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ORIGINAL RESEARCH

CircRNA-PTN Sponges miR-326 to Promote Proliferation in Hepatocellular Carcinoma

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Background: Mounting evidences reveal that circular RNAs (circRNAs) are critical to regulate biological behavior and process of tumor. Our objective is to explore the role of circRNA-PTN (circPTN) and explain the exact mechanism in hepatocellular carcinoma (HCC).

Methods: Real-time polymerase chain reaction assay was used to detect the level of circPTN and miR-326. The proliferation of cell was measured by CCK-8 assay and EdU assay. Western blot assay was performed to assess ErbB/PI3K expression. Luciferase and RNA pull-down assays were carried out to confirm the interaction between circPTN and miR-326.

Results: Our results indicated that circPTN was upregulated in human hepatocellular carcinoma tumor tissues and cell lines, compared with paratumor tissues and immortalized normal liver cell line. circPTN could significantly promote HCC tumor growth according to gain-and loss-of-function assays. Additionally, we determined that circPTN acted as a sponge through interacting with miR-326. Overexpression of miR-326 could rescue the cell proliferation inhibition and ErbB/PI3K downregulation in HCC cells by circPTN. Besides, the effects of miR-326 on HCC were missing when circPTN binding sites were mutated.

Conclusion: Our study indicates that circPTN acts as an oncogenic factor via sponging miR-326 in HCC.

Keywords: circRNA-PTN, HCC, miR-326, proliferation

Introduction

Hepatocellular carcinoma (HCC) is common cancer over the world. Nowadays, HCC is becoming the third leading cause of death associated with cancer.[1] Surgery shows to be the best intervention of HCC, nevertheless the survival rate is poor in HCC patients, due to the lack of accurate clinical markers and specific indicator in the early period.[2] Thus, it is crucial to discover and develop effective biomarkers, providing better diagnosis and treatment in HCC.

Circular RNAs (circRNAs) are defined as a kind of small non-coding RNAs which can form closed-loop structures.[3] It was previously reported to be generated because of splicing errors, recent developments in RNA sequencing technologies have showed that circRNAs are important in gene regulation through sponging miRNAs.[4] Abnormal regulation of circRNAs has been discovered in several diseases, including cancers. More and more studies reported that circRNAs taken part in many biological processes of HCC, including origination,[5] progress,[6] metastasis,[7] and therapy resistance.[8] For instance, the increased hsa_circ_0005075 expression was related to tumor size in HCC, indicating that hsa_circ_0005075

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might promote the tumor growth.[9] A similar study was that the expression of Cdr1as was associated with PIK3CD, a miR-7 target gene, and Cdr1as could function as a sponge to miR-7.[9] Furthermore, circRNAs typically act a sponge of miRNAs. MiR-122, miR-124, miR-125a-b and miR-199 are the major miRNAs studied and involved in the tumorigenesis, angiogenesis and metastases of HCC.[10]

Recently, circPTN was found to be a carcinogenic factor that acts as a sponge to miRNAs in glioma.[11] Based on the study, we screened circPTN expression in tumor tissues and paratumor tissues in HCC patients. We observed that circPTN expression was higher in tumor tissues, as well as HCC cell lines. Based on further study, we performed gain- and loss-of-function assays, and the results showed that circPTN could promote the HCC cell proliferation. We used bioinformatics databases to predict the target of circRNAs, and hypothesized that circPTN could act as a sponge of miR-326. Then, we confirmed this effect using luciferase and RNA pull-down assays. Additionally, we observed that circPTN promoted the expression of ErbB/PI3K via the sponging miR-326. In conclusion, we suggest that circPTN may promote cell proliferation by sponging miR-326 in hepatocellular carcinoma.

Materials and Methods

Tumor Tissues and Clinical Ethics

A total of 30 pairs of tumor tissues and paratumor tissues were obtained from patients with HCC who underwent surgery for excision of a primary tumor from August 2017 to May 2019 in The First Affiliated Hospital of Anhui Medical University. Written informed consent was obtained from the patients before samples collection. All experiments were approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University (Approval No. 2017-KY-98).

Cell Culture

The human HCC cell lines, including Hep3B, MHCC97-L, MHCC97-H, SMMC-7721 and normal liver cell lines, including L02, THLE-3, as well as 293 T cells were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and Beyotime (Hangzhou, China). Cell authentication examination was performed by using STR profiling. The mycoplasma examination was performed by using PCR mycoplasma detection kit (ABM (G238), Richmond, Canada) according to the manufacturer's instructions. All cells were cultured with Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS; Gibco). Actinomycin D (ActD; Sigma-Aldrich, St. Louis, USA, 2 μg/mL) was added into the cell medium to detect the RNA stability.

Plasmids Construction and Transfection

The sequence of a small (~30- 40-nt) region of the AluSq2 element and circPTN was synthesized and cloned into pCDNA3.1 by GENEWIZ (Suzhou, China). The sequence of miRNA mimics and siRNAs was synthesized by GenePharma (Shanghai, China). The sequences were as follows: si-circPTN: 5′-TCAAGAATGCAGGCTCAAC-3′, si-NC 5′-GAGTCTCGTTGCGTTGTAATGATCA-3′. Lipofectamine 3000 (Invitrogen, Carlsbad, USA) was used to transfect siRNA, miRNA or plasmid. OptiMEM (Gibco) were combined with these reagents to form the mix complexes, adding into the wells. After 4 h, the culture medium was removed and changed. RNA and protein isolation were performed at 48 h.

RNase R Treatment and Real-Time PCR

After the total RNA extraction, 1 μg RNA were reverse transcribed into cDNA according to the PrimeScript™ RT Reagent Kit (Takara, Kusatsu, Japan). SYBR Premix Ex Taq™ II (Takara) and biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, USA) were used to perform real-time polymerase chain reaction. To separate cytoplasmic and nuclear RNA, RNAs from cells were isolated with PARIS™ kit (Thermo Fisher Scientific, Rockford, USA). GAPDH and U6 RNA were tested as the control for cytoplasmic RNA and nuclear RNA. For RNase R treatment, 2 μg total RNA was incubated with 10 U RNAse R (Epicentre, Madison, USA) at 37°C for 40 or 60 min, followed by 70°C for 5 min to deactivate the RNase R. Real-time PCR of miR-346 was carried out by using commercial TaqMan miRNA assays (Invitrogen) according to the manufacturer's protocol. Primers for real-time PCR: circPTN forward: 5′-AACTGGAAGTCTGAA GCGAGC-3′ (531–548 bp in exon 2 of PTN mRNA sequence), circPTN reverse: 5′- TGTTGAGCCTGCATT CTTGAG-3′ (887–907 bp in exon 4 of PTN mRNA sequence); GAPDH forward: 5′-GAACGGGAAGCTCA CTGG-3′, GAPDH reverse: 5′-GCCTGCTTCACCACC TTCT-3′; U6 forward: CTCGCTTCGGCAGCACA, U6 reverse: AACGCTTCACGAATTTGCGT.

Western Blot

RIPA (Beyotime, Hangzhou, China) containing protease inhibitors were used to lyse cells at 4°C. After adding loading buffer and boiling, SDS-PAGE was used to run the samples and PVDF membranes were used to transfer the protein (Millipore, Darmstadt, Germany). The bands were incubated with primary antibodies: ErbB (2165S, 1:1000; CST, Danvers, USA), PI3K (ab32089, 1:1000; Abcam, Cambridge), PTN (ab79411, 1:1000; Abcam) and GADPH (sc-47,724, 1:1000; Santa, California, USA) overnight at 4°C. The secondary antibodies (sc-2004, sc-2005, 1:5000; Santa) were incubated for 1 h at room temperature. After washing, the bands were used Image Lab after adding chemiluminescent substrate (ECL; Millipore) to visualize. Image Lab software was used to analyze results.

CCK-8 Assay

CCK-8 kit (Yeasen, Shanghai, China) was used to measure Hep3B and SMMC-7721 cells proliferation. 100 μL cell suspension containing 10^3 cells was cultured in 96-well plate. After 24 h, a working reagent was incubated for 2 h. We carried out this experiment at different points of time.

EdU Assay

Cell resuspension was added in 96-well plate and the cells were cultured with 20 μM EdU. After incubating at 37°C for 2 h, cells were fixed and Hoechst dye 33,342 was used to stain the nuclei. EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher, Waltham, USA) was used to detect the proliferation. Microscope (Leica, Wetzlar, Germany) was used to take images.

RNA Pull-Down Assay

Biotin-labeled wild type (WT) circPTN and mutant (MUT) circPTN (Biotin RNA Labeling Mix; Roche, Mannheim Germany) were treated with streptavidin beads (Thermo Fisher, USA). After 12 h, the mix was centrifuged at speed of 3000 rpm and used Wash buffer I to wash three times. Then, cell lysates were harvested 48 h after transfection and incubated with Dynabeads M-280 Streptavidin (Invitrogen) for 3 h at 4°C according to the manufacturer's protocol. Then, the beads were washed three times with ice-cold lysis buffer and once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0 and 500 mM NaCl).[12] The bound RNAs were purified using TRIzol for the real-time PCR analysis.

Luciferase Reporter Assay

To detect the interaction between circRNA and miRNA. The wild-type circPTN sequences and mutation sequences were placed into the psiCHECK-2 plasmid (Promega, Madison, USA). 293 T cells were cultured to 60% density to co-transfected with plasmid and miRNA mimic. Luciferase activity was detected after 48 h. After transfection, the Luciferase Reporter Assay System (Promega) was applied. Relative luciferase activity was normalized to the Renilla luciferase internal control.

Fluorescence in situ Hybridization

A FAM-labeled probe was used to detect circPTN and a Cy3-labeled probe was used to detect miR-326 were synthesized by GenePharma (Shanghai, China). Then, 4% paraformaldehyde was used to fix cells at room temperature. Subsequently, prehybridization buffer (Riobo biotech, Guangzhou, China) was added into cells at 37°C for 30 min and cells were treated with probes at 37°C overnight. Next day, the cells were washed at 42°C. DAPI was used to stain the nuclei.

Statistical Analysis

Statistical analyses were carried out by using SPSS IBM 20.0. The P-values were determined by using *t*-test (student's *t*-test) or ANOVA (Analysis of Variance). All data in the graphs are showed as mean ± SD.

Results

CircPTN Is Upregulated in HCC Tumor Tissues and HCC Cell Lines

Recently, circRNA-PTN (circPTN) was found to be a new carcinogenic factor and acted as miRNAs sponge in glioma.[11] Based on the study, we hypothesize that circPTN played an important role in HCC. Firstly, we screened the expression of circPTN in human hepatocellular carcinoma tumor tissues, compared with paratumor tissues. As expected, the circPTN expression was higher in HCC tumor tissues (Figure 1A). The expression of circPTN was also detected in HCC cell lines, including Hep3B, SMMC-7721, MHCC97-L, MHCC97-H, compared with normal liver cell line (L02 and THLE-3). These results indicated that the circPTN level was also significantly increased in HCC cell lines (Figure 1B).

CircPTN derives from pleiotrophin (PTN) gene exons 2 to 4 and the length is 452 bp[13] (Figure 1C). And circRNAs could be resistant to RNase R treatment.[14]

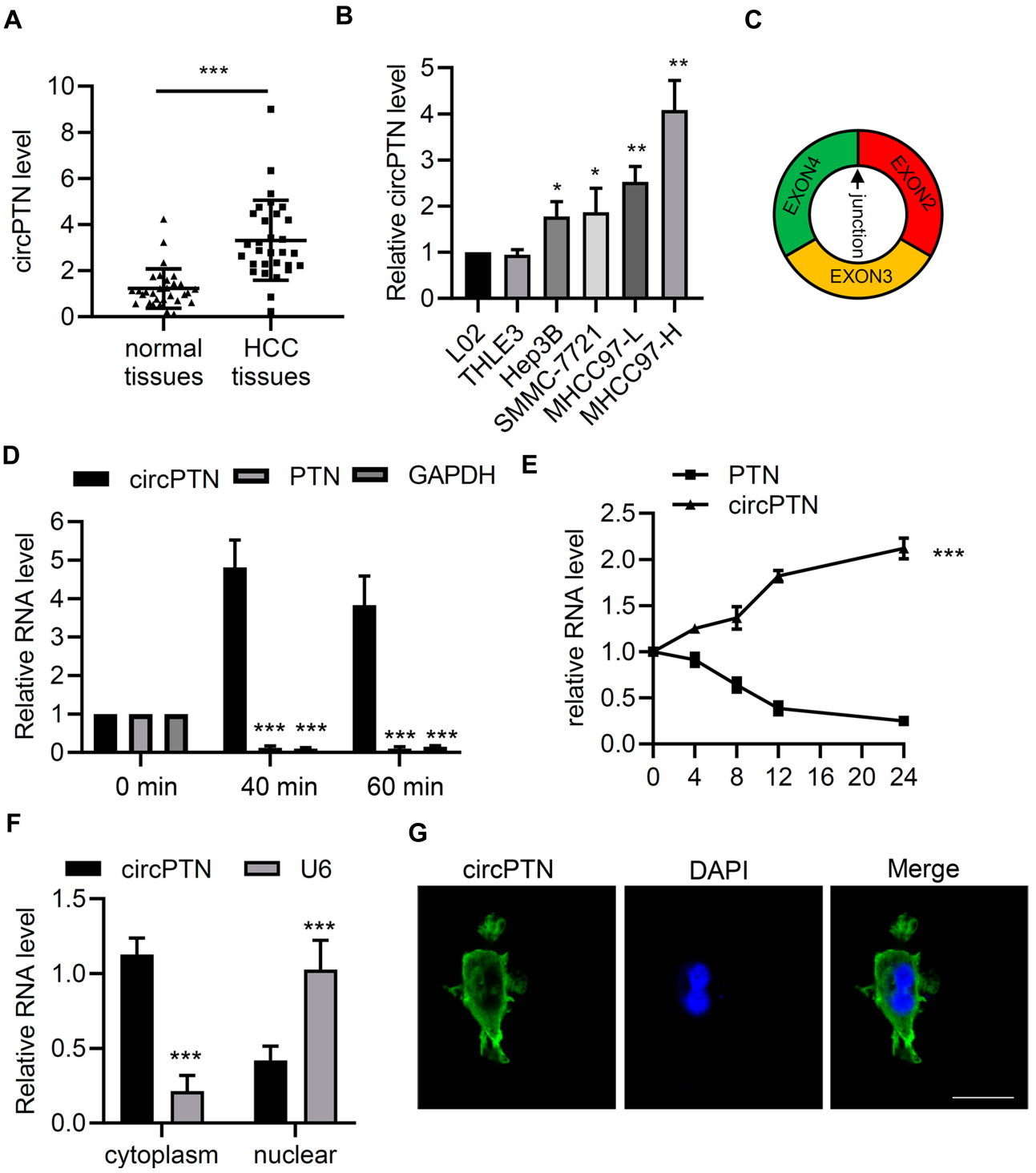

Figure 1 The circPTN level is higher in HCC tumor tissues and HCC cell lines. (**A**) The expression of circPTN was upregulated in tumor tissues. n = 30, ***P < 0.001, t-test. (**B**) The expression of circPTN in HCC cell lines and normal liver cell line (L02 and THLE-3). n = 3, *P < 0.05, **P < 0.01, ANOVA. (**C**) Schematic illustration indicates that the circPTN formed from PTN exons 2–4. (**D**) circPTN was stable with RNase R treatment. n = 3, ***P < 0.001, ANOVA. (**E**) The level of circPTN and PTN in SMMC-7721 cells was measured at different time points after treated with actinomycin D (2 μg/mL). n = 3, ***P < 0.001, ANOVA. (**F**) The real-time PCR suggested that circPTN mainly expressed in the cytoplasm. n = 3, ***P < 0.001, ANOVA. (**G**) FISH indicated that circPTN mainly existed in the cytoplasm in SMMC-7721 cells. Scale bar, 10 μm.
Abbreviations: HCC, hepatocellular carcinoma; ANOVA, analysis of variance; PCR, polymerase chain reaction.

Total RNA of SMMC-7721 cells were treated with RNase R, and real-time PCR showed that circPTN could be unaffected by RNase R, while linear mRNAs were digested by RNase R, including PTN and GADPH (Figure 1D). Then, results indicated that circPTN was more stable than linear PTN mRNA in SMMC-7721

cells when treated with actinomycin D (Figure 1E). As well, we showed that circPTN is mainly placed in the cytoplasm by carrying out cytoplasmic and nuclear RNA real-time PCR and fluorescence in situ hybridization (FISH) assays in SMMC-7721 cells (Figure 1F and G). These results indicated that circPTN located in the cytoplasm and may act as miRNAs sponge.

CircPTN Promotes HCC Proliferation in vitro

We constructed a vector to circularize circPTN in vitro and confirmed that the vector was circularized correctly[15]. Furthermore, we designed and identified the siRNA for circPTN that could target circPTN specifically, but did not affect the linear *PTN* mRNA. We have established a stable overexpression system successfully for circPTN by transfection of the plasmid in Hep3B and SMMC-7721 cells (Figure 2A and B). As well, the level of PTN mRNA did not change in HCC cell lines after treated with si-circPTN (Figure 2C and D) and did not affect the PTN protein expression (Figure 2E). According to CCK-8 and EdU assays, we confirmed that circPTN overexpression promoted cell proliferation, while the knockdown of circPTN reduced proliferation in HCC cell lines (Figure 2F and G). Our results demonstrated that circPTN could promote HCC cells proliferation in vitro.

CircPTN Sponges miR-326

Furthermore, we decided to investigate that the exact mechanism of circPTN promoting the proliferation of HCC cells. Increasing studies suggested that circRNA could function as sponge through binding to miRNAs. We assumed that circPTN could sponge miRNAs to regulate the growth of HCC tumors because circPTN expressed mainly in the cytoplasm. We used *circInteractome*, a database predicting miRNAs sponged by circRNA to show that miR-326 can be sponged by circPTN for the highest score[16] (Figure 3A). To verify this prediction, a luciferase reporter system was constructed by implanting the circPTN sequence into the 3′ UTR of the specific plasmid. We indicated that mimic miR-326 could significantly decrease luciferase activity after transfected with WT and negative control or miRNA mimics at the same time (Figure 3B). Then, we also cloned a mutated sequence into 3′ UTR of a specific plasmid. However, we could not detect significant luciferase activity change when transfection with mutated sequence and miRNA mimic (Figure 3C). Meanwhile, circPTN overexpression plasmid and siRNA for circPTN were transfected into HepG2. It showed that increased circPTN could down-regulate the level of miR-326 and decreased circPTN upregulate miR-326 level (Figure 3D). Next, we confirmed whether circPTN can directly interact with miR-326 by using an RNA pull-down experiment. Biotin-labeled WT-circPTN and MUT-circPTN were hatched with total RNA. Then, the total RNA was extracted. Finally, streptavidin-labeled magnetic bead was served to enrich the biotin. The real-time PCR results indicated that miR-326 was enriched in the WT-circPTN treated group (Figure 3E). Furthermore, we performed FISH assay and verified that circPTN could interact with miR-326 in the cytoplasm in HCC cell lines (Figure 3F).

When confirming that circPTN was able to bind to miR-326, we asked whether circPTN can degrade miR-326 directly. SMMC-7721 cells were transfected with circPTN plasmid to enhance the level of circPTN and were treated with actinomycin D. Then, total cell RNA was extracted and the amount of miR-326 was measured at 0, 12, 24 hrs after treatment. The data indicated that the level of miR-326 was declined in the circPTN overexpression group (Figure 3G). ErbB and PI3K are confirmed to be downstream targets of miR-326[17,18] after transfecting with miR-326 mimic (Figure 3H). To further verify that circPTN could regulate the expression of ErbB and PI3K, we detected the protein levels of ErbB and PI3K when knockdown and overexpression of circPTN. The result indicated that circPTN could regulate the expression of ErbB and PI3K (Figure 3I).

MiR-326 Is Downregulated in HCC Tumor Tissues and HCC Cell Proliferation, and This Effect Is Rescued by circPTN

Once verifying that circPTN was able to bind to miR-326, we tried to illuminate the relationship between circPTN and miR-326 in HCC tumor tissues. Although many studies have suggested that miR-326 is a tumor suppressor which can reduce cell proliferation, we decided to confirm this consequence in our study. Firstly, we measured miR-326 expression in HCC tumor tissues and HCC cell lines. As expected, miR-326 decreased significantly in the HCC tumor tissues (Figure 4A). Then, we used Pearson's correlation analysis to assess the relationship between miR-326 and circPTN. It showed that miR-326 was negatively correlated with the circPTN level (Figure 4B). We also determined that miR-326 decreased in HCC cell lines

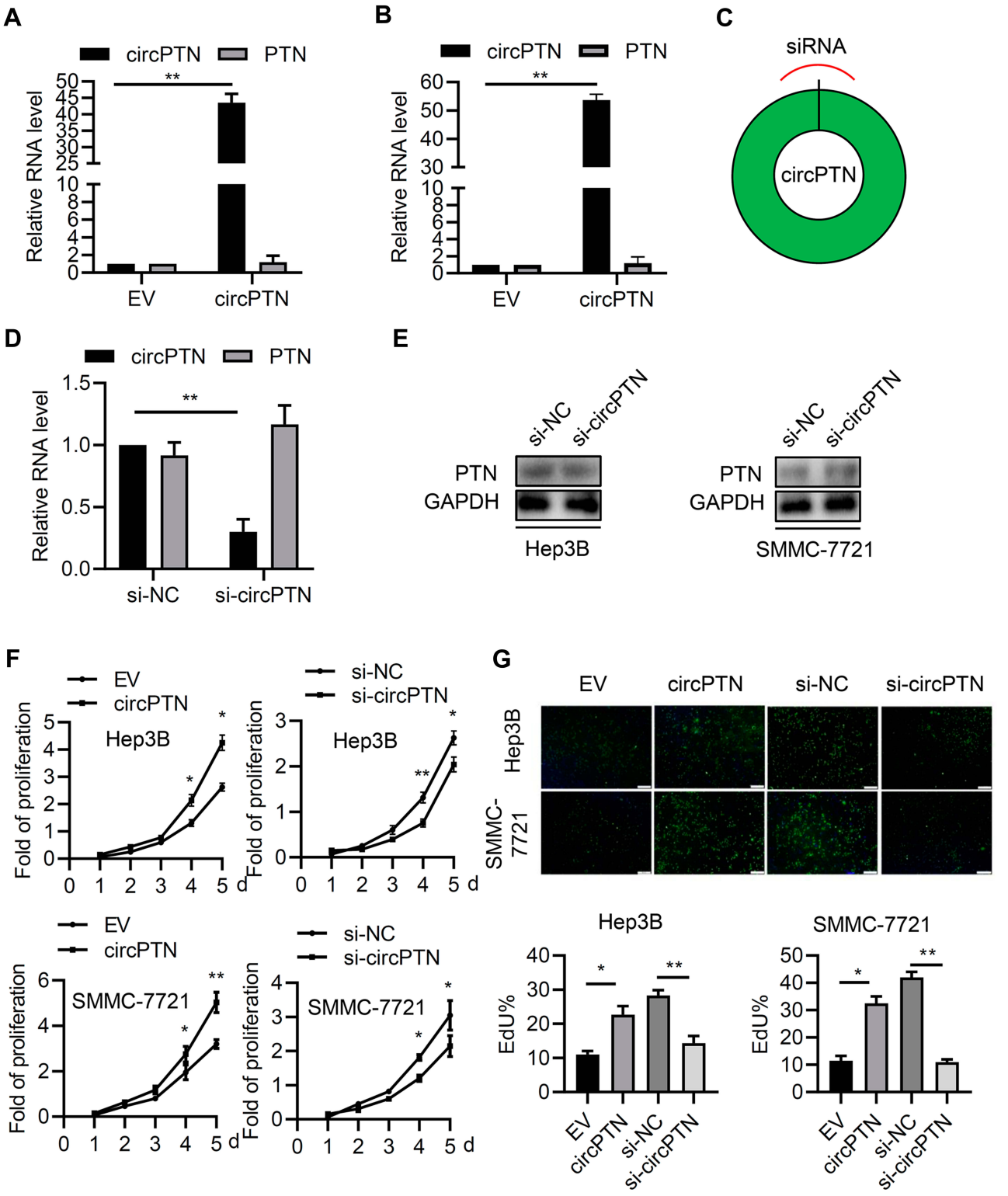

Figure 2 CircPTN promotes HCC cell proliferation in vitro. (**A** and **B**) circPTN was stably overexpressed in Hep3B and SMMC-7721 cells, n = 3, $^{**}P < 0.01$, ANOVA. (**C**) Schematic illustration showed the siRNA binding site for circPTN. (**D**) circPTN can be knocked down by siRNA transfection in Hep3B cells, n = 3, $^{**}P < 0.01$, ANOVA. (**E**) Western blot images showed the PTN protein level when transfected si-NC and si-circPTN in HCC cell lines. (**F**) CCK-8 assay showed that circPTN enhanced cell proliferation of HCC cell lines. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test. (**G**) Images and analyses of EdU assay indicated that overexpression of circPTN upregulated the EdU-positive Hep3B and SMMC-7721 cells. Scale bar, 200 μm. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA.

Abbreviations: HCC, hepatocellular carcinoma; EV, empty vector; si-NC, siRNA of negative control; si-circPTN, siRNA of circPTN; ANOVA, analysis of variance.

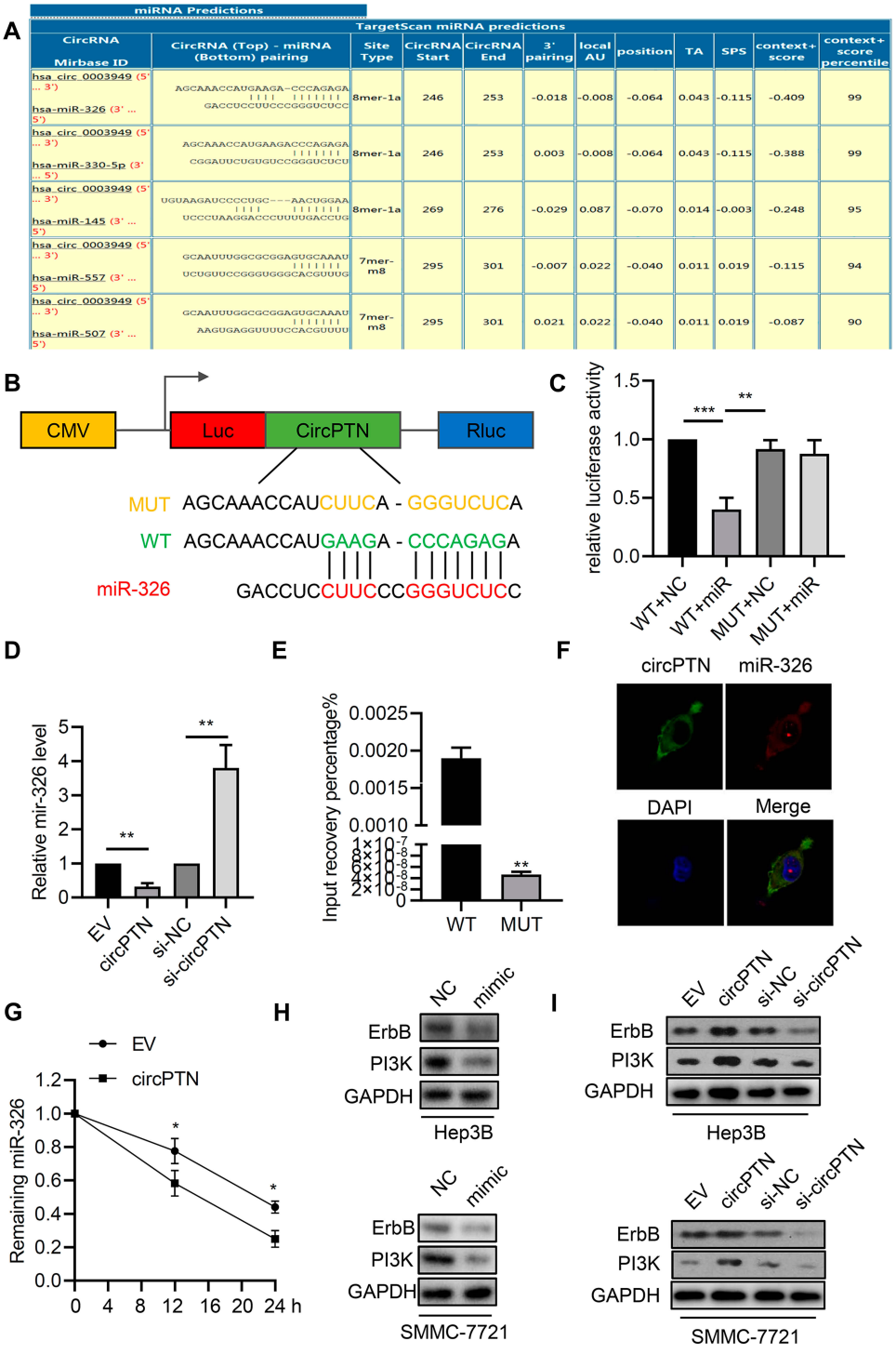

Figure 3 CircPTN sponges miR-326. (**A**) A schematic model showed the potential binding sites for miRNAs and 3-UTR of circPTN. (**B** and **C**) Luciferase reporter assay presented that co-transfection of WT with mimic markedly reduced luciferase activity in 293T cells. n = 3, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA. (**D**) Real-time PCR indicated that miR-326 was regulated by circPTN in HCC cells. n = 3, $^{**}P < 0.01$. (**E**) RNA pull-down assay showed that miR-326 expression in WT-circPTN group was higher than the MUT-circPTN group. n = 3, $^{**}P < 0.01$, *t*-test. (**F**) FAM-labeled circPTN and Cy3-labeled miR-326 showed that circPTN and miR-326 co-localized in the cytoplasm. Scale bar, 10 μm. (**G**) Real-time PCR suggested that miR-326 was significantly less in group circPTN in SMMC-7721 cells treated with 2 μg/mL actinomycin D at different time points, n = 3, $^{*}P < 0.05$, *t*-test. (**H** and **I**) Western blot showed that expression of ErbB and PI3K by regulated by miR-326 and circPTN, n = 3.

Abbreviations: HCC, hepatocellular carcinoma; EV, empty vector; Luc, luciferase; WT, wild-type, MUT, mutation; PCR, polymerase chain reaction; ANOVA, analysis of variance.

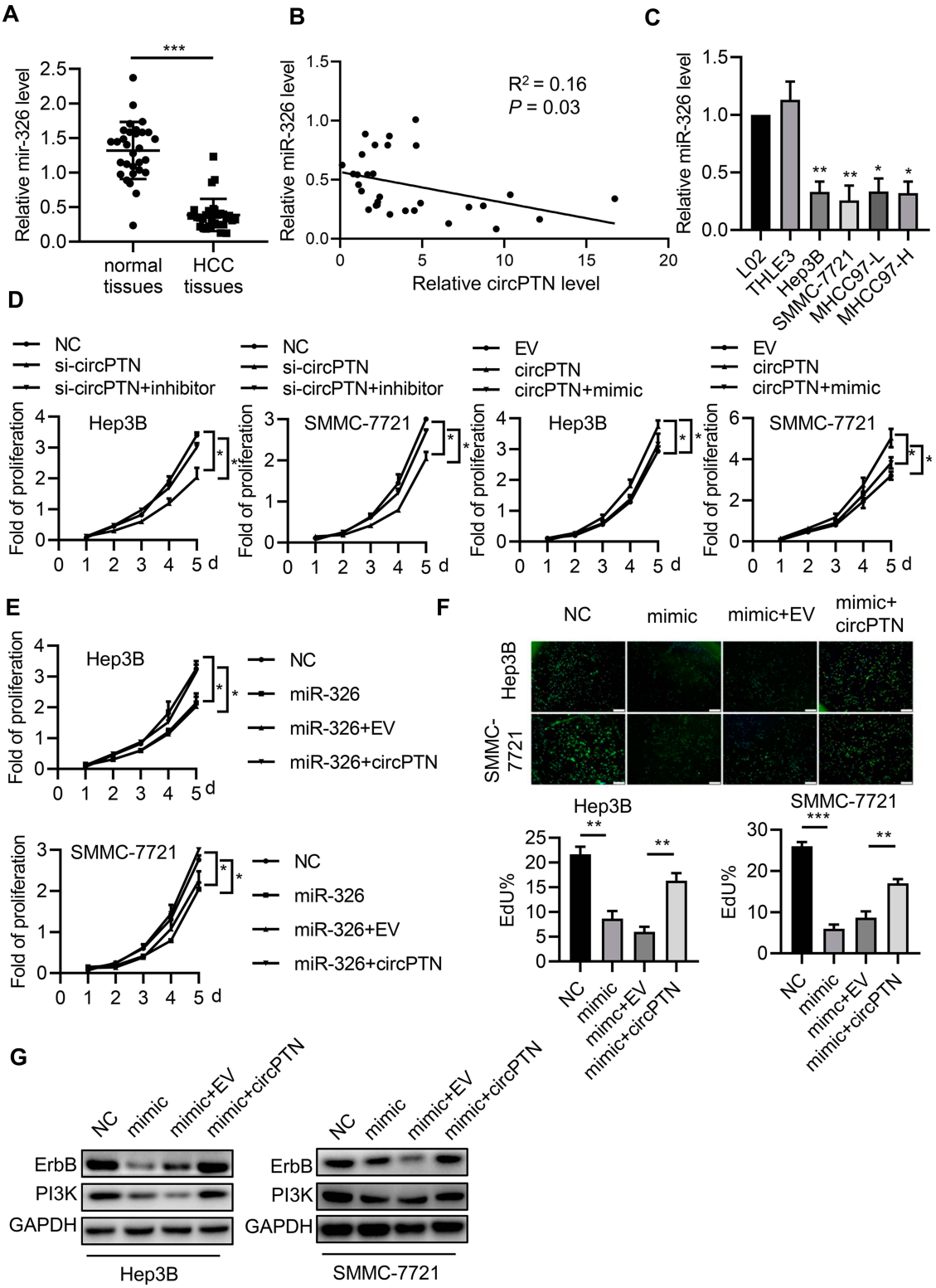

Figure 4 MiR-326 is downregulated in HCC tumor tissues, inhibits HCC cells proliferation, and the effect can be rescued by circPTN. (**A**) The expression of miR-326 was significantly downregulated in HCC tumor tissues. n = 30, ***$P < 0.001$, *t*-test. (**B**) The relationship between the expression of circPTN and miR-326 was analyzed by using Pearson's correlation analysis. (**C**) MiR-326 was significantly downregulated in HCC cell lines. n = 3, *$P < 0.05$, **$P < 0.01$, ANOVA. (**D**) CCK-8 assay indicated miR-326 inhibitor could rescue the decreased proliferation caused by circPTN silencing and miR-326 mimic could downregulate the increased proliferation caused by circPTN overexpression. n = 3, *$P < 0.05$, ANOVA. (**E**) CCK-8 assay showed that miR-326 reduced cell proliferation in HCC cell lines, which could be rescued by circPTN, n = 3, *$P < 0.05$, *t*-test. (**F**) Images and analyses of the EdU assay indicated that miR-326 reduced EdU-positive cells, which could be rescued by overexpression of circPTN in HCC cell lines. Scale bar, 200 μm. n = 3, **$P < 0.01$, ***$P < 0.001$, ANOVA. (**G**) The reduction of ErbB/PI3K expression by miR-326 could be rescued by circPTN overexpression in Hep3B and SMMC-7721 cells.

Abbreviations: HCC, hepatocellular carcinoma; EV, empty vector; NC, negative control; ANOVA, analysis of variance.

(Figure 4C). These results indicated that miR-326 could act as a tumor-suppressive factor.

Next, we transfected si-circPTN and miR-326 inhibitor in HCC cells and performed CCK-8 assay. The results showed that decreased circ-PTN inhibited the proliferation of HCC cells and miR-326 inhibitor could rescue this effect. Besides, the circPTN plasmid was transected into cells with miR-326 mimics, CCK-8 assay indicated that circPTN overexpression promoted HCC cell proliferation, and it could be cancelled by miR-326 mimic (Figure 4D). We constructed an overexpression system of miR-326 through transfecting miRNA mimic in Hep3B, SMMC-7721 cells successfully (Figure 4E). Depending on performing CCK-8 and EdU assays in Hep3B, SMMC-7721 cells, we showed that overexpression of miR-326 could inhibit HCC cell proliferation. Most importantly, we found that the cell proliferation inhibition by miR-326 in Hep3B, SMMC-7721 cells were rescued by upregulating level of circPTN (Figure 4F). In addition, we indicated that the protein levels of ErbB and PI3K downregulated by miR-326 were rescued by overexpressing circPTN (Figure 4G). The results suggested that circPTN could sponge miR-326 to promote the proliferation of HCC.

Discussion

CircRNAs were discovered more than thirty years ago, however it did not attract attention until Hayashi-Takagi et al found that CDR1as sponges miR-7 and had many binding sites.[19,20] Nowadays, more and more studies have reported that circRNA has many other functions, interacting with RNA-binding proteins,[21] regulating gene transcription,[22] as well as translating into proteins.

Recently, circPTN was found to be a cariogenic factor, sponging miRNAs in glioma first time.[11] Then, we screened the expression of circPTN tumor tissues in HCC. By detecting the expression of circPTN in our system of HCC tumor tissues and paratumor tissues, we verified that the expression of circPTN was higher in HCC tumor tissues than that in paratumor tissues. As same with lots of circRNAs, circPTN was also more stable, because of being resistant to RNase R. By performing FISH assays and nuclear-cytoplasmic real-time PCR, we confirmed that circPTN placed in the cytoplasm, suggesting that circPTN could sponge miRNA. We overexpressed and knocked down the level of circPTN, and the result showed that circPTN could promote proliferation in Hep3B, SMMC-7721cells. Then, based on the predictions using *CircInteractome*, miR-326 is one of the miRNAs gaining the highest score. So, we hypothesized that miR-326 may be a target of circPTN. To verify whether the circPTN sponge miR-326, we performed lots of experiments. Firstly, we inserted the wild-type sequences or miR-326 binding sites mutation PTN into specific plasmid to create a luciferase reporter system. It showed that luciferase activity was reduced when co-transfecting mimic miR-326 with the WT, and no luciferase activity change transfected with mutation. Secondly, we used biotin-labeled circPTN to perform RNA-RNA pull-down assays, showing that miR-326 was markedly upregulated in SMMC-7721 cells compared with the normal liver cells. Thirdly, we performed FISH to confirm that circPTN and miR-326 were indicated to be co-localized in the cytoplasm. Fourthly, when treating with actinomycin D, we detected that the speed of miR-326 degradation higher after circPTN was overexpressed. Finally, ErbB and PI3K are targets of miR-326. We observed that circPTN could rescue the decrease of ErbB and PI3K. These two proteins can be degraded by miR-326. Taken together, we established that circPTN could sponge miR-326, increasing the level of ErbB and PI3K. Besides, we confirmed that the decreased proliferation regulated by miR-326 could be rescued by overexpressing circPTN. Hence, circPTN could promote proliferation and upregulated ErbB and PI3K expression by sponging miR-326.

Several studies indicated that the expression of circRNAs is lower in HCC tumor tissues than that in paratumor tissues, including breast cancer,[23] gastric cancer,[24] colorectal cancer,[25] glioma,[26] prostate cancer,[27] and hepatocellular carcinoma.[28] As we know, the level of circRNA is negatively related to proliferation, because of high proliferative activity in many cancer tissues. However, whether alteration of proliferation is associated with level of circRNA is unidentified. Bachmayr-Heyda et al indicated the decreased circRNAs because of the distribution to next-generation during the process of proliferation in cells.[29] It is our hypothesis that proliferation can be influenced by the alteration in circRNA expression. It is reported that circRNA had two effects. One is that circRNA could inhibit proliferation, and the other is dependent on the specific sequence, which affected activities includes miRNA sponge, gene translation and RNA-binding proteins. Thus, further research should be validated the exact mechanism that the level of circRNAs regulated proliferation. We advise to detect the level of circRNAs in tumor tissues to reflect their proliferative activity and use individual

circRNAs as biomarkers to predict prognosis. Additionally, circRNA is generated via backsplicing with direct covalent ligation of 5′ and 3′ ends of RNA.[30] Previous reports showed that several RNA-binding proteins (RBP) regulated backsplicing and were contributed to circRNA biogenesis, such as heterogeneous nuclear ribonucleoprotein, serine-arginine (SR) proteins, MBNL, NUDT21, and FUS.[31] Whether these RBPs are involved in the formation of circPTN needs to be further investigated.

Conclusion

In a word, our study indicates that the expression of circPTN is upregulated in HCC patients. CircPTN could enhance the growth of HCC through the sponge activity on miR-326 and upregulating the expression of ErbB/PI3K. Our data also suggest that circPTN may act as a diagnostic biomarker and a target for HCC.

Ethical Approval

All methods were achieved in agreement with the guideline approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University. All patients gave informed consent and written informed consent was obtained.

Disclosure

Benli Jia and Xiaoqiang Yin are co-first authors. The authors declare that they have no competing interests in this work.

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