Long non-coding RNA PVT1 serves as a competing endogenous RNA for miR-186-5p to promote the tumorigenesis and metastasis of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma is third leading cause of cancer-related death globally. Long non-coding RNA plasmacytoma variant translocation 1 has been reported to be dysregulated and plays a crucial role in various cancers. In this study, we investigated the interactions between plasmacytoma variant translocation 1 and miR-186-5p in the progression of hepatocellular carcinoma and explored the functional significance of plasmacytoma variant translocation 1. It was determined that plasmacytoma variant translocation 1 was significantly higher, while miR-186-5p was statistically lower in the hepatocellular carcinoma tissues than that in the adjacent normal tissues. Using gain-of-function and loss-of-function methods, our results revealed that plasmacytoma variant translocation 1 affected hepatocellular carcinoma cells proliferation, invasion, and migration. It was found that there was direct interaction between miR-186-5p and the binding site of plasmacytoma variant translocation 1 by performing dual-luciferase assay and RNA immunoprecipitation assay. Furthermore, it was identified that plasmacytoma variant translocation 1 regulated the expression of the miR-186-5p target gene, yes-associated protein 1. Taken together, plasmacytoma variant translocation 1 served as an endogenous sponge for miR-186-5p to reduce its inhibiting effect on yes-associated protein 1 and thus promoted the tumorigenesis of hepatocellular carcinoma.

Keywords

Hepatocellular carcinoma, plasmacytoma variant translocation 1, miR-186-5p, yes-associated protein 1, competing endogenous RNA, endogenous sponge

Date received: 10 November 2016; accepted: 11 January 2017

Introduction

Hepatocellular carcinoma (HCC) has been regarded as the fifth most common type of malignant tumor. Invasion, metastasis, and postoperative recurrence are identified as the main causes of death in patients with HCC. More seriously, HCC is frequently diagnosed at an advanced stage and hence has a high lethality.¹ The molecular mechanism underlying HCC carcinogenesis has not been completely elucidated. The development and progression of HCC are often described as a multistage process involving a series of genetic alterations that play the crucial roles in the malignant transformation of the hepatocytes.² Currently, an increasing number of studies...
reported the role of non-coding RNAs (ncRNAs) in the carcinogenesis of HCC, including microRNAs (miRNAs), long non-coding RNA (lncRNAs), and small nucleolar RNAs (snoRNAs).

LncRNAs are a class of RNA molecule with over 200 nucleotides in length and do not code for proteins but bear the ability to regulate the gene expression. Accumulating evidence showed that the dysregulation of lncRNAs might be associated with the development and metastasis of various cancers. Additionally, as another class of ncRNAs with lengths of 17–25 nucleotides, miRNAs could also regulate the gene expression by inducing messenger RNA (mRNA) degradation and/or inhibiting mRNA translation. Similarly, miRNAs are involved in diverse cellular biological processes and are deregulated in various cancers. It is reported that all RNA transcripts bearing miRNA-binding sites can interact and regulate each other’s expression levels by specifically competing for shared miRNAs, acting as competing endogenous RNAs (ceRNAs). Emerging studies demonstrated that lncRNAs might play as the ceRNAs to exert their function on tumors, by binding with miRNAs and regulating target gene expression. Although this ceRNA regulation mode was confirmed in the tumorigenesis of various cancers, the exploration of ceRNA interaction in cancers will enrich our comprehension about the carcinogenesis and tumor progression.

LncRNA plasmacytoma variant translocation 1 (PVT1) locates on chromosome 8q24 which is a region with frequent copy number amplification. Numerous studies have shown that upregulation of PVT1 was associated with tumor progression and reduced survival in various cancers. It has been identified that PVT1 is associated with tumor progression and predicts recurrence in HCC patients. However, its molecular mechanism in HCC remains unknown. According to the bioinformatics prediction, there was a putative binding site between PVT1 and miR-186-5p. Therefore, we hypothesized that PVT1 might exert its oncogenic function via the above-mentioned ceRNA regulation pattern.

In this study, we investigated the expression of PVT1 and miR-186-5p in HCC samples. Using gain-of-function and loss-of-function approaches, we detected the effects of PVT1 on HCC cells proliferation, cell apoptosis, invasion, and migration. Besides, we explored the molecular mechanisms underlying how PVT1 interacts with miR-186-5p. Our data indicated that PVT1 acted as an oncogenic ceRNA by binding miR-186-5p to upregulate yes-associated protein 1 (YAP1) expression, thus promoting the tumorigenesis of HCC.

**Materials and methods**

**Tissue specimens and cell lines**

The paired HCC samples and the adjacent non-tumor tissues were collected following curative surgical resection from 48 patients with HCC in the Zhongnan Hospital of Wuhan University between May 2011 and May 2013. This study was approved by the Hospital’s Protection of Human Subjects Committee, and written informed consent was obtained from all patients. Based on their medical documents, we conducted a 48-month follow-up survival survey. Overall survival (OS) was defined as the interval between resection and death or the last follow-up visit. Curative resection was defined as the removal of all recognizable tumor tissue with a clear microscopic margin. HepG2, Hep3B, Huh-7, HCCLM9, SK-Hep1, and SMMC-7721 cell lines included in this study were purchased from the Cell Bank of Type Culture Collection (CBTCC, Chinese Academy of Sciences, Shanghai, China) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/high-glucose medium (GE Healthcare Life Sciences HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin and streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

**In situ hybridization**

In situ hybridization (ISH) was used to detect PVT1 in 48 pairs of HCC samples. A digoxigenin-UTP-labeled antisense RNA probe (Beijing View Solid Biotechnology, Beijing, China) was derived from 128 to 321 nt of PVT1 by in vitro transcription using the DIG RNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA). The digoxigenin-UTP-labeled sense RNA probe derived from 128 to 321 nt of PVT1 was used as a negative control. ISH was performed using the ISH Kit (Boster Bio-Engineering Company, Wuhan, China).

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from clinical HCC specimens and human HCC cell lines in TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted with random primers using the First Strand cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with an iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA, USA). All reactions were run in triplicate on the iCycler IQ Multi-color Detection System (Bio-Rad). The amplification profile was denatured at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. The comparative cycle threshold (CT) method was applied to quantify the expression levels of mRNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as an endogenous control. Primers used in this study are listed in Table 1.
Construction of the recombinant plasmid

The cDNA encoding PVT1 was PCR amplified by the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) and subcloned into pcDNA3.1 (Invitrogen). The full-length PVT1 was amplified by PCR, and the products were digested and ligated into pMir-REPORT Luciferase Plasmid (Ambion, Life Technologies, Carlsbad, CA, USA). The miR-186-5p binding site mutations were generated using a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutations were verified using sequencing.

Cell transfection

The small interfering RNA (siRNA) targeting PVT1 was purchased from Sigma-Aldrich (St Louis, USA). The miR-186-5p mimic and miR-scramble were designed and synthesized by RiboBio (Guangzhou, China). Transfection was conducted utilizing Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

Western blot

Total proteins were extracted with radioimmunoprecipitation assay (RIPA) Lysate Buffer (Beyotime Institute of Biotechnology, Shanghai, China), separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Subsequently, the PVDF membrane was blocked with 5% skim milk powder in Tris buffered saline with Tween 20 (TBST) at room temperature (RT) for 2 h. Then, the primary antibodies were added and incubated overnight at 4°C. After washing with TBST buffer, the secondary antibodies were added and the membrane was incubated at RT for 2 h. The proteins were visualized by autoradiography using the enhanced chemiluminescence (ECL) reagent (Bio-Rad). The relative expression of interest protein was represented as the grayscale ratio of the protein to GAPDH. The information of antibodies used in this study was presented in Table 2.

Cell proliferation assay

Cells were seeded in each well of a 96-well plate and incubated for 24 h. A volume of 10 µL 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) from the Cell Counting Kit-8 (CCK-8; Boster Bio-Engineering Company) was then added to each well. After incubation at 37°C in 5% CO₂ for 12, 24, 48, 72, and 96 h, the absorbance of each sample was measured at a wavelength of 450 nm using the Thermo Plate Microplate Reader (Rayto Life and Analytical Science Co. Ltd., Shenzhen, China).

Flow cytometry analysis

Cell apoptosis was tested by two-color immunofluorescence staining in the flow cytometric analysis. Cells were trypsinized without ethylenediaminetetraacetic acid (EDTA) followed by two washing steps with phosphate-buffered saline. Cells were then incubated with 5 µL of fluorescein isothiocyanate (FITC) Annexin V (BD Pharmingen) and 5 µL of propidium iodide (BD Pharmingen, San Jose, CA, USA) in 400 µL of 1× binding buffer for 15 min at RT. Data were analyzed by flow cytometry (FACScan®; BD Biosciences, San Jose, CA, USA) equipped with CellQuest Software (BD Biosciences).

Cell invasion assay

Transwell chambers were precoated with 200 µg/mL Matrigel (BD Biosciences) and incubated overnight. Cells were cultured in serum-free medium in the upper chambers of a Transwell (Corning Inc., Corning, NY, USA)

Table 1. Primer sequence and target sequence used in this study.

| Gene          | Primer       | Sequence                              |
|---------------|--------------|---------------------------------------|
| PVT1          | Forward primer | 5’-ATAGATCCTGCCCTGTTTGC-3’            |
|               | Reverse primer | 5’-CATTTCTCTGCTGCCGTTTC-3’            |
| miR-186-5p    | Stem-loop primer | 5’-gttgctctggctgaggtcttcaggatttgccagccagacacagacacagccagccagc-3’ |
|               | Forward primer | 5’-cgccgAAAGAATTCTCCTTT-3’           |
|               | Reverse primer | 5’-gccgaggtcaggtt-3’                   |
| YAPI          | Forward primer | 5’-ACGACTTCTCCTAACAGTGT-3’            |
| GAPDH         | Reverse primer | 5’-TGAAAGAGATCCCTCCCTCCAAT-3’         |
| pcDNA3.1-PVT1 | Forward primer | 5’-GGCTGTGTGTCACTTTTCTCAGTGG-3’       |
|               | Reverse primer | 5’-GGGTCCTTCGTCATTTTGTCACTTTTCTCAGTG-3’ |
| miR-186-5p mimic | Forward primer | 5’-CAGAAUUCUCUCUUUGGGGC-3’           |
| miR-scramble  | Reverse primer | 5’-UUUCGAGAAGCAGUGUCAGUTTT-3’         |
USA) plate, which are separated from the lower chambers with permeable 8.0 µm polycarbonate membranes. The lower chamber was filled with 500 µL DMEM containing 10% FBS. After incubation for 24 h, the cells remaining on the upper membrane were carefully removed, and the others that had invaded through the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of stained cells was manually counted under a phase-contrast microscope from three different visual fields.

**Cell migration assay**

Cells were cultured in six-well plates. Confluent cell monolayers were disrupted by standardized wound scratching using a sterile 10 µL pipette tip and incubated in serum-free medium. The size of the wound was measured after 24 h and photographed using a phase-contrast microscope.

**Dual-luciferase assay**

Cells were cultured overnight until 60%–70% confluence. Cells were co-transfected with pMir-Reporter-PVT1-WT or pMir-Reporter-PVT1-MUT and miR-186-5p mimic or miR-scramble. Lipofectamine 3000 was used according to the manufacturer’s instructions. After 48 h, cells were harvested for luciferase detection using the dual-luciferase reporter gene assay system (Promega, Madison, WI, USA).

**RIP assay**

RNA immunoprecipitation (RIP) assay was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer’s protocol. Cells at 80%–90% confluence were collected and were lysed using RIP lysis buffer. Next, the cell extracts were incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody or negative control IgG. The samples were incubated with Proteinase K to digest the protein and subsequently the precipitated RNA was obtained. The purified RNA was used for qPCR analysis.

### Statistical analysis

All experiments were performed in triplicate and data were presented as mean ± SD. All statistical analyses were performed with SPSS version 22.0 software (IBM, Chicago, IL, USA). The expressions of PVT1 and miR-186-5p in HCC samples were compared with adjacent normal tissues utilizing the paired sample t-test. The associations between PVT1 and miR-186-5p expression and clinicopathological variables were evaluated using the chi-square test or Fisher’s exact test. The Kaplan–Meier test was used to estimate OS. The p < 0.05 was considered to be statistically significant.

### Results

**PVT1 was significantly upregulated, whereas miR-186-5p was statistically downregulated in HCC tissues**

We detected the expression levels of PVT1 and miR-186-5p in 48 pairs of HCC tissues and the adjacent normal tissues utilizing the qPCR (Figure 1(a), (b), (d), and (e)). The results showed that PVT1 was significantly upregulated, while miR-186-5p was statistically downregulated in tumor tissues (p < 0.001; Figure 1(c) and (f)). Moreover, in order to evaluate the correlation between PVT1 expression, miR-186-5p expression, and clinicopathological variables, patients were divided into the high-expression group (n = 24) and the low-expression group (n = 24) based on the median expression level of all tumor tissues. Specifically, as presented in Table 3, PVT1 expression levels in HCC were significantly associated with vascular invasion (p < 0.05), liver cirrhosis (p < 0.05), and tumor–node–metastasis (TNM) stage (p < 0.05). Similarly, miR-186-5p expression levels were statistically correlated with tumor size (p < 0.05), vascular invasion (p < 0.05), and TNM stage (p < 0.05). Additionally, an inverse correlation was observed between PVT1 and miR-186-5p expression levels by using Pearson’s test ($r^2 = 0.102$, p < 0.05; Figure 1(g)). Kaplan–Meier analysis of the 48-month follow-up survival survey demonstrated that high PVT1 expression was significantly associated with poor OS (p = 0.035;
Figure 1(h)), and OS was significantly shorter in low miR-186-5p expression than that in high miR-186-5p expression (p = 0.023; Figure 1(i)). Furthermore, compared with other expression pattern combinations, the group of both high PVT1 and low miR-186-5p expression (n = 15) presented a significantly poorer OS (p = 0.004; Figure 1(j)), and the 1-, 2-, 3- and 4-year survival rates were dramatically less than that of high PVT1 group or low miR-186-5p group. These findings suggested that the overexpression of PVT1 and/or the downregulation of miR-186-5p might be associated with tumorigenesis and poor prognosis in patients with HCC.

**PVT1 promoted the proliferation and inhibited the apoptosis of HCC cells**

qRT-PCR was performed to detect PVT1 expression in six human HCC cell lines (HepG2, Hep3B, Huh-7, HCCLM9, SK-Hep1, and SMMC-7721). The highest expression level of PVT1 was found in SK-Hep1 cells when compared with the other cell lines (p < 0.05; Figure 2(a)). Therefore, the SK-Hep1 cell line was selected for the remaining experiments in this study. We next determined the function of PVT1 through gain-of-function and loss-of-function approaches. PVT1 overexpression plasmid or PVT1-siRNA was transfected into SK-Hep1 cells. The overexpression or knockdown effect of PVT1 was confirmed by qPCR assay (Figure 2(b)). Then, the CCK-8 assay showed that the proliferation ability of SK-Hep1 cells presented a significant growth via upregulating PVT1 and a statistical decline after the depletion of PVT1 (Figure 2(c)). Subsequently, flow cytometry analysis was performed to further examine the effect of PVT1 on the apoptosis of SK-Hep1 cells, indicating that the proportion of cells in early apoptosis significantly decreased with the overexpression of PVT1 and statistically ascended by the silencing of PVT1 (Figure 2(d) and (e)).

| Variable                        | Total no. (n = 48) | PVT1 High expression (n = 24) | PVT1 Low expression (n = 24) | miR-186-5p High expression (n = 24) | miR-186-5p Low expression (n = 24) | p value |
|---------------------------------|-------------------|-----------------------------|-----------------------------|-------------------------------------|-----------------------------------|---------|
| Gender                          |                   |                             |                             |                                     |                                   |         |
| Male                            | 37                | 18                          | 19                          | 0.7313                              | 20                                 | 17       | 0.4936 |
| Female                          | 11                | 6                           | 5                           |                                     | 4                                  | 7        |
| Age (years)                     |                   |                             |                             |                                     |                                   |         |
| <60                             | 20                | 9                           | 11                          | 0.5582                              | 12                                 | 8        | 0.2416 |
| ≥60                             | 28                | 15                          | 13                          |                                     | 12                                 | 16       |
| Tumor size (cm)                 |                   |                             |                             |                                     |                                   |         |
| <5                              | 19                | 7                           | 12                          | 0.1400                              | 14                                 | 5        | 0.0079*|
| ≥5                              | 29                | 17                          | 12                          |                                     | 10                                 | 19       |
| Vascular invasion               |                   |                             |                             |                                     |                                   |         |
| Present                         | 32                | 20                          | 12                          | 0.0305*                             | 11                                 | 21       | 0.0022*|
| Absent                          | 16                | 4                           | 12                          |                                     | 13                                 | 3        |
| PVTT                            |                   |                             |                             |                                     |                                   |         |
| Present                         | 8                 | 5                           | 3                           | 0.7008                              | 2                                  | 6        | 0.2448 |
| Absent                          | 40                | 19                          | 21                          |                                     | 22                                 | 18       |
| Serum AFP (ng/mL)               |                   |                             |                             |                                     |                                   |         |
| <200                            | 10                | 4                           | 6                           | 0.7238                              | 7                                  | 3        | 0.2865 |
| ≥200                            | 38                | 20                          | 18                          |                                     | 17                                 | 21       |
| Liver cirrhosis                 |                   |                             |                             |                                     |                                   |         |
| Present                         | 23                | 16                          | 7                           | 0.0093*                             | 10                                 | 13       | 0.3861 |
| Absent                          | 25                | 8                           | 17                          |                                     | 14                                 | 11       |
| Differentiation                 |                   |                             |                             |                                     |                                   |         |
| Well                            | 8                 | 2                           | 6                           | 0.3010                              | 5                                  | 3        | 0.4648 |
| Moderate                        | 31                | 17                          | 14                          |                                     | 16                                 | 15       |
| Poor                            | 9                 | 5                           | 4                           |                                     | 3                                  | 6        |
| TNM stage                       |                   |                             |                             |                                     |                                   |         |
| I–II                            | 18                | 5                           | 13                          | 0.0171*                             | 14                                 | 4        | 0.0065*|
| III–IV                          | 30                | 19                          | 11                          |                                     | 10                                 | 20       |

AFP: α-fetoprotein; PVTT: portal vein tumor thrombus; TNM: tumor–node–metastasis.

*p < 0.05.
Figure 1. Expressions of PVT1 and miR-186-5p in HCC samples. (a and b) Relative PVT1 expression in tumor tissues (n = 48) compared with adjacent non-tumor tissues (n = 48). PVT1 expression was examined by qPCR and normalized to GAPDH expression. (c) PVT1 expression was higher in tumor tissues compared with the paired adjacent non-tumor tissues. (d and e) Relative miR-186-5p expression in HCC tissues (n = 48) compared with adjacent normal tissues (n = 48). (f) MiR-186-5p expression was lower in HCC tissues compared with the paired adjacent normal tissues. (g) Bivariate correlation analysis of the negative association between PVT1 expression level and miR-186-5p expression level in 48 pairs of HCC tissues. (h and i) Kaplan–Meier analysis of overall survival (months) based on PVT1 and miR-186-5p expression levels in 48 patients with HCC, respectively. The median level was used as the cut off. Patients with HCC were divided into high-expression group and low-expression group. (j) Compared with other expression pattern combinations, high PVT1 and low miR-186-5p expression showed a significantly poorer overall survival (months; *p < 0.05).
Figure 2. The functional analysis of PVT1 in HCC cells. (a) PVT1 expression levels in various human HCC cell lines (HepG2, Hep3B, Huh-7, HCCLM9, SK-Hep1, and SMMC-7721). (b) The PVT1 expression levels were determined by qPCR when SK-Hep1 cells were transfected with pcDNA3.1-PVT1 or PVT1-siRNA. (c) CCK-8 assay was applied to detect the proliferation of SK-Hep1 cells. (d and e) Flow cytometry assays were performed to analyze the apoptosis of SK-Hep1 cells after treatment with pcDNA3.1-PVT1 or PVT1-siRNA and stained with apoptosis markers (FITC-Annexin V and PI). (f and g) The ability of cancer cell invasion was measured by utilizing Transwell assay after overexpression or depletion of PVT1 in SK-Hep1 cells (original magnification 100×). (h and i) The ability of cancer cell migration was measured by using wound-healing assay when PVT1 was upregulated or silenced in SK-Hep1 cells (original magnification ×100). Data represented the mean ± SD from three independent experiments (*p < 0.05; NS: no significance).
Effects of PVT1 on the invasion and migration of HCC cells

Cell invasion and migration assays were performed to investigate how PVT1 affected these characteristics in SK-Hep1 cells. The Transwell chamber assay illustrated that the number of SK-Hep1 cells that had passed through the polycarbonate membrane of the chamber witnessed a significant rise after the overexpression of PVT1 and a remarkable drop when PVT1 was downregulated (p < 0.05; Figure 2(f) and (g)). Following that, the wound-healing assay showed that enhanced expression of PVT1 dramatically increased the distance traveled of migrated cells. In contrast, PVT1 knockdown significantly reduced the distance traveled of migrated cells (p < 0.05; Figure 2(h) and (i)). Overall, the results demonstrated that silencing PVT1 might be able to inhibit the invasion and migration abilities of HCC cells.

PVT1 interacted with miR-186-5p and functioned as its sponge in HCC cells

An ISH assay first confirmed that PVT1 was primarily located in the cytoplasm (Figure 3(a)). It has been identified that there are interactions between the lncRNAs and miRNAs in the tumorigenesis of various cancers.\(^\text{17,18}\) To address the regulatory mechanism of PVT1 in HCC cells, we performed the bioinformatics analysis using StarBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) software, which predicted that there was a putative binding site between PVT1 and miR-186-5p (Figure 3(b)). In order to verify the influence of PVT1 on miR-186-5p expression, SK-Hep1 cells were transfected with pcDNA3.1-PVT1 or PVT1-siRNA. The cells with PVT1 overexpression showed significantly decreased miR-186-5p expression (Figure 3(c)). Conversely, the cells with PVT1 knockdown presented statistically increased
miR-186-5p expression (Figure 3(c)). Next, to determine whether there was direct interaction between PVT1 and miR-186-5p, the full-length PVT1 was cloned into pMir-Reporter plasmid named pMir-Reporter-PVT1-WT. Similarly, the mutant construct without the putative binding site was also generated and termed pMir-Reporter-PVT1-MUT. The plasmids were transfected into SK-Hep1 cells together with miR-186-5p mimic or the negative control. The data revealed that luciferase activity of pMir-Reporter-PVT1-WT was significantly reduced by miR-186-5p mimic (Figure 3(d)). By contrast, the luciferase activity of pMir-Reporter-PVT1-MUT presented no statistical changes (Figure 3(d)). In other words, miR-186-5p could bind to PVT1 via the miRNA recognition site. Subsequently, based on the prediction in StarBase v2.0, we observed that this site could also bind the Argonaute 2 (Ago2) protein (Figure 3(e) and (f)). Previous study showed that miRNAs exert their gene silencing functions via RNA-induced silencing complex (RISC) containing Ago2.19 Emerging evidence demonstrated that lncRNAs might serve as the sponge of miRNAs through binding the miRNAs and Ago2.12 Therefore, we hypothesized that PVT1 and miR-186-5p might interact in the same way. To clarify the speculation, RNA binding protein immunoprecipitation (RIP) assay was conducted in SK-Hep1 cell extracts utilizing the antibody against Ago2. The levels of PVT1 and miR-186-5p were detected by qPCR. The results illustrated that both PVT1 and miR-186-5p were detected by qPCR. The results illustrated that both PVT1 and miR-186-5p were detected by qPCR. The results illustrated that both PVT1 and miR-186-5p were detected by qPCR. The results illustrated that both PVT1 and miR-186-5p were detected by qPCR.

**PVT1 regulated the expression of the miR-186-5p target gene YAP1**

Recent study has shown that miR-186-5p is able to inhibit HCC tumorigenesis by directly targeting YAP1 and downregulating Hippo signaling pathway.20 Specifically, the 3′-UTR of YAP1 mRNA was proved to be a direct target of miR-186-5p by conducting luciferase reporter assays. Since PVT1 served as an endogenous sponge for miR-186-5p in HCC, we supposed that it could modulate YAP1 expression to some extent. As expected, the expression of YAP1 mRNA increased or decreased with the overexpression or silencing of PVT1, respectively (Figure 4(a)). Moreover, the YAP protein level was detected in SK-Hep1 cells after transfection with pcDNA3.1-PVT1 and/or miR-186-5p mimic. The cells with PVT1 overexpression showed significantly ascending YAP1 expression. On the contrary, the cells with miR-186-5p upregulation presented statistically descending YAP1 expression. Intriguingly, when SK-Hep1 cells were co-transfected with pcDNA3.1-PVT1 and miR-186-5p mimic, the YAP1 protein level remained statistically unchanged (Figure 4(b) and (c)), which indicated that PVT1 overexpression completely abolished the decline in YAP1 expression level induced by upregulating miR-186-5p. Together, these data demonstrated that PVT1 regulated the expression of YAP1.

**Discussion**

LncRNAs PVT1 has been identified to play an oncogenic role in various cancers. Precisely, it was reported that increased expression of PVT1 in pancreatic ductal adenocarcinoma (PDAC) was correlated with tumor progression, and it might be an independent prognostic factor for poor OS rate in patients with PDAC.21 Moreover, overexpression of PVT1 in gastric carcinoma promoted the development of multidrug resistance.22 Zheng et al.23 found that upregulation of PVT1 could induce epithelial–mesenchymal transition (EMT) by regulating the expressions of E-cadherin, N-cadherin, and vimentin in esophageal cancer.

In this study, we systematically investigated potential interactions between PVT1 and miR-186-5p in HCC. Our results illustrated that PVT1 was significantly upregulated, while miR-186-5p was statistically downregulated in tumor tissues. In addition, PVT1 expression levels were significantly associated with vascular invasion, liver cirrhosis, and TNM stage. Similarly, miR-186-5p expression levels were statistically correlated with tumor size, vascular invasion, and TNM stage. Next, through using gain-of-function and loss-of-function methods, our data revealed that PVT1 affected HCC cells proliferation, invasion, and migration. We observed an inverse correlation between PVT1 and miR-186-5p, which stimulated our interest to determine whether there was an endogenous ceRNA mechanism involved between PVT1 and miR-186-5p. To begin with, our preliminary data showed that PVT1 was able to regulate the expression of miR-186-5p in HCC. Subsequently, we performed the bioinformatics analysis to seek putative binding site between them. Following that, it was proved that miR-186-5p could bind the PVT1 by conducting the dual-luciferase assay. Most importantly, we found a direct interaction between PVT1 and miR-186-5p using co-immunoprecipitation with the Ago2 antibody in SK-Hep1 cells.

YAP1, located chromosome 11q22, is the core member of the Hippo pathway. Previous study reported that YAP1 was upregulated in over 60% of HCC patients and was a promising drug target for the inhibition of HCC tumorigenesis.24 Ruan et al.20 found that miR-186-5p could directly bind the 3′-UTR of YAP1 mRNA and consequently suppressed the expression of YAP1. Our further results demonstrated that overexpression of PVT1...
counteracted the decline in YAP1 expression level induced by upregulating miR-186-5p, suggesting that PVT1 absorbed miR-186-5p and released YAP1 from miR-186-5p. Based on the previous work, we proposed an endogenous sponge model including PVT1 and miR-186-5p in HCC (Figure 4(d) and (e)), which may contribute to a better comprehension of tumorigenesis and metastasis in HCC.

In summary, this study showed a novel mechanism of PVT1 in tumorigenesis and metastasis of HCC. PVT1 has the potential to be developed as a clinically promising biomarker for malignant phenotype of HCC. Specifically, PVT1 was confirmed to function as an endogenous sponge for miR-186-5p to reduce its inhibiting effect on YAP1 and thus promoted the tumorigenesis of HCC. More importantly, the exploration of ceRNA mechanism will help us to enhance the understanding of cancer pathogenesis and provide us with novel insights into molecular cancer therapy.

Acknowledgement
T.L. and X.Y. contributed equally to this work.

Compliance with ethical standards

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by the Science and Technology Project of Wuhan (grant no. 2013060501010153) and the Key Project supported by Health and Family Planning Commission of Hubei Province (grant no. WJ2017Z007).

Informed consent
Informed consent was obtained from all individual participants included in the study.
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