


# P7TP3 inhibits tumor development, migration, invasion and adhesion of liver cancer through the Wnt/ $\beta$ -catenin signaling pathway

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## Funding information

The National Science and Technology Major Project, Grant/Award Number: 2017ZX10302201-005-004 and 2017ZX10202202-005-008; the Beijing Municipal Administration of Hospitals' Ascent Plan, Grant/Award Number: DFL20151701; the National Key Research and Development Program of China, Grant/Award Number: No. 2017YFC0908100 / No. 2017YFC0908104 and 2017YFC0908104; the Beijing Municipal Administration of Hospitals, Grant/Award Number: XMLX201711

## Abstract

The effect of hepatitis C virus p7 trans-regulated protein 3 (P7TP3) in the development of hepatocellular carcinoma (HCC) is still unknown. The present study aimed to investigate the role and mechanism of P7TP3 in HCC. P7TP3 was significantly decreased in HCC tissues when compared with corresponding liver tissues immediately around the tumor (LAT) from seven HCC patients. Fewer and smaller colonies originated from HepG2-P7TP3 cells when compared to HepG2-NC cells. Overexpression of P7TP3 in HepG2 cells significantly repressed the growth of HCC xenografts in nude mice. Furthermore, wound-healing tests, Transwell assays, Matrigel Transwell assays, adhesion assays, CCK-8 assays, flow cytometry and western blotting analysis showed that P7TP3 protein expression inhibited migration, invasion, adhesion, proliferation and cell cycle progression in HCC cell lines. Moreover, P7TP3 suppressed the activity of the Wnt/ $\beta$ -catenin signaling pathway, and was restored by Wnt3a, which is an activator of the Wnt/ $\beta$ -catenin signaling pathway. Consistently,  $\beta$ -catenin was highly expressed by P7TP3 silencing, and restored by XAV939, an inhibitor of the Wnt/ $\beta$ -catenin signaling pathway. Finally, microRNA (miR)-182-5p suppressed the expression of target gene *P7TP3* by directly interacting with the 3'-UTR region. Taken together, *P7TP3*, the direct target gene of miR-182-5p, inhibited HCC by regulating migration, invasion, adhesion, proliferation and cell cycle progression of liver cancer cell through the Wnt/ $\beta$ -catenin signaling pathway. These findings provide strong evidence that P7TP3 functions as a new promising tumor suppressor in HCC.

## KEYWORDS

liver cancer, miR-182-5p, P7TP3, Wnt,  $\beta$ -catenin signaling pathway

## 1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is one of the leading causes of cancer death worldwide.<sup>1</sup>

HCC is generally related to other factors such as viral- and alcohol-related liver diseases, and nonalcoholic fatty liver disease (NAFLD).<sup>2</sup> Multimodal therapies are available for treatment of this heterogeneous malignancy, which includes surgical resection, radiofrequency ablation, liver transplantation, systemic therapy such as sorafenib,

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cytotoxic chemotherapy, immunotherapy and oncolytic viral therapy.<sup>3</sup> But, unfortunately, the mechanisms of HCC development are still poorly investigated. Therefore, there is an urgent need to gain insights into the novel targets by elucidating the complicated molecular mechanisms of HCC development.

The *P7TP3* gene, also known as TMEM50B (GenBank accession No. DQ286229), is located at 21q22.11, is 447-bp long, and encodes a 158-residue protein.<sup>4</sup> Hepatitis C virus (HCV) p7 transactivated target genes were screened and cloned using suppression subtractive hybridization and bioinformatic techniques, and a series of p7 protein trans-regulated target genes were identified including several unknown genes such as *P7TP3*.<sup>5</sup> The *P7TP3* protein contains four transmembrane-spanning domains with cytoplasmic carboxy- and amino-terminals.<sup>6</sup> Rosen et al<sup>6</sup> have developed a six-gene array-based prediction model to diagnose benign versus malignant thyroid lesions, which includes *P7TP3*. However, the function and mechanism of *P7TP3* on tumor growth and development in thyroid cancer are still unknown. Moldrich et al<sup>7</sup> have indicated that *P7TP3* regulates the development of intracellular endoplasmic reticulum and Golgi apparatus membrane protein, proving its importance in brain development. Kong et al<sup>8</sup> have identified 11 significantly enriched genes, including *P7TP3*, in Down syndrome samples by identifying differently expressed genes (DEG) and transcription factor motifs. To date, *P7TP3* has rarely been studied, except for sequencing and localization. In the present study, *P7TP3* was confirmed as the direct target gene of microRNA (miR)-182-5p and tumor suppressor for HCC.

MicroRNA-182 emerged as a high priority micro-RNA in HCC, and has been proven to be associated with other cancer types.<sup>9</sup> miR-182 expression is significantly upregulated in HCC patients,<sup>10</sup> and dysregulated during the transition of NAFLD to non-alcoholic steatohepatitis (NASH)-HCC in mice.<sup>11</sup> In a chemically induced HCC mouse model, miR-182 was overexpressed.<sup>12</sup> In addition, the expression of *Cited2* increased liver cell proliferation, resulting in the progression of HCC that was suppressed by miR-182-5p.<sup>13</sup> In HCC, natural killer (NK)-cell cytotoxicity against HCC was also augmented by miR-182.<sup>14</sup> More importantly, miR-182 might exert biological effects through the Wnt/ $\beta$ -catenin signaling pathway. For example, upregulation of miR-182 in human osteosarcoma inhibited cancer progression and activated the Wnt/ $\beta$ -catenin signaling pathway by targeting *HOXA9*.<sup>15</sup> miR-182-5p also contributed to HCC metastasis by activating the Wnt/ $\beta$ -catenin signaling pathway through *FOXO3a*.<sup>9</sup>

Aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway contributes to various diseases. In the absence of Wnt, Axin-serine/threonine glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ )-adenomatous polyposis coli (APC), which is the cytoplasmic destruction complex, binds to  $\beta$ -catenin, and then undergoes phosphorylation. Phosphorylated  $\beta$ -catenin is ubiquitinated by  $\beta$ -TrCP and is then degraded by the proteasome.<sup>16</sup> Wnt induces the binding of axin and low-density lipoprotein receptor-related protein, which destroys the degradation complex and stabilizes  $\beta$ -catenin.  $\beta$ -Catenin then accumulates in the cytoplasm and enters the nucleus to regulate gene transcription proteins, such as cyclin D1, c-myc and MMP-7.<sup>17</sup>

Based on these findings, in the present study, *P7TP3* was confirmed as a direct target gene of miR-182-5p and as a tumor suppressor in HCC, inhibiting migration, invasion, adhesion, proliferation and cell cycle progression by downregulation of the Wnt/ $\beta$ -catenin signaling pathway.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and tissues

Inclusion criteria included patients with pathologically confirmed diagnosis of HCC. Diagnosis was made according to the guidelines for standardized pathological diagnosis of primary HCC, 2015.<sup>18</sup> Seven patients in Beijing Ditan Hospital, Capital Medical University were enrolled in this study. Written informed consent was obtained before sample collection. The study was approved by the Human Ethics Committee of the Institute Research Ethics Committee of Beijing Ditan Hospital, Capital Medical University.

### 2.2 | Animal model

Animal experiments were approved by the Institute of Research Ethics Committee of Beijing Ditan Hospital. Mice were maintained in the Institute of Zoology, Chinese Academy of Sciences, in a specific pathogen-free environment. Male nude mice were purchased from Beijing Vital River Laboratory Animal Technology Company, Beijing, China. The nude mice (weighing approximately 18–20 g and aged 6 weeks) were randomly divided into HepG2-NC and HepG2-*P7TP3* groups.<sup>19</sup> The left upper armpit of the mice in the HepG2-*P7TP3* group was s.c. injected with 0.1 mL of  $1 \times 10^6$ /mL cell suspension.<sup>20</sup> Tumor size was continuously observed and measured with a Vernier caliper. Tumor volume was calculated by the formula:  $V = (\text{length} \times \text{width} \times 2)/2$ .

### 2.3 | Cell culture and cell treatment

HepG2 cells transfected with lentivirus for overexpression of *P7TP3* (HepG2-*P7TP3*) or its negative control (HepG2-NC) were purchased from Genaray Biotech. HepG2 cells and Huh7 cells were stored in our laboratory. The cells were cultured in DMEM supplemented with 10% FBS, containing 100 U/mL penicillin G, and 100  $\mu$ g/mL streptomycin (SV30010; Thermo Scientific) at a constant temperature of 37°C in a humid atmosphere of 5% CO<sub>2</sub>.

### 2.4 | Plasmids and siRNA oligonucleotides

The Coding Sequence (CDS) for amino acids in protein fragment of *P7TP3* was amplified by PCR using genomic DNA of L02 cells as a template. The amplified product was cloned into pcDNA 3.1/myc-His(-) vector. Wild-type (WT) and mutant (MUT) pmirGLO-*P7TP3* 3'-UTR

vectors were constructed by Genaray Biotech. The chemically synthesized miR-182-5p mimic and negative mimic control (mimic-NC) were purchased from RiboBio. The CDS fragment of P7TP3 was as follows:<sup>4</sup>

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ATGGCAGGCTTCCTAGATAATTTTCGTTGGCCAGAATG
TGAATGTATTGACTGGAGTGAGAGAAGAAATGCTGTGGCAT
CTGTTGTCGCAGGTATATTGTTTTTACAGGCTGGTGGATAA
TGATTGATGCAGCTGTGGTGTATCCTAAGCCAGAACAGTTGA
ACCATGCCTTTACACATGTGGTGTATTTCCACATTGGCTTT
CTTCATGATAAATGCTGTATCCAATGCTCAGGTGAGAGGT
GATAGCTATGAAAGCGGCTGTTTAGGAAGAACAGGTG
CTCGAGTTTGGCTTTTCATTGGTTTCATGTTGATGTTTGGGTC
ACTTATTGCTTCCATGTGGATTCTTTTTGGTGCATATGTT
ACCCAAAATACTGATGTTTATCCGGGACTAGCTGTGTTT
TTTCAAATGCACCTATATTTTTTAGCACTCTGATCTACAAA
TTTGAAGAACCGAAGAGCTATGGACCTGA.
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## 2.5 | Western blot analysis

Protein was evaluated by 12% Bis-Tris Gel/MOPS (Invitrogen) in MOPS SDS running buffer (Thermo Fisher) for 2 hours. After transferring the protein electrophoretically for 2 hours, the gels were transferred onto the PVDF membranes (Millipore), and were incubated with 5% skimmed milk (2321000; Becton, Dickinson and Company, America) for 1.5 hours. After that, the primary antibodies (Table S1) were added and maintained at 4°C overnight followed by the addition of secondary antibodies (Zhongshan Jinqiao) for 1.5 hours. Immunoreactive bands were analyzed by BioID software (Vilber Lourmat).

## 2.6 | RNA extraction and quantitative reverse transcription PCR

Total RNA was isolated by total RNA Kit (Omega) and was reverse transcribed into single-stranded cDNA (Prime Script RT reagent Kit; TaKaRa). The cDNA was then subjected to quantitative reverse transcription PCR (RT-qPCR) amplification (ABI) using gene-specific primers (Hong Xun) (Table S2).

## 2.7 | Proliferation assay

Cell Counting Kit-8 (CCK-8 Kit) was purchased from Dojindo. Optical density was observed at 450 nm.

## 2.8 | Luciferase activity assay

HepG2 cells were transiently transfected with P7TP3 3'-UTR promoter plasmids, and miR-182-5p mimics/mimic-NC or miR-182-5p inhibitor/inhibitor-NC for 24 hours. For the TOP/FOP-flash system, TOP-flash plasmids and FOP-flash plasmids (TOP-flash/

FOP-flash = 50/1) were transiently transfected into HepG2 cells for 24 hours. *Renilla* luciferase vector plasmids served as controls. Dual-luciferase reporter assay kit was purchased from Promega.

## 2.9 | Colony formation assay

Cells were generated by seeding  $1 \times 10^4$  cells per well in ultra-low adhesion in a 4-cm cell culture dish and subsequently incubated for 10-14 days. Colonies were observed under a microscope.<sup>21</sup>

## 2.10 | Wound-healing assay

HepG2 cells were cultured in 12-well plates and then transfected with plasmids for 24 hours. Three straight scratches were drawn in each well using a 10- $\mu$ L sterile pipette tip. The old medium was then exchanged with DMEM without FBS. Marked fields were detected at 0, 24, 48 and 72 hours to assess the rate of wound healing. Differences in the migration areas were considered to be the mobility.<sup>21,22</sup>

## 2.11 | Transwell migration assay and Matrigel invasion assay

Medium (100  $\mu$ L) without FBS containing  $1 \times 10^5$  cells was seeded in the upper layer of Transwell plates with an 8- $\mu$ m pore membrane (Corning), whereas the bottom chambers contained 600  $\mu$ L DMEM containing 20% FBS. Cells in the upper chamber that did not pass through the membrane were gently wiped with a cotton swab after 24 hours.<sup>21</sup> Cells in the bottom chamber that migrated were fixed with ethanol for 20 minutes at room temperature and stained with crystal violet for 20 minutes. For Matrigel invasion assay, the upper chamber of the bottom membrane was coated with 100  $\mu$ L Matrigel (1:5 dilution) for 12 hours before conduction of the above experiment.

## 2.12 | Flow cytometry

Cells were treated with the Annexin V-FITC/7-AAD kit (BioLegend) and then detected by FACSCalibur Flow Cytometer (BD Biosciences). DNA content was detected by 7-AAD. Data were analyzed by Flow Jo 7.6.1 or ModFit LT 3.1.

## 2.13 | Statistical analysis

Results from at least three independent experiments were taken and are presented as mean  $\pm$  standard error of mean. Data calculation was done using Student's *t* test. Statistical analysis was done using SPSS 17.0 software. Differences of  $P < .05$  were considered to be statistically significant.

### 3 | RESULTS

#### 3.1 | P7TP3 was downregulated in HCC tissues when compared with liver tissues immediately around the tumor

To assess the differences of P7TP3 expression between HCC and normal liver tissues, HCC tissues and the corresponding liver tissues immediately around the tumor (LAT) from seven patients with HCC were collected. Expression of P7TP3 was assessed in the two groups by real-time quantitative PCR (Figure 1A) and western blotting (Figure 1B). Compared with LAT, the expression of P7TP3 was significantly decreased in HCC tissues, indicating its crucial role in HCC.

Owing to significant downregulation of P7TP3 in HCC tissues, P7TP3 was hypothesized to play a tumor suppressor role in liver cancer. Thus, the effects of P7TP3 on colony formation and tumor development were assessed.

#### 3.2 | P7TP3 inhibited colony formation and tumor development

Cell lines from the subsequent trials were selected and defined the potential role of P7TP3 in liver cancer to investigate the relative expression of P7TP3 in liver cell lines (Figure S1A). As a normal liver cell line, L02 served as control. Results showed that P7TP3 was low in HepG2 cells, and so it was used for overexpression experiments (Figure S1B,C). In contrast, Huh7 cells showed high expression of P7TP3 intrinsically and were used for gene silencing experiments (Figure S1D,E). P7TP3-overexpressing HepG2 cells were generated by transfection with P7TP3-expressing lentivirus as detected by fluorescence microscopy (Figure 2A), western blotting (Figure 2B), and RT-qPCR (Figure 2C). Fewer and smaller colonies originated from HepG2-P7TP3 cells when compared to HepG2-NC cells (Figure 2D). Xenograft assay in immunodeficient nude mice with HepG2-P7TP3 cells was carried out to test the tumorigenicity of P7TP3 in vivo (Figure 2E). As shown in Figure 2F, HepG2-P7TP3 cells showed significant repression of the development of HCC xenografts in nude mice.

These results suggested that P7TP3 inhibited colony formation and tumor development. However, no metastatic nodule was observed in lungs and other organs during xenograft assay in immunodeficient nude mice. Thus, liver cancer cell lines were used to investigate whether or not P7TP3 conferred potential ability in vitro.

#### 3.3 | P7TP3 inhibited HCC cell migration, invasion and adhesion

HepG2 cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid or siRNA-NC/siRNA-P7TP3. After treatment for 24 hours, the cells were scratched with straight lines, followed by detection of cell migration using wound-healing test at 0, 24, 48 and 72 hours. Difference in the migration area was

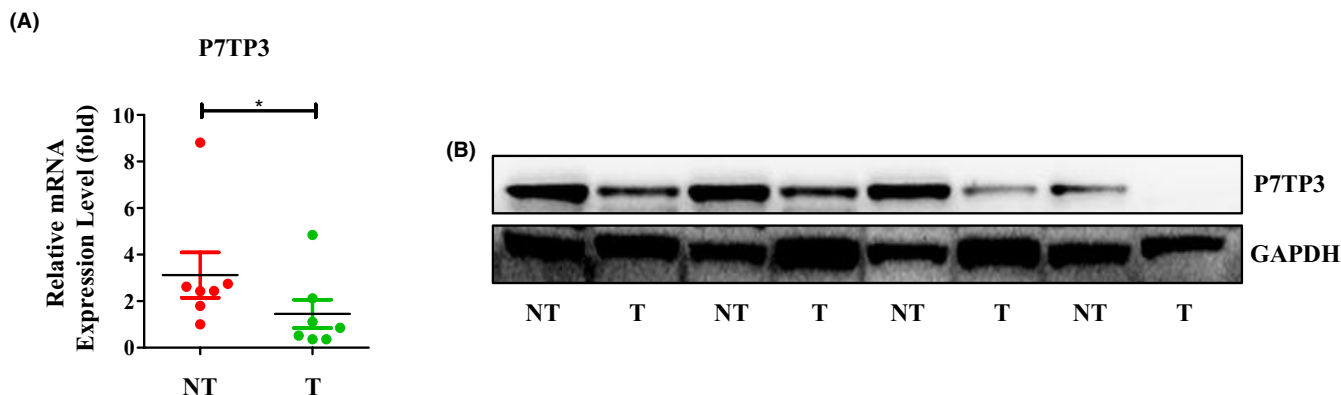
considered to show mobility, which was significantly inhibited by P7TP3 (Figure 3A). Consistently, the mobility was increased after P7TP3 silencing (Figure 3B). HepG2 cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid, whereas Huh7 cells were transfected with siRNA-NC/siRNA-P7TP3. Cells were harvested with a two-chamber assay after 24 hours. The cells that migrated from the upper chambers were counted, and this was considered to be the migration rate. P7TP3 overexpression suppressed HepG2 cell migration, whereas knockdown of P7TP3 promoted Huh7 cell migration (Figure 3C). Next, the cells were harvested for a Matrigel invasion assay after 24 hours. P7TP3 overexpression cells showed a remarkable decrease in the invasive ability, whereas P7TP3 silencing cells showed a significant increase when compared with the control cells (Figure 3D). HepG2 cells infected with lentivirus overexpressing P7TP3 were subjected to cell adhesion assay. Results showed that P7TP3 significantly inhibited cell adhesion ability (Figure 3E).

Epithelial cells, with adherens junctions, desmosomes and tight junctions, form a tight line of defense. Mesenchymal cells that are loosely organized lack cell adhesion and cell polarity, enabling invasion and metastasis. Epithelial mesenchymal transition (EMT) refers to the transformation of epithelial cells to mesenchymal cells, conferring the ability of cell invasion and metastasis. Hence, total protein and RNA were obtained after transfection for 48 hours. N-cadherin, a marker of mesenchymal cells, was significantly lowered by P7TP3 at both mRNA and protein levels (Figure S2A,B). Meanwhile, E-cadherin, a marker of epithelial cells, was significantly lowered by P7TP3 silencing (Figure S2C,D). Genes related to invasion and metastasis, such as *snail*, *vimentin* and *MMP-7*, were upregulated followed by EMT. These results indicated that P7TP3 inhibited migration, invasion and adhesion in HCC cells.

#### 3.4 | P7TP3 inhibited HCC proliferation and cell cycle progression

As described earlier, P7TP3 dramatically inhibited the growth of HCC xenografts in nude mice, which indicates that P7TP3 has the potential ability to regulate proliferation and progression of the cell cycle. To verify this inference, protein levels of Bcl-2 and Bax were confirmed by western blotting analysis. P7TP3 accelerated the apoptosis of HCC cells and slowed down the proliferation of HCC cells (Figure 4A). Furthermore, a similar difference in proliferation rate was also observed by CCK-8 assay (Figure 4B). P7TP3 overexpression increased the rates of apoptosis of HCC cells, which were decreased by P7TP3 silencing (Figure 4C).

Next, we examined whether P7TP3 also affected cell cycle progression, using flow cytometry. P7TP3 overexpression induced a reduction of cell population in the G2 phase, whereas P7TP3 silencing caused increased numbers of cells in the G2 phase and S phase (Figure S3). By western blotting, expression of cell cycle-related proteins was detected. Expression of cyclins A, B, D1, E and CDK2 protein levels was significantly inhibited by P7TP3 overexpression, whereas the opposite results were obtained when P7TP3 expression



**FIGURE 1** p7 Trans-regulated protein 3 (P7TP3) expression is downregulated in hepatocellular carcinoma (HCC) tissues. HCC tissues and the corresponding liver tissues immediately around the tumor (LAT) from seven patients with HCC were collected. A, Expression of P7TP3 at mRNA level in liver tissues of HCC patients was detected ( $n = 7$ ). HCC tissues (T) were compared with LAT (NT). B, Total protein was subjected to western blotting with antibody against P7TP3. Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$

was silenced (Figure 4D). Cyclins A, D1, E and CDK2, well-known cell cycle regulators that function as G1/S cell cycle checkpoints, promoted cell entry from G1 phase into S phase (Figure 4E). Therefore, G1/S cell cycle arrest was induced by P7TP3 in HCC cells.

These results showed that P7TP3 inhibited HCC by inhibiting colony formation, tumor development and HCC cell migration, invasion, and adhesion. Next, the mechanism underlying the anti-HCC effect of P7TP3 was systematically investigated in HCC cell lines.

### 3.5 | P7TP3 inhibited HCC by regulating the Wnt/ $\beta$ -catenin signaling pathway

Several signaling pathways regulated by P7TP3 involved in cell proliferation, apoptosis, and tumorigenesis were screened (Figure S4A,B). The results confirmed that cyclin D1, a nuclear target gene of the Wnt/ $\beta$ -catenin signaling pathway, was simultaneously decreased or increased when P7TP3 was overexpressed or silenced (Figure 4D). Therefore, it was suggested that P7TP3 attenuated HCC through the Wnt/ $\beta$ -catenin signaling pathway. At rest, the degradation complex that consists of Axin2, GSK3 $\beta$  kinase and APC phosphorylated  $\beta$ -catenin undergoes ubiquitination and then degradation by the proteasome. The nucleated  $\beta$ -catenin that binds to the transcription factor TCF/LEF forms a complex that initiates transcription of downstream regulatory genes.<sup>16</sup> To validate this hypothesis, the expression of Wnt3a,  $\beta$ -catenin, GSK3 $\beta$ , axin2, TCF-1 and TCF-4, which was regarded as the key active molecule of the Wnt/ $\beta$ -catenin signaling pathway, was detected (Figure 5A,B). Meanwhile, when P7TP3 was overexpressed, *cyclin D1*, *c-myc* and *MMP-7*, nuclear target genes of  $\beta$ -catenin, were significantly decreased, indicating that P7TP3 suppressed the activity of the Wnt/ $\beta$ -catenin signaling pathway (Figure 5C,D).

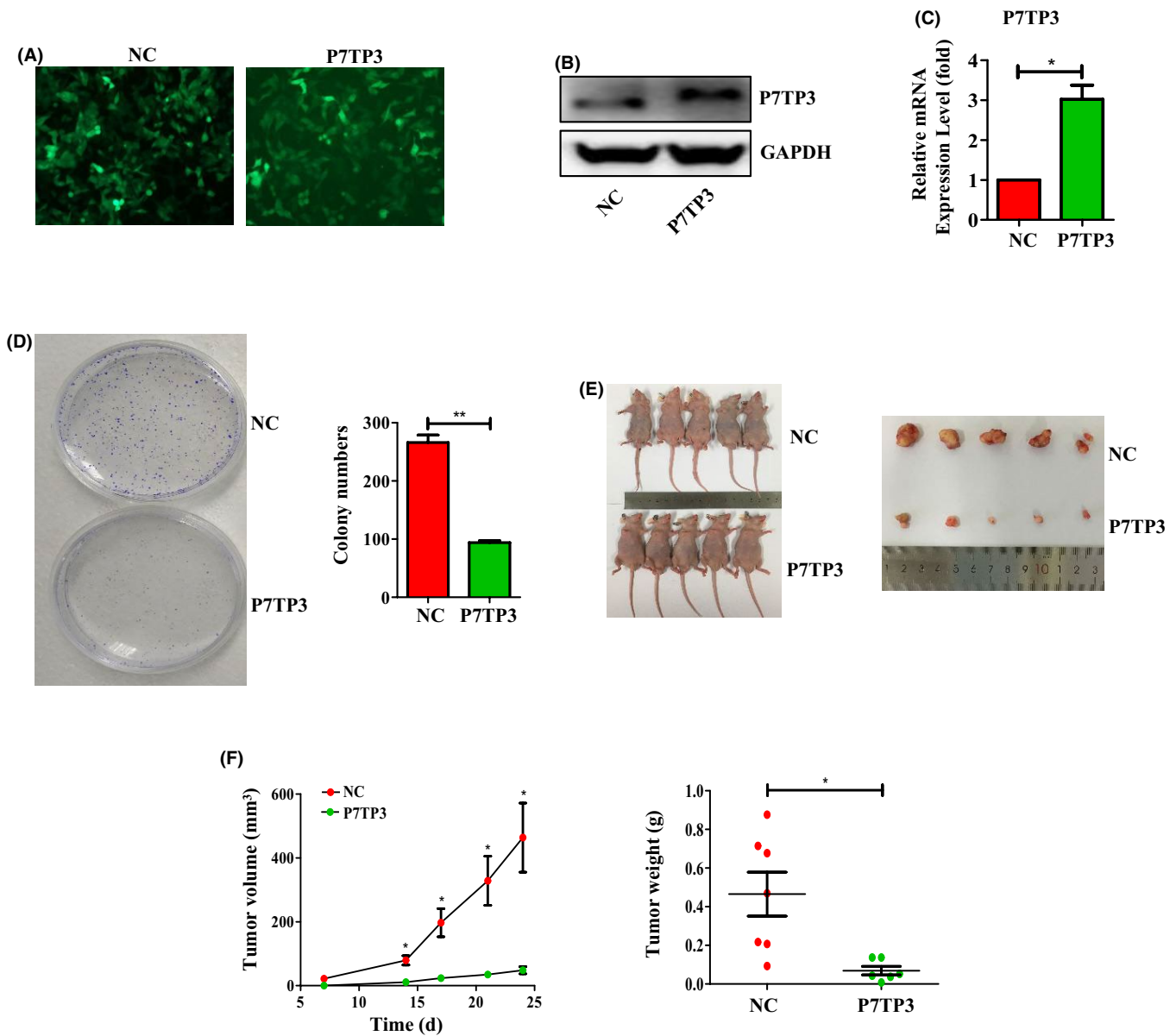
The TOP/FOP-Flash system was used for determining  $\beta$ -catenin-mediated transcriptional activity. The TOP-Flash plasmid was constructed by cloning the TCF/LEF-DNA binding site (AGATCAAAGGGGTA) into the firefly luciferase reporter vector. At the same time, the system also designed a control plasmid

containing a mutated TCF/LEF-DNA binding site, namely the FOP-Flash plasmid for reducing the error. Activation of Wnt/ $\beta$ -catenin signaling pathway was strongly inhibited by P7TP3 overexpression in HepG2 cells, and vice versa, when P7TP3 gene expression was silenced in Huh7 cells (Figure 5E).

Recombinant human Wnt3a, which is an activator of the Wnt/ $\beta$ -catenin signaling pathway, was added to HepG2 cells at a concentration of 10 or 100 ng/mL for 24 hours. XAV939, an inhibitor of the Wnt/ $\beta$ -catenin signaling pathway, was added to HepG2 cells at concentrations of 0.1, 0.5, 5 or 10  $\mu$ mol/L for 24 hours. Total protein obtained underwent western blotting analysis to confirm optimal concentration. According to the expression of  $\beta$ -catenin, the optimal dosage of Wnt3a and XAV939 in HepG2 cells was 10 ng/mL and 10  $\mu$ mol/L, respectively, and this dosage was used in subsequent experiments (Figure S5A,B). The TOP/FOP-Flash system showed that Wnt3a activated the Wnt signaling pathway, whereas XAV9 inhibited the Wnt signaling pathway activity (Figure S5C). To verify if Wnt3a and XAV939 affected the basal activity of control cells, the effects of Wnt3a and XAV939 on cell viability in L02 cells (the normal hepatocyte cell line) were examined by CCK-8 kit and annexin V-FITC/7-AAD kit (Figure S5D,E). Cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid or siRNA-NC/siRNA-P7TP3 for 24 hours, followed by stimulation with or without Wnt3a or XAV939 for 24 hours. Effect of P7TP3-induced reduction of  $\beta$ -catenin was restored by Wnt3a (Figure S5F). Consistently,  $\beta$ -catenin, which was highly expressed by silencing P7TP3, was restored by XAV939 (Figure S5G).

Next, whether the Wnt/ $\beta$ -catenin signaling pathway affected the response of HCC cell migration, invasion and adhesion to P7TP3 were examined. Inhibition of migration, invasion and adhesion derived from HepG2-P7TP3 was eliminated by Wnt3a treatment. Also, inhibition of migration and invasion derived from HepG2-siP7TP3 versus HepG2-siNC was compared by treatment with XAV939 (Figure 6). Thus, depletion of  $\beta$ -catenin might compromise the development of HCC, which could be completed by P7TP3. Taken together, these data suggested that P7TP3 might be a novel target for liver cancer prevention and treatment.





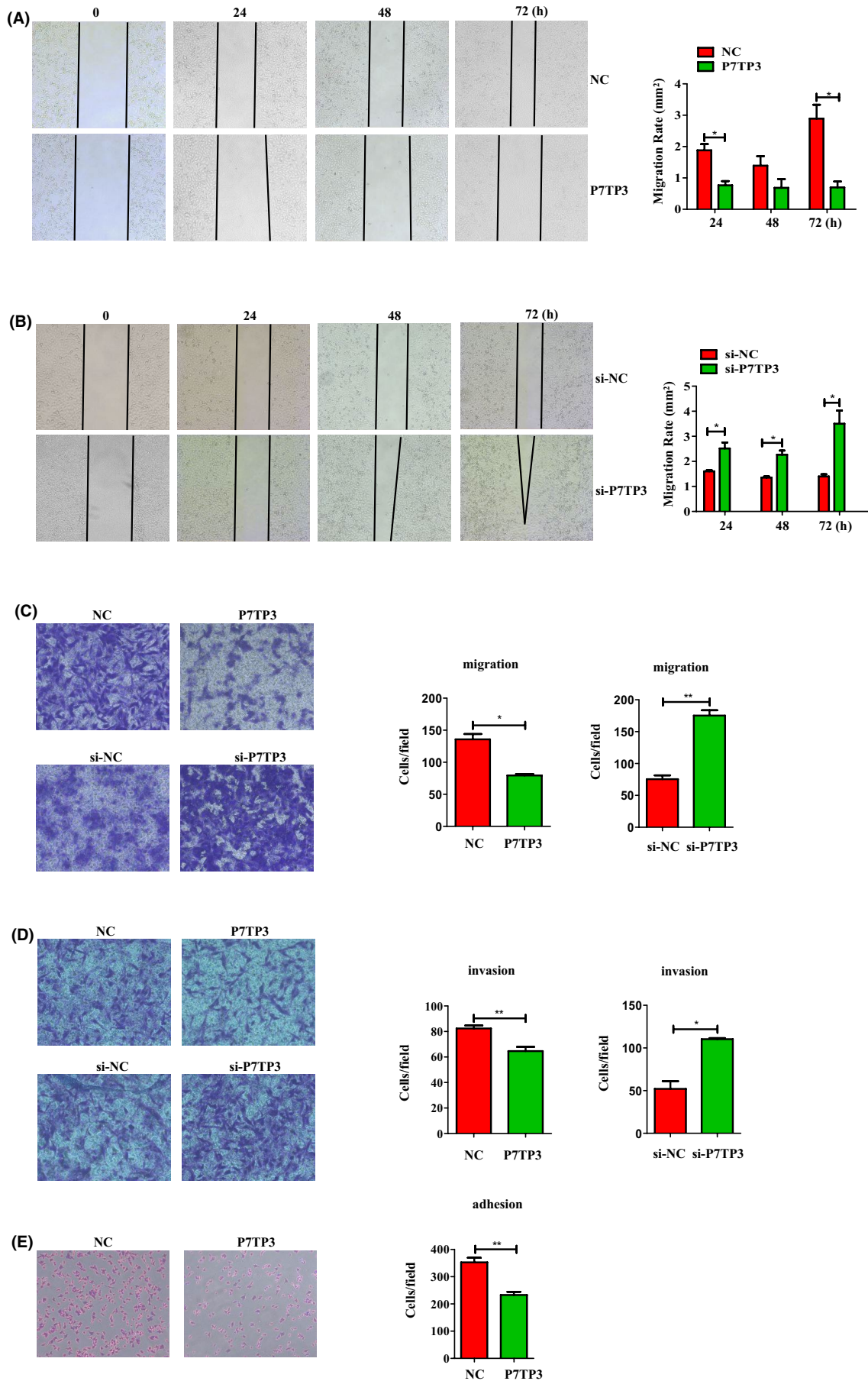
**FIGURE 2** p7 Trans-regulated protein 3 (P7TP3) expression inhibited colony formation and tumor development. A, The lentivirus packaging P7TP3 and its negative control (NC) were expressed by GFP, and were transfected to HepG2 cells. The stable P7TP3 and the control cells (P7TP3 and NC) were observed by fluorescence microscopy (200 $\times$ ). B, Total protein was extracted. Protein levels of P7TP3 were analyzed by western blotting. C, Total RNA was extracted. Expression of P7TP3 at mRNA level was analyzed by RT-qPCR ( $n = 3$ ). D, Colony formation assays were conducted ( $n = 3$ ). E, F, HepG2 cells infected with P7TP3 were inoculated s.c. into nude mice to evaluate tumor volume and tumor weight ( $n = 7$ ). Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$ , \*\* $P < .01$

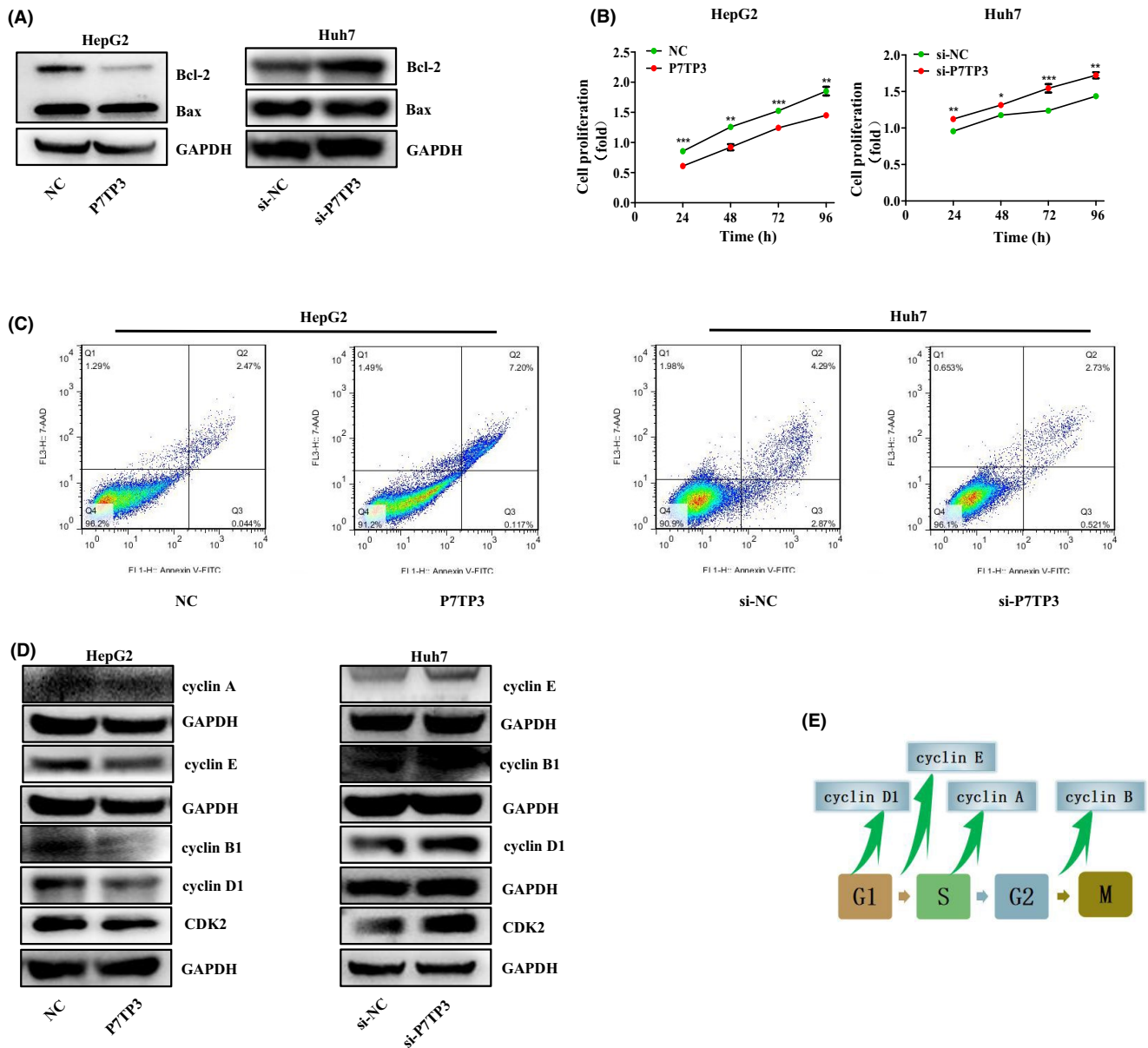
### 3.6 | MiR-182-5p directly interacted with 3'-UTR of P7TP3

To determine the molecular mechanism of P7TP3 on HCC, software online forecasting was carried out. TargetScan, micro-RNA.

org, miRDB and TargetMiner all predicted that miR-182-5p potentially binds to sequence in the 3'-UTR of P7TP3. TargetScan, micro-RNA.org, miRDB and TargetMiner predicted potential binding of miR-182-5p to sequence in 3'-UTR of P7TP3. More importantly, miR-182-5p promoted HCC progression, and activated the

**FIGURE 3** p7 Trans-regulated protein 3 (P7TP3) expression inhibited hepatocellular carcinoma cell migration, invasion and adhesion. A, HepG2 cells and (B) Huh7 cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid, and siRNA-NC/siRNA-P7TP3, respectively. After treatment for 24 h, the cells were scratched with straight lines to detect cell migration at 0, 24, 48 and 72 h (100 $\times$ ). Migration rate was measured ( $n = 3$ ). C, After treatment for 24 h, the cells were subjected to Transwell assays (200 $\times$ ). Migration rate was measured ( $n = 3$ ). D, After treatment for 24 h, the cells were subjected to Matrigel Transwell assays (200 $\times$ ). Invasion rate was measured ( $n = 3$ ). E, HepG2 cells were infected with adenovirus to detect cell adhesion ability (200 $\times$ ) ( $n = 3$ ). Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$ , \*\* $P < .01$





**FIGURE 4** p7 Trans-regulated protein 3 (P7TP3) expression inhibited hepatocellular carcinoma proliferation and cell cycle progression. HepG2 cells and Huh7 cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid, and siRNA-NC/siRNA-P7TP3, respectively. A, Total protein was extracted and underwent western blotting with apoptosis-related genes Bcl-2 and Bax after 48 h. B, Effects of P7TP3 on proliferation were evaluated using CCK-8 kit in HepG2 cells and Huh7 cells ( $n = 5$ ). C, Apoptosis was detected by flow cytometry using the annexin V-FITC/7-AAD kit. D, Regulation of the expression of cell cycle genes by P7TP3 was examined using western blotting. E, Cyclin family proteins in cell cycle progression. Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

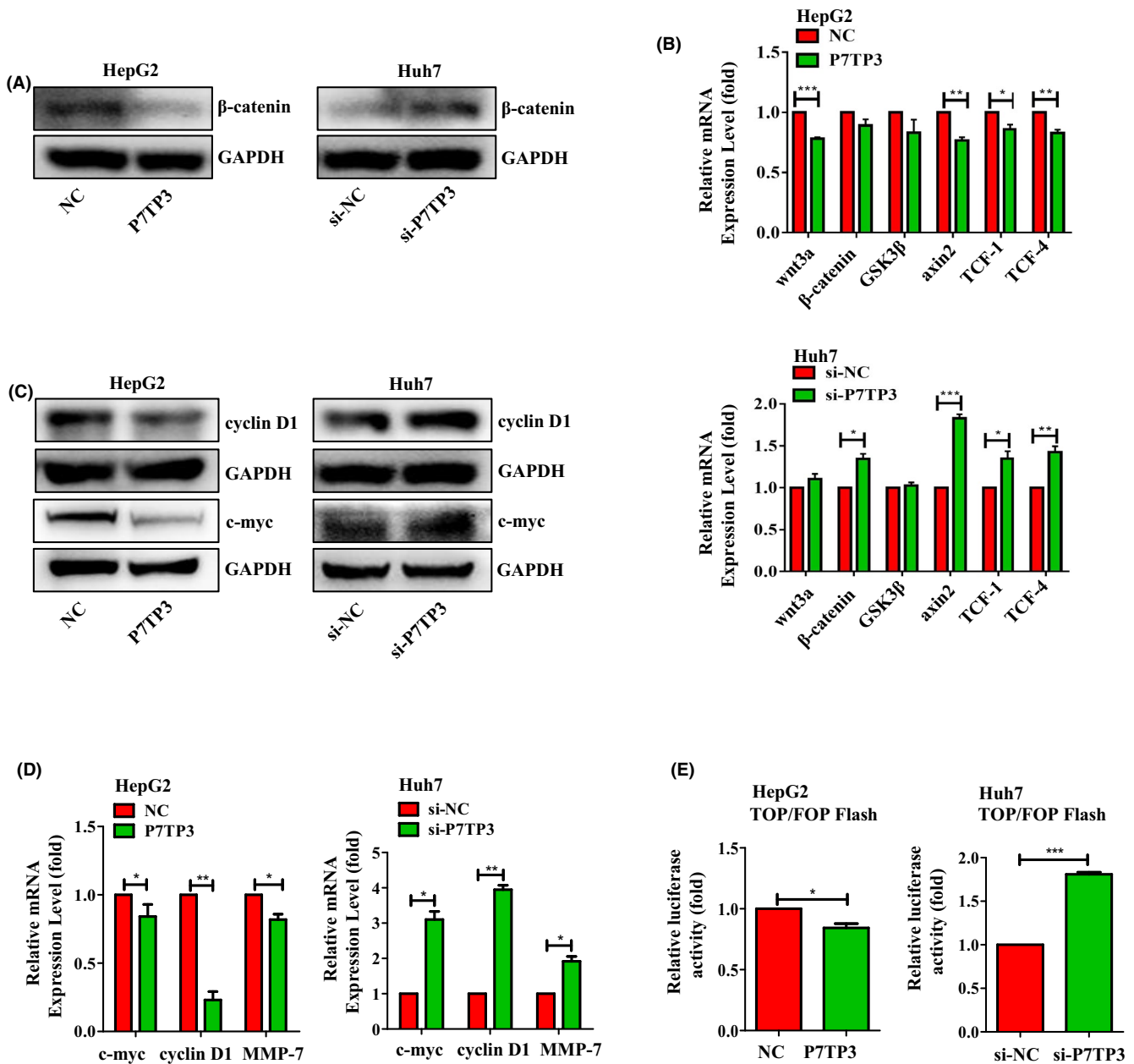
Wnt/ $\beta$ -catenin signaling pathway via FOXO3a.<sup>9</sup> According to these results, miR-182-5p inhibited HCC by targeting P7TP3 through the Wnt/ $\beta$ -catenin signaling pathway. To verify the hypothesis, HepG2 cells were transiently transfected with miR-185-5p mimics/mimic-NC or miR-185-5p inhibitor/inhibitor-NC. Consistent with the online prediction, this study showed a significant decrease in the expression of P7TP3 in miR-185-5p mimics or miR-185-5p inhibitor group at the protein level, but not at the mRNA level (Figure 7A,B). The pmirGLO-luciferase reporter containing either the WT or mutated (MUT) miR-182-5p binding sequences in 3'-UTR of P7TP3 were constructed (Figure 7C). Activity of firefly luciferase was significantly reduced in the WT group (Figure 7D), but no significant inhibition

was observed in the MUT group (Figure 7E), whereas miR-182-5p significantly increased the activity of firefly luciferase with WT but not with mutant 3'-UTR of P7TP3 (Figure 7F,G). The results strongly indicated that P7TP3 was the direct target gene of miR-182-5p.

To determine the relationship of miR-182-5p with the Wnt signal pathway, activity of the Wnt signal pathway was detected by western blot (Figure S6A), RT-qPCR (Figure S6B) and dual-luciferase reporter assays (Figure S6C). Consistent with previous findings,<sup>15</sup> miR-182-5p mimics significantly upregulated Wnt signaling activity, whereas miR-182-5p inhibitors dramatically inhibited it.

P7TP3 was the direct target gene of miR-182-5p, and functions as a powerful cancer suppressor by targeting the Wnt/ $\beta$ -catenin





**FIGURE 5** p7 Trans-regulated protein 3 (P7TP3) expression inhibited Wnt/ $\beta$ -catenin signaling pathway. HepG2 cells and Huh7 cells were transiently transfected with pcDNA 3.1/myc-His(-)-P7TP3 plasmid, and siRNA-NC/siRNA-P7TP3, respectively. After treatment for 48 h, total protein and RNA were extracted. A, Western blotting analysis and (B) RT-qPCR analyses of Wnt3a,  $\beta$ -catenin, GSK3 $\beta$ , axin2, TCF-1 and TCF-4 were conducted ( $n = 3$ ). C, D, Protein and mRNA levels of nuclear target genes of cyclin D1, c-myc and MMP-7 were detected ( $n = 3$ ). E, HepG2 cells were transiently transfected with TOP-Flash plasmids and FOP-flash plasmids (TOP-flash/FOP-flash = 50/1), together with pcDNA 3.1/myc-His(-)-P7TP3 plasmid, or siRNA-NC/siRNA-P7TP3 for 24 h. Then, dual-luciferase reporter assays were carried out ( $n = 3$ ). Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

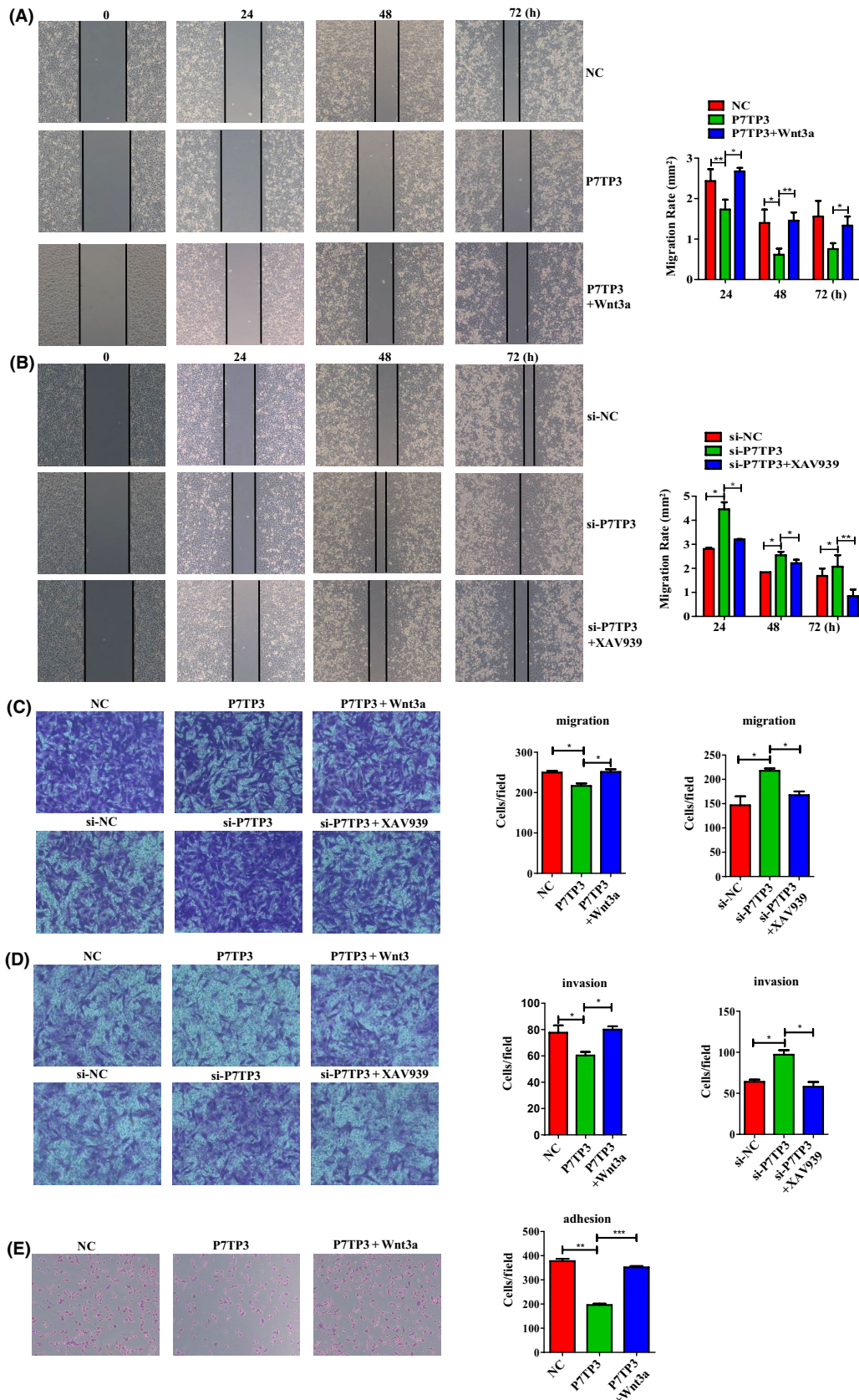
signaling pathway. This, in turn, inhibits tumor development, migration, invasion, proliferation, and progression of the cell cycle in HCC through c-myc, MMP-7 and cyclin D1 proteins (Figure 8).

## 4 | DISCUSSION

In the present study, P7TP3 was reported to be a tumor suppressor gene and a direct target gene of miR-182-5p. P7TP3 inhibited tumor

development, migration, invasion, proliferation, and progression of the cell cycle in HCC. Activity of the Wnt/ $\beta$ -catenin signaling pathway can be downregulated by P7TP3. This study is the first to report these findings and provides new insights into the role of P7TP3 as a tumor suppressor gene in HCC.

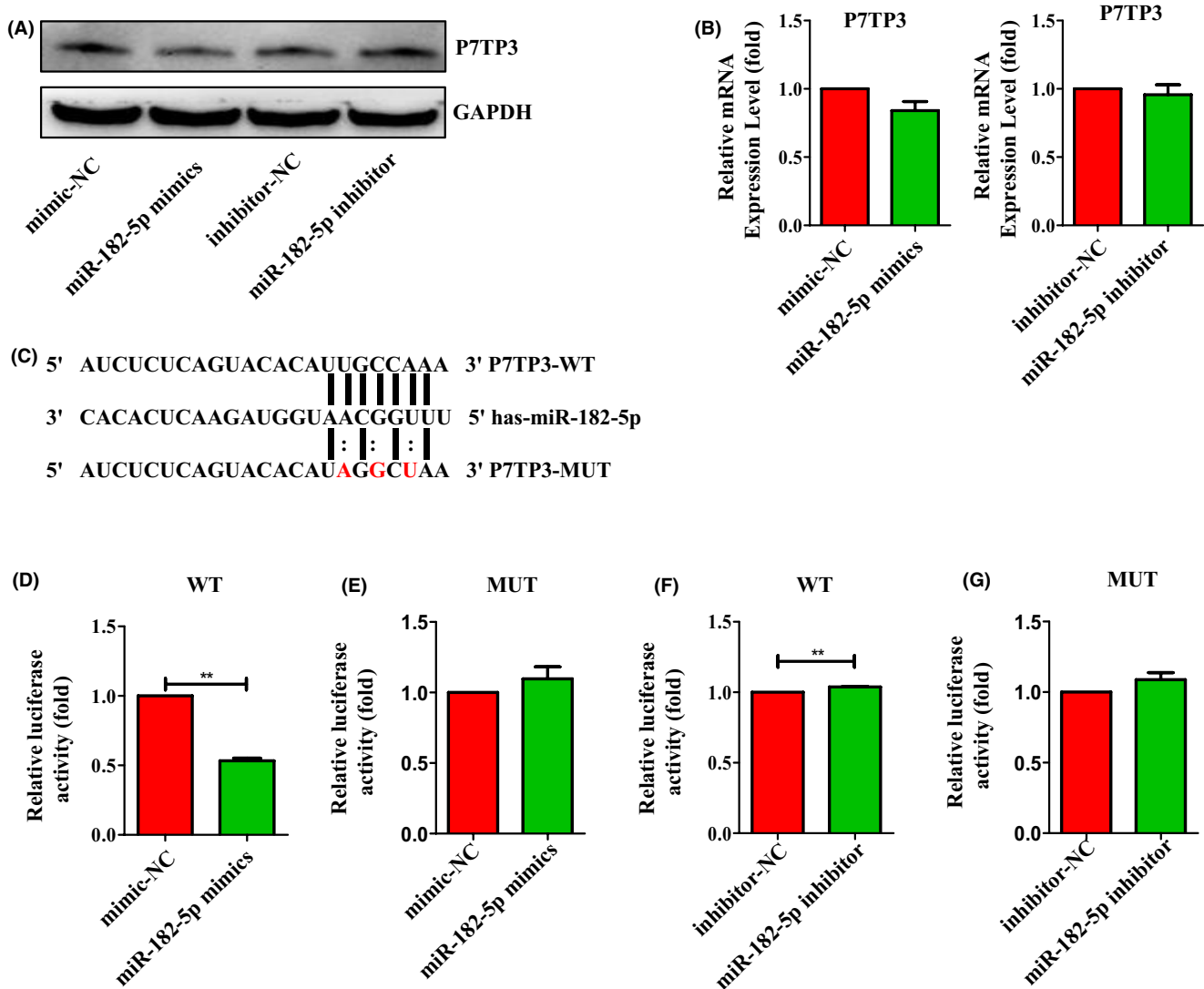
Transmembrane protein (TMEM), a type of protein that spans biological membranes, often extends through the plasma membrane or is located on the biological membrane.<sup>23</sup> TMEM are differentially regulated in many types of cancers and are predicted to be closely



related to cancer pathogenesis. TMEM have been partly described as tumor suppressors, and are generally downregulated in tumor tissues when compared to the corresponding tissues immediately

around the tumor, such as TMEM45A<sup>24</sup> and TMEM97.<sup>25</sup> In contrast, few TMEM are upregulated in cancer, and these function as oncogenes. For example, TMEM17<sup>26</sup> and TMEM45B<sup>27,28</sup> are associated

**FIGURE 6** p7 Trans-regulated protein 3 (P7TP3) expression inhibited hepatocellular carcinoma through the wnt/ $\beta$ -catenin signaling pathway. P7TP3 was overexpressed or knocked down in HepG2 cells and Huh7 cells, respectively. After 24 h, HepG2 cells were treated with Wnt signaling pathway activator, wnt3a (10 ng/mL) for 24 h, while the Huh7 cells were treated with wnt pathway inhibitor XAV939 (10  $\mu$ mol/L). A, B, Wound-healing tests were conducted (100 $\times$ ). Migration rate was measured ( $n = 3$ ). C, Transwell assays were conducted (200 $\times$ ). Migration rate was measured ( $n = 3$ ). D, Invasion rate was measured by Matrigel Transwell assays (200 $\times$ ) ( $n = 3$ ). E, Cell adhesion was detected using HepG2 cells transfected with adenovirus (200 $\times$ ) ( $n = 3$ ). Results are presented as mean  $\pm$  standard error of mean. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

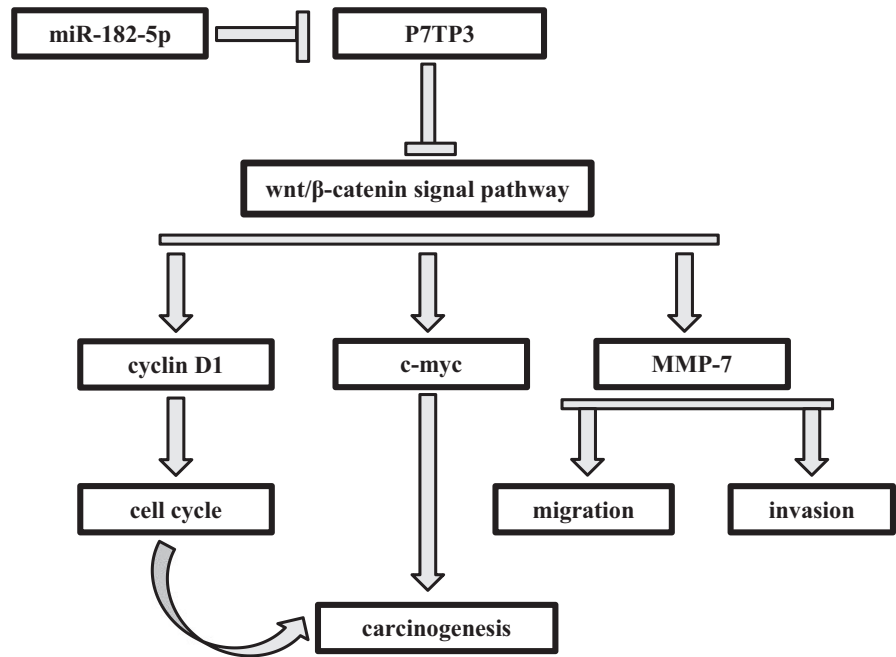


**FIGURE 7** miR-182-5p promoted hepatocellular carcinoma by regulating the expression of p7 trans-regulated protein 3 (P7TP3). HepG2 cells were transiently transfected with miR-185-5p mimics/mimic-NC or miR-185-5p inhibitor/inhibitor-NC for 48 h, respectively. A, P7TP3 protein level showed significant changes when transfected with miR-185-5p mimics or miR-185-5p inhibitor, and P7TP3 protein level was analyzed by western blotting. B, P7TP3 mRNA level was analyzed by RT-qPCR. MiR-182-5p directly interacts with 3'-UTR of P7TP3: (C) prediction of binding sequences involving miR-182-5p and seed matched in P7TP3 3'-UTR and (D, E, F and G) Luciferase reporter vectors were generated by inserting the wild-type (WT) or mutant (MUT) 3'-UTR fragment of P7TP3 into pmirGLO plasmid. WT or MUT plasmids were cotransfected with miR-185-5p mimics/mimic-NC or miR-185-5p inhibitor/inhibitor-NC. After 24 h, luciferase reporter assays were carried out ( $n = 3$ ). Luciferase reporter assays were carried out 24 h after transfection with WT or MUT plasmids were cotransfected with miR-185-5p mimics/mimic-NC or miR-185-5p inhibitor/inhibitor-NC ( $n = 3$ ). Results are presented as mean  $\pm$  standard error of mean. \*\* $P < .01$

with poor prognosis in cancer. However, the TMEM family involves various proteins whose functions are mostly unknown, and P7TP3 is one of them. In the present study, the role and mechanism of P7TP3 in HCC were confirmed for the first time, further enriching the research on TMEM.

P7TP3 expression was downregulated in HCC tissues when compared to that in the corresponding LAT. However, no statistical relationship between P7TP3 and HCC was observed in the network database (data not shown). P7TP3 is a newly discovered gene, which has rarely been studied so far. Thus, there is little information on

**FIGURE 8** Schematic image showing tumor development, migration, invasion, proliferation, and progression of the cell cycle in hepatocellular carcinoma through c-myc, MMP-7 and cyclin D1 proteins



P7TP3 in the database to validate our findings. However, the background of patients in the database was not the same as that in our research. Our patients were all Asians, which, in turn, might lead to inconsistencies.

Moreover, miR-182-5p is considered a potential carcinogen as well as a prognostic factor in HCC. Until now, several target genes have been scanned for miR-182-5p in HCC, such as LINC01018<sup>29</sup> and FOXO3a.<sup>9</sup> We herein provided a novel target gene for miR-182-5p (Figure 7), which acts as a promising tumor suppressor. In HCC, miR-182-5p directly binds to the 3'-UTR of P7TP3 mRNA by the seed sequences (Figure 7C), and silences P7TP3 by inhibiting translation of P7TP3 mRNA (Figure 7A). However, miR-182-5p is partially matched to P7TP3 mRNA rather than fully matched to it and therefore does not degrade P7TP3 mRNA (Figure 7B).

Compared to L02, which is a normal liver cell line, the expression of endogenous P7TP3 was lowered in HepG2 cells but was higher in Huh7 cells. Therefore, gene overexpression and silencing experiments were carried out in HepG2 and Huh7 cells, respectively (Figure S1). Human liver cancer cell lines are diverse and are used for various experimental purposes. HepG2 cell line is derived from hepatoblastoma, whereas Huh7 cell line is derived from highly differentiated HCC. These experiments were conducted in two liver cancer cell lines simultaneously to confirm the data. Unfortunately, the cell adhesion ability of Huh7 cells remained weak, as it was suspended in cell culture dish after straight scratches were drawn with a 10- $\mu$ L sterile pipette tip. So, wound-healing assays were done in HepG2 cells only (Figure 3A,B). Lentiviral packaging Huh7 cells silencing P7TP3 were entrusted to Generay Biotech. But, so far, the cell line has not been successfully constructed. After more than 2 months of hard work, the lentivirus has successfully been built. After the lentivirus initially infects Huh7 cells, fluorescence activity can be detected by fluorescence microscopy (Figure S7A). The

morphology of Huh7 cells is also normal (Figure S7B). However, it is difficult for Huh7 cells to survive for several days after lentiviral transfection. In addition, limited techniques have led to the fact that the experimental results of HepG2 cells do not clearly distinguish the cell cycle. Therefore, we verified the effect of P7TP3 on cell cycle progression in Huh7 cells only (Figure S3).

P7TP3 attenuated HCC through the Wnt/ $\beta$ -catenin signaling pathway. To confirm the mechanism of P7TP3 during HCC, several pathways regulated by P7TP3 that are involved in cell proliferation, apoptosis, and tumorigenesis, including the Wnt/ $\beta$ -catenin signaling pathway, AMPK signaling pathway, mTOR signaling pathway, NF-kappa B signaling pathway and transforming growth factor (TGF) $\beta$ /smad3 signaling pathway, were screened (Figures S4 and S5). Besides cyclin D1, nuclear target genes of the Wnt/ $\beta$ -catenin signaling pathway were decreased or increased simultaneously when P7TP3 was overexpressed or silenced (Figure 4D). Therefore, it has been proposed that P7TP3 attenuated HCC through the Wnt/ $\beta$ -catenin signaling pathway. P7TP3 may indirectly inhibit the transcription of wnt3a and  $\beta$ -catenin (Figure 4C), and regulate post-translationally by  $\beta$ -catenin protein degradation (Figure 4A,B), thereby inhibiting transcription of nuclear target genes that bind to  $\beta$ -catenin (Figure 4D,E). In order to prove this hypothesis, there is much work still to be done.

Hepatocellular carcinoma is the final outcome of various chronic liver diseases such as fatty liver, and so prevention of HCC progression remains a priority. In fact, we also observed that expression of P7TP3 in the liver tissues of a CCl<sub>4</sub>-induced liver fibrosis mouse model or in the liver tissues of a high fat diet-induced fatty liver mouse model was significantly higher than that of the control group. This indicated that P7TP3 was involved in the development of fatty liver or liver fibrosis to HCC (data not shown). Therefore, the effect of P7TP3 on fibrosis or fatty liver, the role of P7TP3 in the



development of fatty liver or liver fibrosis to HCC, and the development of new anti-HCC drugs targeting P7TP3 should be explored in future.

The following questions were unanswered by our study, requiring further exploration. First, does P7TP3 promote angiogenesis by maintaining endothelial cell survival? Second, can P7TP3 be used as an early diagnostic marker for HCC? Third, can P7TP3 be used as a novel marker for assessing the risk of HCC metastasis?

In conclusion, together, these results suggested that P7TP3 inhibited migration, invasion, adhesion, proliferation and cell cycle progression of liver cancer cells. Consequently, P7TP3 might be a potential tumor suppressor in HCC. We look forward to investigating the potent alternatives for treating liver cancer.

## ACKNOWLEDGMENTS

This study was supported by the National Key Research and Development Program of China (No. 2017YFC0908100 / No. 2017YFC0908104), the Beijing Municipal Administration of Hospitals (XMLX201711), the Beijing Municipal Administration of Hospitals' Ascent Plan (DFL20151701) and the National Science and Technology Major Project (No. 2017ZX10302201-005-004 and No. 2017ZX10202202-005-008). Support was also provided by the Program of Beijing Advanced Innovation Center for Big Data-Based Precision Medicine and the Beijing Key Laboratory of Emerging Infectious Diseases, Beijing, China.

## DISCLOSURE

Authors declare no conflicts of interest for this article.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Zhao J, Wang Y, Han M, et al. P7TP3 inhibits tumor development, migration, invasion and adhesion of liver cancer through the Wnt/ $\beta$ -catenin signaling pathway. *Cancer Sci*. 2020;111:994–1007. <https://doi.org/10.1111/cas.14243>