH-6 tumors with PVT1 overexpression exhibited a higher growth rate than control ones (Figure 3A). Conversely, the growth rate of HuH-6 tumors with PVT1 knockdown was pronouncedly suppressed compared with those expressing shRNA

control (Figure 3B). The overexpression or knockdown of PVT1 in HuH-6 tumors was subsequently confirmed by qRT-PCR analysis (Figure 3C). Moreover, consistently, the immunohistochemistry analysis showed that HuH-6 tumors with PVT1 overexpression displayed stronger expression Ki-67, a marker of proliferation,²² than control tumors, and oppositely, PVT1 knockdown resulted in a weaker Ki-67 expression in HuH-6 tumors (Figure 3D). Hence, PVT1 not only promotes hepatoblastoma cell proliferation in vitro, but also boosts its growth in vivo.

PVT1 activates STAT3 and cell cycle progression

It has been recently reported that PVT1 activates STAT3 signaling pathway in gastric cancer.²³ Besides, STAT3 activation regulates cell cycle progression and proliferation in cancers,^{24,25} including hepatoblastoma.¹² To test whether STAT3 is involved in PVT1-promoted hepatoblastoma cell proliferation, we first checked its activation status in response

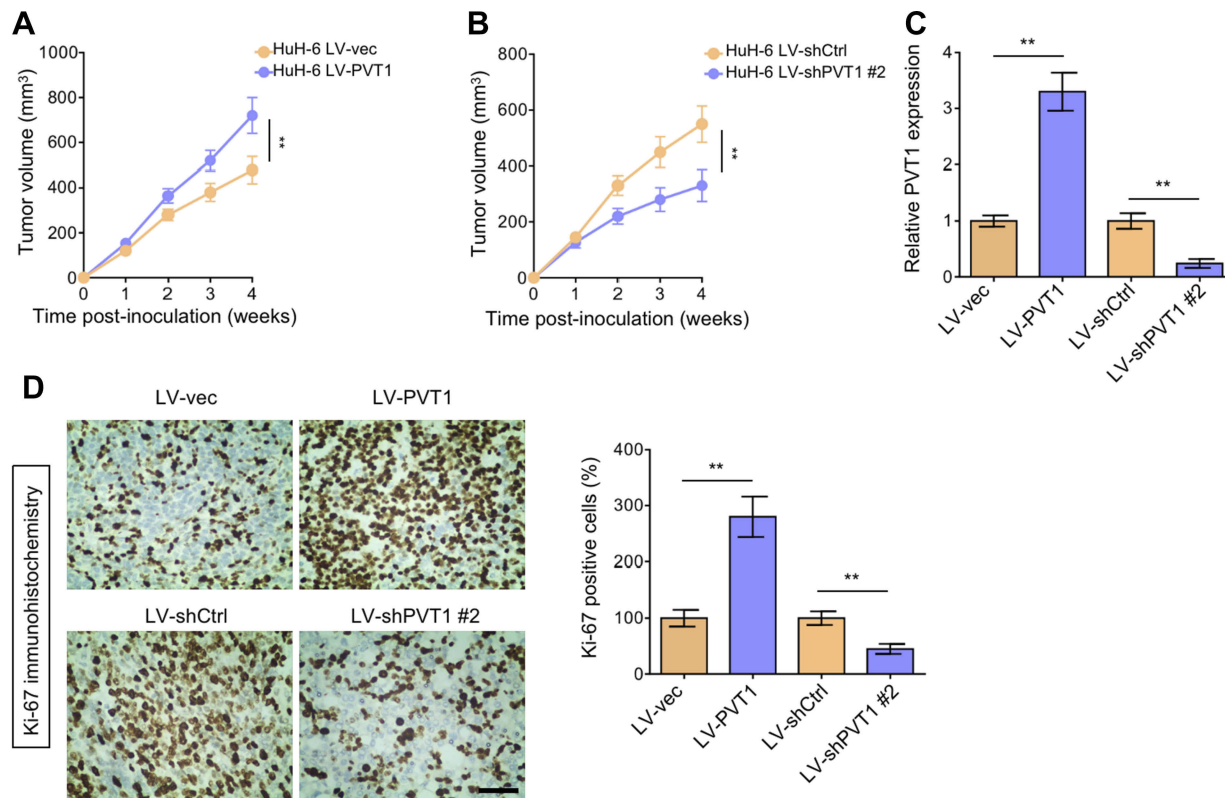


Figure 3 PVT1 promotes hepatoblastoma cell growth in vivo. (A–B) HuH-6 cells stably overexpressing Lenti-vec or Lenti-PVT1 (A), or expressing shCtrl or shPVT1 (B), were subcutaneously inoculated into the flank of athymic nude mice (n=9). Tumor volumes were measured by a caliper every week until 4 weeks after inoculation. (C) PVT1 expression in the resected tumors as shown in (A–B) was determined by qRT-PCR analysis (n=3). (D) Immunohistochemistry analysis of Ki-67 on tumor sections. Scale bar, 100 μ m. The representative images (left) and quantitative analysis of Ki-67 positive cells (right) from 12 random fields of each group were depicted. Data are mean \pm SD. **P<0.01.

to PVT1 manipulation. Western blot analysis revealed that in both HepG2 and HuH-6 cells, the level of phosphorylation of STAT3 (p-STAT3) was starkly elevated when PVT1 was overexpressed (Figure 4A). In reverse, PVT1 knockdown resulted in decreased level of p-STAT3 in these cells (Figure 4B), indicating that PVT1 activates STAT3 in HepG2 and HuH-6 cells. Following STAT3 activation, the p-STAT3 will dimerize and translocate to the nucleus, whereby upregulating numerous critical genes are involved in cell cycle progression and proliferation, such as cyclin D1, Myc and cyclin B1.^{24–27} Keeping pace with STAT3 activation, we found that the transcript level of cyclin D1, Myc and cyclin B1 was increased upon PVT1 overexpression (Figure 4C), and reversely, PVT1 knockdown decreased their expression (Figure 4D). Furthermore, in concert with these results, cell cycle distribution analysis showed that PVT1 knockdown retarded (Figure 4E) and its overexpression promoted (Figure 4F) cell cycle progression of HepG2 and HuH-6 cells. Collectively, these data indicate that PVT1 activates STAT3 and accelerates cell cycle progression of hepatoblastoma cells.

STAT3 inhibition abrogates PVT1 effects on cell cycle progression and proliferation of hepatoblastoma

Finally, we asked whether STAT3 activation contributes to PVT1-promoted hepatoblastoma cell proliferation. To this end, we inhibited STAT3 in HepG2 and HuH-6 cells with static, a small-molecule inhibitor of STAT3 activation and dimerization.²⁸ The inhibition of STAT3 by static was confirmed by decreased p-STAT3 level (Figure 5A) as well as suppressed expression of cyclin D1, Myc and cyclin B1 (Figure 5B) in HepG2 and HuH-6 cells overexpressing PVT1. Notably, along with inhibited STAT3 activation, PVT1-accelerated cell cycle progression was abrogated in HepG2 and HuH-6 cells (Figure 5C). Accordingly, PVT1-promoted proliferation of HepG2 (Figure 5D) and HuH-6 (Figure 5E) cells also vanished in the presence of static treatment. Taken together, these findings identify STAT3 activation-induced cell cycle progression as a critical mechanism by which PVT1 promotes hepatoblastoma cell proliferation.

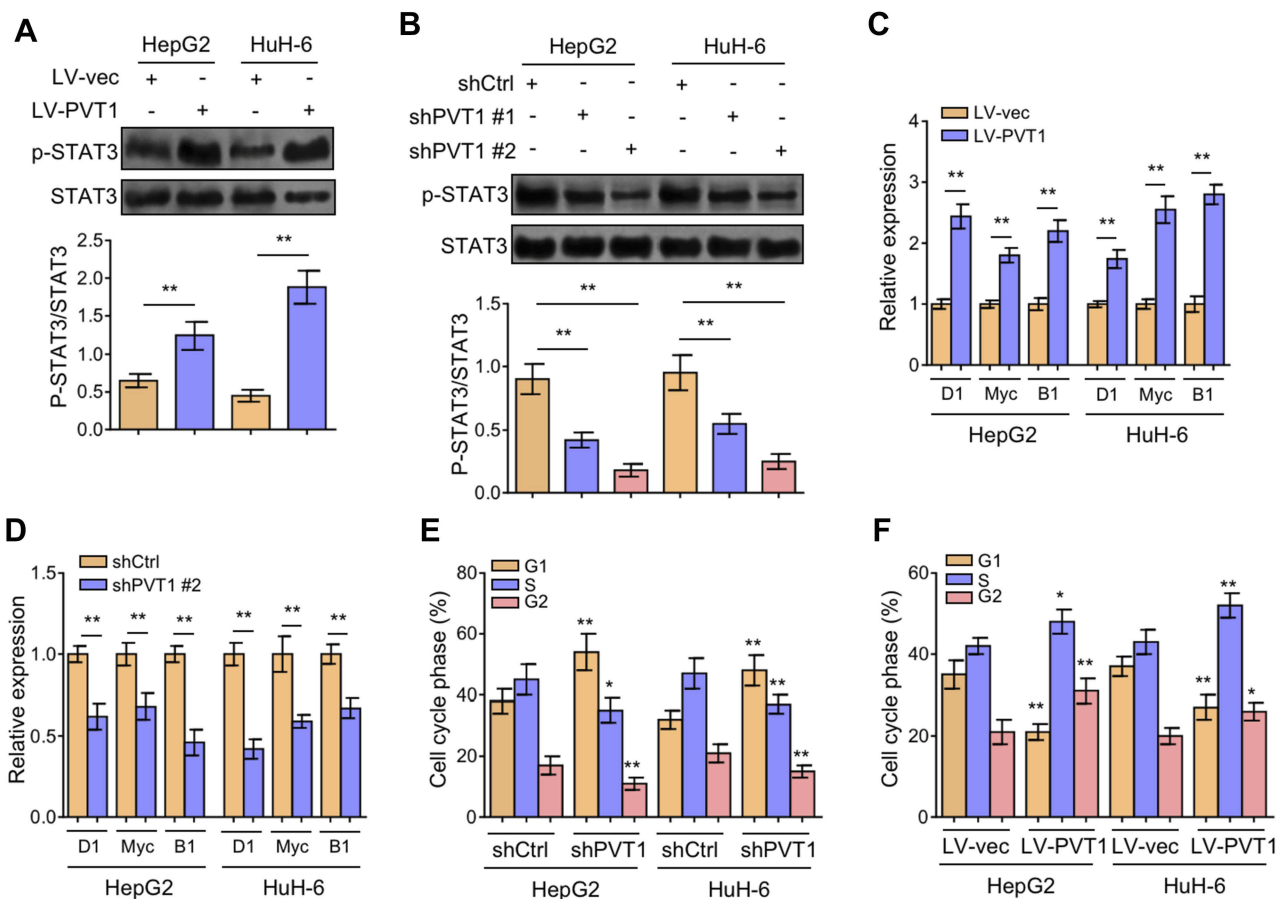


Figure 4 PVT1 activates STAT3 and promotes cell cycle progression. **(A)** Western blotting analysis of p-STAT3 and STAT3 in HepG2 and HuH-6 cells stably expressing Lenti-vec or Lenti-PVT1. **(B)** Western blotting analysis of p-STAT3 and STAT3 in HepG2 and HuH-6 cells stably expressing shCtrl or PVT1 shRNA (shPVT1 #1 and shPVT1 #2). The representative images (top) and quantification of p-STAT3 level relative to STAT3 (bottom) are shown. **(C-D)** qRT-PCR analysis of mRNA levels of cyclin D1, myc, and cyclin B1 in HepG2 and HuH-6 cells shown as in **(A-B)**. **(E-F)** The cell cycle distribution of HepG2 and HuH-6 cells shown as in **(A-B)** was evaluated by FACS analysis. Data are mean \pm SD (n=3). **P<0.01; *P<0.05 versus control.

Abbreviations: STAT3, signal transducer and activator of transcription 3; FACS, fluorescence activated cell sorter.

Discussion

Hepatoblastoma is a common liver malignancy in infants and children.²⁹ Due to the development of effective neoadjuvant chemotherapy and surgical approaches, the clinical outcome of patients with hepatoblastoma has dramatically improved over the last three decades.⁴ However, for a high proportion of patients who are diagnosed with advanced hepatoblastoma, including primary refractory disease and metastatic disease, the treatment effectiveness is still very limited and the prognosis remains poor.³⁰ Therefore, there is an unmet clinical need to improve early diagnosis and retard the aggressive progression of hepatoblastoma. In recent years, numerous studies have reported the dysregulation of lncRNAs in hepatoblastoma and correlated it to patient prognosis,^{10,11,31,32} and a genome-wide screening analysis has also been carried out to identify lncRNAs which may serve as potential targets for hepatoblastoma.⁹ In this study,

we for the first time report that lncRNA PVT1 is upregulated in hepatoblastoma tissues and that its expression is positively correlated with advanced progression of hepatoblastoma and also predicts the poor prognosis of patients. We next explored the possible involvement of PVT1 in hepatoblastoma progression and show that PVT1 promotes hepatoblastoma cell proliferation both in vitro and in vivo. We further reveal that the promoted hepatoblastoma cell proliferation by PVT1 is dependent on STAT3 activation-induced cell cycle progression. Therefore, our study may identify PVT1 as a novel prognostic factor for hepatoblastoma and also uncover it as a positive regulator in hepatoblastoma cell proliferation.

Although hepatoblastoma is the most common hepatic cancer in infants and children, its incidence is rare, and currently there are no validated prognostic biomarkers for hepatoblastoma patients.³³ The large-scale efforts have been paid to proliferate the prognostic biomarkers for hepatoblastoma, and

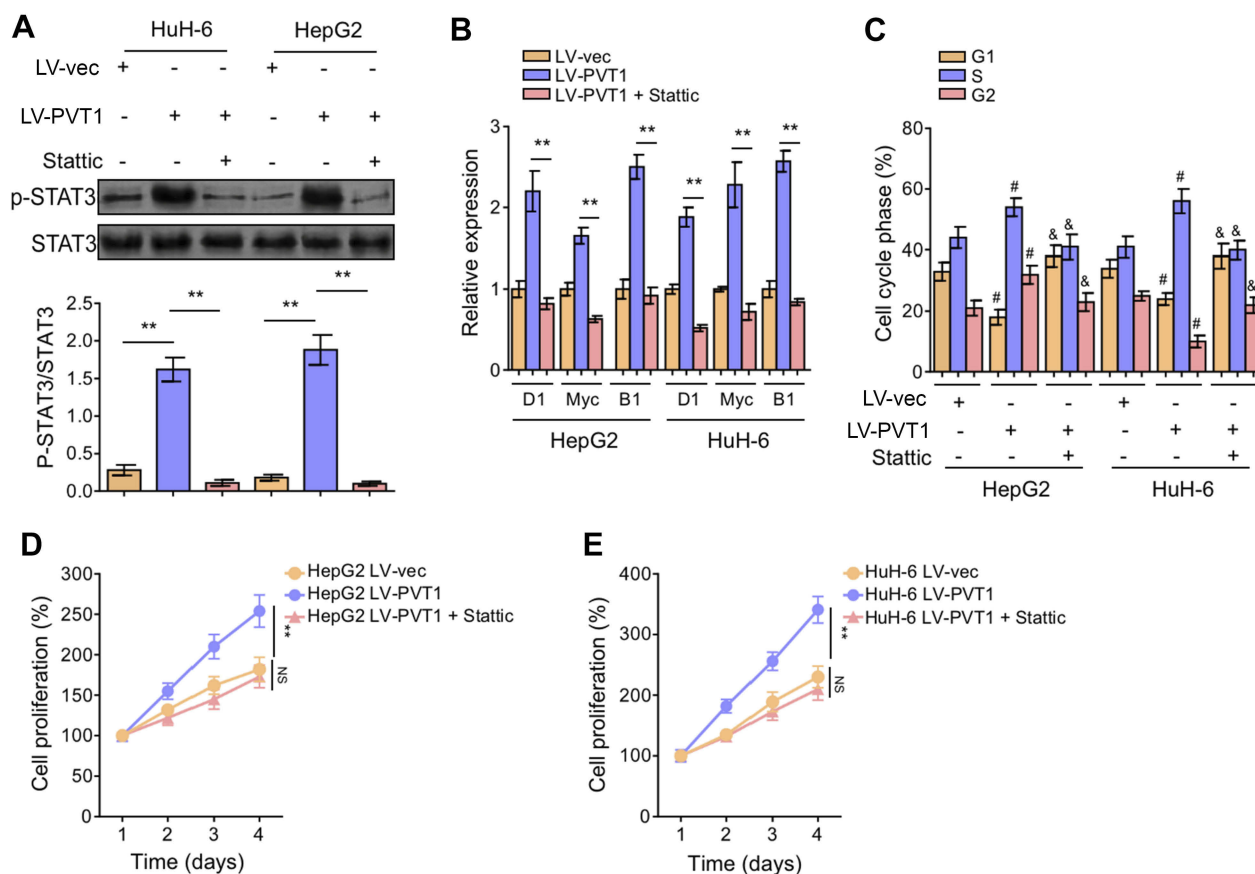


Figure 5 PVT1 promotes hepatoblastoma cell proliferation through activating STAT3-mediated cell cycle progression. **(A-E)** HepG2 and HuH-6 cells stably expressing Lenti-vec or Lenti-PVT1 were treated with vehicle control or STAT3 inhibitor stattic. **(A)** The levels of p-STAT3 and STAT3 were determined by Western blotting analysis. The representative images (top) and quantification of p-STAT3 level relative to STAT3 (bottom) are shown. **(B)** The mRNA levels of cyclin D1, myc, and cyclin B1 were determined by qRT-PCR analysis. **(C)** The cell cycle distribution was evaluated by FACS analysis. #, $P < 0.01$, Lenti-PVT1 versus Lenti-vec; &, $P < 0.01$, Lenti-PVT1 plus stattic versus Lenti-PVT1 plus vehicle control. **(D-E)** The cell proliferation of HepG2 **(D)** and HuH-6 **(E)** cells was monitored by CCK-8 assay during the course of continuous cell culture. Data are mean \pm SD ($n=5$). ** $P < 0.01$; NS, not significant.

several candidates were proposed, such as LIN28B, AFP, NOTCH1, NRF2, CTNNB1, and HNF1A². Moreover, lately, in one comprehensive microarray data analysis for profiling the expression of lncRNAs, seven lncRNAs were identified to be of prognostic value, including LINC01314.³¹ Previous studies have also shown that the increased expression of PVT1 is associated with poor prognosis of multiple human cancers, such as colorectal cancer,³⁴ gastric cancer,²⁰ pancreatic cancer,³⁵ non-small cell lung cancer,³⁶ cervical cancer³⁷ and hepatocellular carcinoma.³⁸ We found that PVT1 expression was significantly higher in hepatoblastoma tissues than noncancerous counterparts, and that PVT1 expression was even higher in hepatoblastoma tissues with metastasis. Further Kaplan–Meier analysis showed that patients with high expression of PVT1 had shorter overall survival time than those with low-PVT1 expression. Given its association with hepatoblastoma aggressiveness and poor prognosis, we suppose that PVT1 may be used as a biomarker to improve

risk stratification and assist treatment decisions for hepatoblastoma patients at diagnosis and predict clinical prognosis. However, it should be noted that the investigated clinical sample size in the present study is very limited. More studies which include larger sample size are required to validate our findings in the future. Moreover, PVT1 expression is markedly upregulated in three hepatoblastoma cell lines compared with a nonmalignant liver cell line, further suggesting a connection between PVT1 and hepatoblastoma pathogenesis. It has been demonstrated that PVT1 is a downstream target of Myc proteins,³⁹ and a high Pearson correlation between PVT1 and MYC expression levels has been revealed by an analysis of The Cancer Genome Atlas (TCGA) project.¹⁴ Additionally, PVT1 was also reported as a p53-inducible target gene.⁴⁰ Myc activation is involved in the pathogenesis of hepatoblastoma,⁴¹ and the overexpression of p53 protein was infrequently observed in hepatoblastoma.⁴² Therefore, we doubt that PVT1 upregulation in hepatoblastoma tissues and cell lines

may be due to the regulation by Myc and/or p53. Addressing this issue may provide insights into the molecular mechanism that controls PVT1 expression in hepatoblastoma.

The proliferation of hepatoblastoma cells can be modulated by lncRNAs, like OIP5-AS1,⁴³ and LUCAT1.¹² Through utilizing hepatoblastoma cell lines HepG2 and HuH-6, and a mouse xenograft model, we observed that PVT1 promoted hepatoblastoma cell proliferation in vitro and tumor growth in vivo. Thus, we supplement PVT1 as another lncRNA which plays a regulatory role in hepatoblastoma cell proliferation and growth, and in turn, therapeutic targeting of PVT1 may have potential clinical benefit in interfering hepatoblastoma growth and progression. In fact, except for hepatoblastoma, PVT1 seems to play a pro-proliferative function in a broad range of human cancers,^{13,14} which suggests its wide application as a therapeutic target in cancer therapy. PVT1 plays versatile functions in cancer, including those activities in regulating apoptosis,⁴⁴ migration and invasion,⁴⁵ stemness¹⁵ and drug resistance.⁴⁶ The functional roles and underlying mechanisms of PVT1 in hepatoblastoma are largely uncharacterized, it would be interesting to test whether and how PVT1 regulates other malignant phenotypes in hepatoblastoma.

Acting as an oncogene, STAT3 is persistently activated in numerous human cancers and transformed cells.⁴⁷ STAT3 expression is also higher in hepatoblastoma tissues than that in adjacent nontumor tissues.¹² The well-established roles for transcription factor STAT3 in cancer include promoting cancer cell proliferation, survival, invasion and immunosuppression, which render STAT3 to be one of the most promising targets for cancer therapy.⁴⁸ STAT3 promotes proliferation through G1 and G2/M cell cycle progression via inducing the expression of regulators involved in cell cycle machinery, including cyclin D1, cyclin B1 and c-Myc.²⁴ We show that PVT1 activates STAT3 and promotes cell cycle progression in hepatoblastoma cells, and more importantly, the treatment of STAT3 inhibitor stattic abrogates PVT1-induced cell cycle progression and proliferation of hepatoblastoma cells, thus establishing a concept that STAT3 activation-mediated cell cycle progression is a downstream event through which PVT1 promotes hepatoblastoma cell proliferation. Coincidentally, in gastric cancer, PVT1 was found to promote angiogenesis via activating the STAT3/VEGFA axis.²³ Furthermore, it has been well demonstrated that PVT1 is able to directly interact with p-STAT3 in the nucleus so as to increase its protein stability by preventing

degradation.²³ Given this closely relevant clue, we conjecture it is very likely that PVT1 activates STAT3 in hepatoblastoma cells by increasing the stability of p-STAT3, which is consistent with our observation that PVT1 elevates p-STAT3 level in hepatoblastoma cells.

In summary, we identify the PVT1/STAT3 axis as a positive regulator for cell cycle progression and proliferation of hepatoblastoma cells. According to our findings, antagonizing PVT1/STAT3 axis may display efficacy in retarding the growth and progression of hepatoblastoma.

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Disclosure

The authors report no conflicts of interest in this work. Dr Zhenqin Luo has nothing to disclose. Dr Peiguo Cao has nothing to disclose.

References

1. Spector LG, Birch J. The epidemiology of hepatoblastoma. *Pediatr Blood Cancer*. 2012;59(5):776–779. doi:10.1002/pbc.24215
2. Sumazin P, Chen Y, Trevino LR, et al. Genomic analysis of hepatoblastoma identifies distinct molecular and prognostic subgroups. *Hepatology*. 2017;65(1):104–121. doi:10.1002/hep.28888
3. Czauderna P, Garnier H. Hepatoblastoma: current understanding, recent advances, and controversies. *F1000Research*. 2018;7:53. doi:10.12688/f1000research
4. Meyers RL, Tiao G, de Ville de Goyet J, Superina R, Aronson DC. Hepatoblastoma state of the art: pre-treatment extent of disease, surgical resection guidelines and the role of liver transplantation. *Curr Opin Pediatr*. 2014;26(1):29–36. doi:10.1097/MOP.00000000000000042
5. Trobaugh-Lotrario AD, Meyers RL, O'Neill AF, Feusner JH. Unresectable hepatoblastoma: current perspectives. *Hepat Med*. 2017;9:1–6. doi:10.2147/HMER.S89997
6. Trobaugh-Lotrario AD, Katzenstein HM. Chemotherapeutic approaches for newly diagnosed hepatoblastoma: past, present, and future strategies. *Pediatr Blood Cancer*. 2012;59(5):809–812. doi:10.1002/pbc.24219
7. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet*. 2009;10(3):155–159. doi:10.1038/nrg2521
8. Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. *Mol Cancer*. 2011;10:38. doi:10.1186/1476-4598-10-93
9. Dong R, Jia D, Xue P, et al. Genome-wide analysis of long noncoding RNA (lncRNA) expression in hepatoblastoma tissues. *PLoS One*. 2014;9(1):e85599. doi:10.1371/journal.pone.0085599
10. Dong R, Liu GB, Liu BH, et al. Targeting long non-coding RNA-TUG1 inhibits tumor growth and angiogenesis in hepatoblastoma. *Cell Death Dis*. 2016;7(6):e2278. doi:10.1038/cddis.2016.143

11. Dong R, Liu XQ, Zhang BB, Liu BH, Zheng S, Dong KR. Long non-coding RNA-CRND: a novel regulator of tumor growth and angiogenesis in hepatoblastoma. *Oncotarget*. 2017;8(26):42087–42097. doi:10.18632/oncotarget.14992
12. Wang X, Guo S, Zhao R, Liu Y, Yang G. STAT3-activated lncRNA LUCAT1 drives cell proliferation, migration and invasion in hepatoblastoma through regulating miR-301b/STAT3 axis. *Hum Gene Ther*. 2018;30:702–713.
13. Cui M, You L, Ren X, Zhao W, Liao Q, Zhao Y. Long non-coding RNA PVT1 and cancer. *Biochem Biophys Res Commun*. 2016;471(1):10–14. doi:10.1016/j.bbrc.2015.12.101
14. Colombo T, Farina L, Macino G, Paci P. PVT1: a rising star among oncogenic long noncoding RNAs. *Biomed Res Int*. 2015;2015:304208. doi:10.1155/2015/304208
15. Wang F, Yuan JH, Wang SB, et al. Oncofetal long noncoding RNA PVT1 promotes proliferation and stem cell-like property of hepatocellular carcinoma cells by stabilizing NOP2. *Hepatology*. 2014;60(4):1278–1290. doi:10.1002/hep.27239
16. Gou X, Zhao X, Wang Z. Long noncoding RNA PVT1 promotes hepatocellular carcinoma progression through regulating miR-214. *Cancer Biomark*. 2017;20(4):511–519. doi:10.3233/CBM-170331
17. Saiki S, Sasazawa Y, Imamichi Y, et al. Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition. *Autophagy*. 2011;7(2):176–187. doi:10.4161/auto.7.2.14074
18. Guo X, Keyes WM, Papazoglu C, et al. TAp63 induces senescence and suppresses tumorigenesis in vivo. *Nat Cell Biol*. 2009;11(12):1451–1457. doi:10.1038/ncb1988
19. Wan L, Sun M, Liu GJ, et al. Long noncoding RNA PVT1 promotes non-small cell lung cancer cell proliferation through epigenetically regulating LATS2 expression. *Mol Cancer Ther*. 2016;15(5):1082–1094. doi:10.1158/1535-7163.MCT-15-0707
20. Kong R, Zhang EB, Yin DD, 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*. 2015;14:82. doi:10.1186/s12943-014-0278-9
21. Zhao L, Kong H, Sun H, Chen Z, Chen B, Zhou M. LncRNA-PVT1 promotes pancreatic cancer cells proliferation and migration through acting as a molecular sponge to regulate miR-448. *J Cell Physiol*. 2018;233(5):4044–4055. doi:10.1002/jcp.26072
22. Telugu RB, Chowhan AK, Rukmangadha N, et al. Histopathological and immunohistochemical evaluation of meningiomas with reference to proliferative markers p53 and Ki-67. *J Clin Diag Res*. 2016;10(1):EC15–19. doi:10.7860/JCDR/2016/15661.7117
23. Zhao J, Du P, Cui P, et al. LncRNA PVT1 promotes angiogenesis via activating the STAT3/VEGFA axis in gastric cancer. *Oncogene*. 2018;37(30):4094–4109. doi:10.1038/s41388-018-0250-z
24. Bollrath J, Pheesse TJ, von Burstin VA, et al. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell*. 2009;15(2):91–102. doi:10.1016/j.ccr.2009.01.002
25. Aggarwal BB, Kunnumakara AB, Harikumar KB, et al. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci*. 2009;1171:59–76. doi:10.1111/j.1749-6632.2009.04911.x
26. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene*. 2000;19(21):2468–2473. doi:10.1038/sj.onc.1203476
27. Zhao J, Lin W, Cao Z, et al. Total alkaloids of *Rubus aleaefolius* Poir. inhibit the STAT3 signaling pathway leading to suppression of proliferation and cell cycle arrest in a mouse model of hepatocellular carcinoma. *Oncol Rep*. 2013;30(3):1309–1314. doi:10.3892/or.2013.2585
28. Schust J, Sperl B, Hollis A, Mayer TU, Berg T. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem Biol*. 2006;13(11):1235–1242. doi:10.1016/j.chembiol.2006.09.018
29. Czauderna P, Haeberle B, Hiyama E, et al. The Children's Hepatic tumors International Collaboration (CHIC): novel global rare tumor database yields new prognostic factors in hepatoblastoma and becomes a research model. *Eur J Cancer*. 2016;52:92–101. doi:10.1016/j.ejca.2015.09.023
30. Venkatramani R, Furman WL, Fuchs J, Warmann SW, Malogolowkin MH. Current and future management strategies for relapsed or progressive hepatoblastoma. *Paediatr Drugs*. 2012;14(4):221–232. doi:10.2165/11597740-000000000-00000
31. Lv B, Zhang L, Miao R, et al. Comprehensive analysis and experimental verification of LINC01314 as a tumor suppressor in hepatoblastoma. *Biomed Pharmacother*. 2018;98:783–792. doi:10.1016/j.biopha.2018.01.013
32. Liu S, Xie F, Xiang X, et al. Identification of differentially expressed genes, lncRNAs and miRNAs which are associated with tumor malignant phenotypes in hepatoblastoma patients. *Oncotarget*. 2017;8(57):97554–97564. doi:10.18632/oncotarget.22181
33. Purcell R, Childs M, Maibach R, et al. Potential biomarkers for hepatoblastoma: results from the SIOPEL-3 study. *Eur J Cancer*. 2012;48(12):1853–1859. doi:10.1016/j.ejca.2011.10.019
34. Takahashi Y, Sawada G, Kurashige J, et al. Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers. *Br J Cancer*. 2014;110(1):164–171. doi:10.1038/bjc.2013.698
35. Huang C, Yu W, Wang Q, et al. Increased expression of the lncRNA PVT1 is associated with poor prognosis in pancreatic cancer patients. *Minerva Med*. 2015;106(3):143–149.
36. Cui D, Yu CH, Liu M, Xia QQ, Zhang YF, Jiang WL. Long non-coding RNA PVT1 as a novel biomarker for diagnosis and prognosis of non-small cell lung cancer. *Tumour Biol*. 2016;37(3):4127–4134. doi:10.1007/s13277-015-4261-x
37. Iden M, Fye S, Li K, Chowdhury T, Ramchandran R, Rader JS. The lncRNA PVT1 contributes to the cervical cancer phenotype and associates with poor patient prognosis. *PLoS One*. 2016;11(5):e0156274. doi:10.1371/journal.pone.0156274
38. Ding C, Yang Z, Lv Z, et al. Long non-coding RNA PVT1 is associated with tumor progression and predicts recurrence in hepatocellular carcinoma patients. *Oncol Lett*. 2015;9(2):955–963. doi:10.3892/ol.2014.2730
39. Carramusa L, Contino F, Ferro A, et al. The PVT-1 oncogene is a Myc protein target that is overexpressed in transformed cells. *J Cell Physiol*. 2007;213(2):511–518. doi:10.1002/jcp.21133
40. Barsotti AM, Beckerman R, Laptenko O, Huppi K, Caplen NJ, Prives C. p53-Dependent induction of PVT1 and miR-1204. *J Biol Chem*. 2012;287(4):2509–2519. doi:10.1074/jbc.M111.322875
41. Cairo S, Wang Y, de Reynies A, et al. Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. *Proc Natl Acad Sci U S A*. 2010;107(47):20471–20476. doi:10.1073/pnas.1009009107
42. Chen TC, Hsieh LL, Kuo TT. Absence of p53 gene mutation and infrequent overexpression of p53 protein in hepatoblastoma. *J Pathol*. 1995;176(3):243–247. doi:10.1002/path.1711760306
43. Zhang Z, Liu F, Yang F, Liu Y. Knockdown of OIP5-AS1 expression inhibits proliferation, metastasis and EMT progress in hepatoblastoma cells through up-regulating miR-186a-5p and down-regulating ZEB1. *Biomed Pharmacother*. 2018;101:14–23. doi:10.1016/j.biopha.2018.02.026
44. Zhuang C, Li J, Liu Y, et al. Tetracycline-inducible shRNA targeting long non-coding RNA PVT1 inhibits cell growth and induces apoptosis in bladder cancer cells. *Oncotarget*. 2015;6(38):41194–41203. doi:10.18632/oncotarget.5880

45. Huang C, Liu S, Wang H, Zhang Z, Yang Q, Gao F. LncRNA PVT1 overexpression is a poor prognostic biomarker and regulates migration and invasion in small cell lung cancer. *Am J Transl Res*. 2016;8(11):5025–5034.
46. Zhang XW, Bu P, Liu L, Zhang XZ, Li J. Overexpression of long non-coding RNA PVT1 in gastric cancer cells promotes the development of multidrug resistance. *Biochem Biophys Res Commun*. 2015;462(3):227–232. doi:10.1016/j.bbrc.2015.04.121
47. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. *Cell*. 1999;98(3):295–303. doi:10.1016/s0092-8674(00)81959-5
48. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer*. 2014;14(11):736–746. doi:10.1038/nrc3818

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