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ORIGINAL PAPER

Circular RNA expression and circPTPRM promotes proliferation and migration in hepatocellular carcinoma

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Abstract

Circular RNAs (circRNAs) play a critical role during hepatocellular carcinoma (HCC) development. CircRNA PTPRM (circPTPRM) has not been reported to cause disease and its role in HCC is unclear. This study explored circRNA expression and the function of circPTPRM in HCC. RNA sequencing (RNA-seq) was performed on 3 randomly selected pairs of HCC tissues and their corresponding adjacent non-tumor tissues. Three differentially expressed circRNAs, circPTPRM, circSMAD2 and circPTBP3 were selected and verified by real-time quantitative reverse transcription-polymerase chain reactions in 30 pairs of tissue samples, In vitro cultured hepatoma cells, and normal liver cells. Clinical data analysis was performed to select target circRNAs. Anti-target circRNA siRNAs were transfected into hepatoma cell lines, and the biological behavior of hepatoma cells following silencing of the target circRNA were detected by cell proliferation, plate cloning, and transwell assays. There were 86 differentially expressed circRNAs from RNA-seq, of which 53 were significantly upregulated and 33 were significantly downregulated in HCC. CircPTPRM expression was significantly upregulated in HCC tissue ($p=0.023$) based on the analysis of 30 paired samples. CircPTPRM expression positively correlated with HCC recurrence and metastasis ($p=0.039$). CircPTPRM silencing reduced HCC cell proliferation, migration and invasion. CircRNAs were differentially expressed in HCC samples. CircPTPRM was significantly upregulated in HCC and may function during the tumorigenesis and metastasis of HCC.

Keywords Hepatocellular carcinoma · Circular RNA · RNA sequencing · circPTPRM

Introduction

Non-coding RNAs play an important role in the development and progression of hepatocellular carcinoma (HCC) [1, 2], and include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNA (circRNAs). MiRNAs have been intensely investigated in cancer studies [3–7]. The variety of lncRNAs [8–11] and the complex mechanisms enabling lncRNA expression are an important factor during HCC therapeutic failure. Due to the development of high-throughput sequencing [12] and bioinformatics technology [13, 14], circRNAs have emerged as a new focus in HCC research.

CircRNAs regulate gene expression by interacting with lncRNA and mRNAs [15–17]. CircRNAs can be used as sponges by targeting miRNAs [18–20] and regulating gene transcription through inhibiting RNAP II extension [15, 21]. CircRNAs directly participate in protein synthesis [22, 23]. In addition, circRNAs show a variety of other functions, including the generation of pseudo-genes derived from circRNAs [24], alternative splicing processes [25], histone modifications [26], and RNA maturation [27]. CircRNAs play an increasingly important role in various tumors. Some circRNAs are differentially expressed in tumor tissue and paracancerous tissue through gene expression profile analyses, which closely correlates with tumor type, staging, and clinical manifestation. These findings suggest that circRNAs play important roles in tumor metabolism, apoptosis, proliferation and metastasis.

The circRNA PTPRM (circPTPRM; circBase [28] ID: Hsa_circ_0007144) is located in chr18:8076452-8143777. Its parental gene is Protein Tyrosine Phosphatase Receptor Type M (PTPRM), a member of the Protein Tyrosine

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Phosphatase (PTP) family. PTP regulates many cell biology processes, including cell growth, differentiation, cell cycle regulation, communication, gene transcription, immune responses and oncogenic transformation [29–31]. PTPRM is structurally similar to cell–cell adhesion molecules and displays homophilic binding, which regulates cell–cell adhesion in epithelial and cancer cells, and is a key component of tumor development [32–34]. In this study, we found that circPTPRM was significantly upregulated in HCC and, therefore, investigated its biological behavior and clinical significance.

Methods

Patients

Thirty HCC samples and their corresponding adjacent non-tumorous liver (ANL) tissue were obtained from the First Affiliated Hospital of China Medical University between May 2017 and January 2018. HCC tissues were obtained from the geometric center of tumors. If the central portion of the tissue was necrotic, the site around the necrotic area and closest to the geometric center was taken. ANL tissues were obtained ~ 1 cm away from the edge of the HCC tumor. HCC diagnosis was confirmed by 2 experienced pathologists upon histological examination. No patients received preoperative radiotherapy, chemotherapy or targeted therapy prior to surgery. Tissue specimens were snap-frozen and stored at − 80 °C until use. Clinical information and human HCC tissues were obtained under the approval of the Institutional Ethics Committee of China Medical University (2017-151-2). Written informed consent was obtained from each subject.

Cell culture

Immortalized hepatoma cell lines, Huh7, HepG2, SMMC-7721, BEL-7402 and one human normal liver cell line HL7702, were obtained from the First Affiliated Hospital of China Medical University. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Bovogen, Australia) and were grown in a humidified atmosphere with 5% CO_2 at 37 °C.

Real-time quantitative reverse transcription-polymerase chain reactions

Total RNA was extracted from tissue samples and cultured cells using RNAisoTM plus (Takara, Japan) as per the manufacturer's instructions. cDNA was synthesized by reverse transcription using Prime ScriptTM RT Master Mix (TaKaRa, Japan). Real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCR) were performed using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan). Primers for circPTPRM, CircSMAD2, circPTBP3 and GAPDH were synthesized by Sangon Biotech (Shanghai, China). All qRT-PCR primer sequences are shown in Table 1.

RNA sequencing

Extracted mRNA was enriched with oligo (dT) magnetic beads. RNA-sequencing (RNA-seq) libraries were produced and subjected to quality inspection by Agilent 2100. Libraries were quantified by qRT-PCR. Samples were sequenced using Illumina Hiseq 4000. RNA-seq was performed using Aksomics (Shanghai, China).

Cell transfection

Transfections were performed using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's protocols. siRNA of circPTPRM were designed and synthesized by GenePharma (Shanghai, China), and targeted to the junction region of circPTPRM. siRNA sequences are were as follows: circPTPRM-1, 5′- GUAAUGAAGAAAAGUCCC ATT-3′ (sense) and 5′-UGGGACUUUU CUUCAUUACTT-3′ (antisense); circPTPRM-2, 5′-GAAAAGUCCCAGGUG CUGUTT-3′ (sense) and 5′-ACAGCACCUGGGACUUUU CTT-3′ (antisense).

MTT assay

Approximately, 5000 cells per well were seeded in 96-well plates in media containing 10% FBS and incubated for

Table 1 qRT-PCR primers

Primer name	Forward primer(5′–3′)	Reversed primer(5′–3′)
circPTPRM	GGGCATCTTGCTGTTCGTGA	TTCAGTGGGAACAGCACCTG
circSMAD2	TTCCAGAAACGCCACCTCCT	TACCAAAGGCAGCAAGCCAC
circPTBP3	GTCAGTGCCGTCCAATCAGG	AGGTCCGTTAATGATGCCAGAAG
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT

qRT-PCR real-time quantitative reverse transcription-polymerase chain reactions

0, 24 h, 48 h, 72 h, and 120 h. On the indicated days, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazolium-romide (MTT) reagent (Sangon, China) was added to each well, and cells were incubated for 4 h at 37 °C. Supernatants were then removed and 100 µL of DMSO (Sangon, China) was added per well to dissolve formazan crystals. Absorbance levels were measured at 490 nm using an automatic microplate reader (Gene, HK).

Colony-formation assays

After transfection, 1000 cells per well were seeded into 6-well plates. Cells were incubated for 10 days, fixed in 4% paraformaldehyde, and stained with 0.1% crystal violet. Colonies were counted when ≥ 50 cells.

Migration and invasion assay

For invasion assays, transwell (Costar, USA) and Matrigel (Solarbio, China) systems were used according to the manufacturers' instructions. Aliquots of 20,000 cells were seeded into upper chambers precoated with Matrigel, and cultured in serum-free DMEM. Lower compartments were filled with DMEM supplemented with 10% FBS as a chemoattractant. After incubation for 48 h, cells remaining in the upper chamber were removed, and cells in the lower insert were fixed. Cells were stained with 0.1% crystal violet and counted under a microscope (Nikon, Japan). Results from three independent experiments were averaged. For migration assays, cells were seeded into the upper chambers without Matrigel coating for 24 h. Assays were then performed as per the invasion assays. Cell numbers in five widefields were counted following crystal violet staining.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 software. All experiments were repeated 3 times. Parametric data were derived from triplicate experiments and are presented as mean ± SEM. Differences between each group were analyzed using a Student's *t* test. The association between relative expression and clinicopathological parameters was evaluated by χ^2 tests or Fisher's exact tests when appropriate. Significance was defined as $p < 0.05$.

Results

Identification of circular RNAs by RNA-seq analysis in human liver samples

We detected 3303 distinct circRNAs from 3 pairs of human HCC and their ANL tissues using RNA-seq. Amongst them, 1367 were upregulated and 1259 were downregulated (Fig. 1a). Expression analysis showed that a series of circRNAs were differentially expressed in HCC compared to ANL tissues. Amongst the 86 differentially expressed circRNAs, 53 were upregulated and 33 were downregulated in HCC (Fig. 1b, c). The top 15 up- and downregulated circRNAs are shown in Table 2. Following a comprehensive analysis of cellular functions, circPTPRM, circSMAD2 (CircBase ID: hsa_circ_0000847) and circPTBP3 (CircBase ID: hsa_circ_0008192) with significantly upregulated expression were selected for further studies. Complete raw data sets were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE125469.

CircPTPRM, circSMAD2 and circPTBP3 expression in HCC tissue and cell lines

To analyze selected circRNA expression patterns, 30 pairs of HCC and ANL tissues were assessed by qRT-PCR. The upregulation of circPTPRM was observed in 73.3% (22/30) of HCC tissues, which was significantly higher than paired ANL tissues ($p = 0.023$, Fig. 2a, b). circSMAD2 expression in HCC tissues was significantly higher than paired samples ($p = 0.0026$, Fig. 2a, b). No statistically significant differences in circPTBP3 expression in HCC and ANL tissues were observed ($p = 0.107$, Fig. 2a, b).

On the cellular level, in comparison to HL7702, circPTPRM expression was higher in all hepatoma cell lines, including Huh7, HepG2, SMMC-7721 and BEL7402 ($p < 0.05$, Fig. 2c). CircSMAD2 showed high expression levels in Huh7, HepG2 and SMMC-7721 cells in contrast with HL7702 ($p < 0.05$, Fig. 2c), in which no expressional changes compared to BEL7402 were observed ($p = 0.115$, Fig. 2c). CircPTBP3 showed high expression in Huh7, SMMC-7721 and BEL7402 cells in contrast with HL7702 ($p < 0.05$, Fig. 2c), whilst expression HepG2 cells showed no statistically significant differences to HL7702 ($p = 0.079$, Fig. 2c).

Association of circPTPRM, circSMAD2 and circPTBP3 expression with clinicopathological features

To explore the clinical significance of circPTPRM, circSMAD2 and circPTBP3 in HCC patients, cases were divided according to the correlation between circPTPRM, circSMAD2 or circPTBP3 expression and clinicopathological characteristics (Table 3). CircPTPRM expression positively associated with HCC recurrence and metastasis ($p = 0.039$). Kaplan–Meier analysis was used to evaluate patient recurrence and metastasis-free survival rates relative to circPTPRM expression up to December 31, 2018.

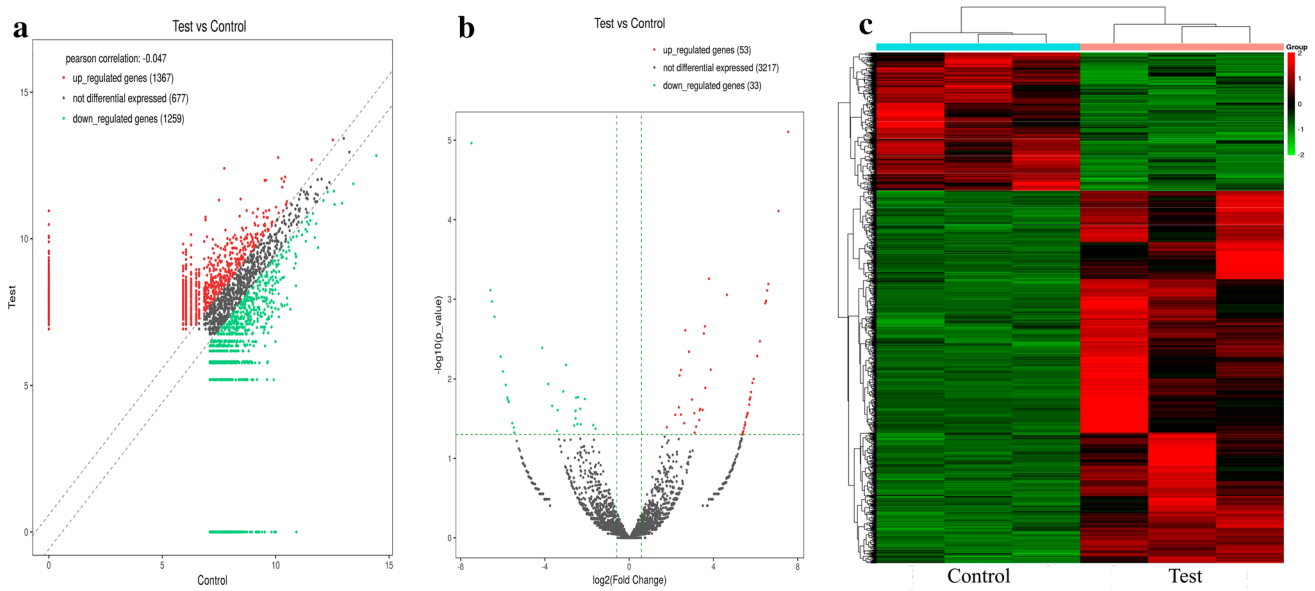

Fig. 1 Identification of circular RNAs in human liver samples. **a** Scatter plot demonstrating variations in circRNA expression in HCC (*Y* axis) versus control (*X* axis) samples. Values are averaged, normalized signal values (log 2) of circRNA expression in HCC. Diagonal dashed lines delineate upper, lower genes (1.5-fold change) and unchanged genes. **b** Volcano plot visualizing differential circRNA expression in HCC. X-axis represents fold changes (log 2), and *y*-axis represents *p* values (− log 10). Vertical green lines are upregulated (right) and downregulated (left), respectively. Green parallel lines correspond to the *p* value thresholds. **c** Cluster analysis of significantly differentially expressed genes between HCC and control samples. Each row represents a gene, whilst each column represents a sample. Red represents upregulated genes, green represents downregulated genes, and black represents undifferentiated genes. HCC, hepatocellular carcinoma; Test, HCC tissues; Control, adjacent non-tumorous liver tissues

The results indicated that higher expression of circPTPRM in HCC patients correlated with tumor recurrence and metastasis ($p = 0.040$, Fig. 2d). However, no correlation with age, gender, HBsAg, serum AFP, HCC history, cirrhosis, tumor number, size, or histological differentiation were observed. circSMAD2 expression was related to TNM stage ($p = 0.014$), and circPTBP3 was related to HCC recurrence and metastasis ($p = 0.007$). These results indicate that circPTPRM is upregulated in HCC, and plays a role during HCC development.

CircPTPRM silencing suppresses HCC cell proliferation and invasion

qRT-PCR was used to detect circPTPRM expression in Huh7 and SMMC-7721 cells which contains higher circPTPRM content. In over-confluent cells, circPTPRM expression was significantly reduced ($p < 0.01$, Fig. 3a). To examine the functional roles of circPTPRM in HCC, we silenced its expression in Huh7 and SMMC-7721 cells using circPTPRM-siRNA-1 and circPTPRM-siRNA-2. circPTPRM silencing was confirmed by qRT-PCR 48 h post-transfection (Fig. 3b). MTT and colony-formation assays were performed to detected proliferation. Compared to control groups, cancer cell proliferation was suppressed in circPTPRM low-expression groups by MTT analysis 72 h and 120 h post-transfection ($p < 0.01$, Fig. 3c). Consistent with the MTT data, colony-formation assay showed that low-circPTPRM expression led to a significant reduction of colony numbers in HCC cells ($p < 0.01$, Fig. 3d). These results confirmed that circPTPRM accelerates proliferation in HCC.

To investigate the functions of circPTPRM during cell migration and invasion, transwell assays were performed in HCC cells. For reasons of expense, we only selected SMMC-7721 cells in transwell. circPTPRM silencing in SMMC-7721 cells significantly suppressed cell invasion and migration compared to negative control groups ($p < 0.01$, Fig. 3e). These findings confirmed that circPTPRM accelerates the migration and invasion of HCC cells.

Discussion

CircRNAs are key regulators of transcription and translation. Studies on circRNA have focused on miRNA sponges to isolate miRNA regulated genes at the post-transcriptional level. The relationship between circRNA and HCC is gradually emerging. Han et al. [35] found that circMTO1 promotes the expression of the tumor suppressor p21 through acting as an miR-9 sponge, and inhibiting the proliferation and invasion

Table 2 Top 15 differentially expressed circRNAs from RNA-sequencing analysis

circRNA position	Length	Gene_Name	log2FC	*p* value
chr6:160103505-160109274:−	**462**	**SOD2**	**7.546200868**	**7.86125E−06**
chr7:111127293-111201979:−	**314**	**IMMP2L**	**7.088929709**	**7.78353E−05**
chr8:112015642-112039662:−	**523**	**RP11-946L20.2**	**6.605672003**	**0.00064394**
chr8:97892025-97892233:+	**208**	**CPQ**	**6.550901757**	**0.000776168**
chr1:21415630-21437876:−	**137**	**EIF4G3**	**6.500231599**	**0.001053203**
chr3:142455220-142467302:+	**460**	**TRPC1**	**6.461447777**	**0.001125531**
chr18:45391429-45423180:−	**783**	**SMAD2**	**6.205574033**	**0.003351505**
chr10:32740519-32762951:+	**847**	**CCDC7**	**6.081705468**	**0.005151238**
chr10:32759991-32762951:+	**286**	**CCDC7**	**6.077232731**	**0.005198823**
chr19:53158813-53164096:−	**209**	**ZNF83**	**5.914378031**	**0.009976258**
chr15:41648236-41669502:+	**667**	**NUSAP1**	**5.856057195**	**0.011187695**
chr8:124089350-124117704:+	**1132**	**TBC1D31**	**5.782710898**	**0.014604149**
chr18:8076452-8143777:+	**859**	**PTPRM**	**5.750616386**	**0.017065791**
chr9:115024714-115060196:−	**558**	**PTBP3**	**5.726557353**	**0.01800762**
chr7:17908029-17937069:−	**825**	**SNX13**	**5.721795448**	**0.018151383**
chr5:38523520-38530768:−	*580*	*LIFR*	*−7.48343365*	*1.09128E−05*
chr6:53986244-54067031:+	*2622*	*MLIP*	*−6.58370167*	*0.000770845*
chr13:46577273-46619648:−	*950*	*ZC3H13*	*−6.51166035*	*0.001070962*
chr16:56385295-56388993:+	*370*	*GNAO1*	*−6.38876161*	*0.001652804*
chr6:53986244-54034370:+	*2448*	*MLIP*	*−6.09945152*	*0.005247578*
chr10:52220432-52350007:−	*371*	*SGMS1*	*−5.9895155*	*0.00806852*
chr12:100598717-100601564:+	*311*	*ACTR6*	*−5.86082592*	*0.011875651*
chr4:22748918-22750583:+	*921*	*GBA3*	*−5.78441572*	*0.017187205*
chr10:51584615-51585599:+	*984*	*NCOA4*	*−5.74844417*	*0.018395172*
chr16:72092152-72093087:+	*177*	*HP*	*−5.74403327*	*0.018453511*
chr14:70245898-70246077:−	*179*	*SLC10A1*	*−5.71751374*	*0.019480091*
chr16:48381417-48382201:+	*399*	*LONP2*	*−5.54954768*	*0.03615926*
chr7:50358643-50367353:+	*174*	*IKZF1*	*−5.47049946*	*0.040977643*
chr1:161205636-161206388:−	*271*	*NR1I3*	*−5.45200642*	*0.047419985*
chr5:113740134-113740553:+	*419*	*KCNN2*	*−4.12751454*	*0.004051871*

Bold values: differentially upregulated; italic values: differentially downregulated

of HCC cells. Shi et al. [36] showed that CircARSP91 inhibits its tumor growth of HCC cells in vitro and in vivo through the AR/ADAR1/CircARSP91 signaling axis. Zhu et al. [37] revealed that circ_0067934 directly inhibits the ability of miR-1324 to target FZD5 and downregulate Wnt/-catenin signaling. Thus, promoting the proliferation, migration and invasion of HCC cells. Zhang et al. [38] also found that circ_104075 acts as a ceRNA to upregulate YAP expression by absorbing miR-582-3p, and stimulating YAP-dependent tumorigenesis by regulating HNF4a.

From sequencing data, 3303 circRNAs were found in HCC and their paired ANL tissues. Although sequencing was limited to a small sample size, circRNA was differentially expressed in HCC, implying a role during HCC development.

In this study, we demonstrated that circPTPRM is significantly upregulated in human HCC tissues and cell lines. The upregulation of circPTPRM was significantly associated with HCC recurrence and metastasis.We detected relative circPTPRM expression at cell densities of 80%, 100%, and over-confluent cells, and found that circPTPRM maintains high expression during rapid cell growth. The relative expression decreased when cells were over-confluent for an extended time period, prompting us to investigate the role of circPTPRM during cell proliferation. Using MTT, colony-formation and transwell assays, circPTPRM silencing suppressed the proliferation, migration and invasion ability of HCC cells in vitro, indicating a crucial role during HCC development. We speculate that circPTPRM is related to the recurrence and metastasis of HCC, which supports the data obtained from clinical analysis.

In this study, circSMAD2 levels were significantly higher than those of paracancer tissues, whilst the expression of circPTBP3 did not significantly differ to paracancer tissues. Further clinical data revealed that circSMAD2 expression was associated with TNM stage. However, Zhang et al. [39]

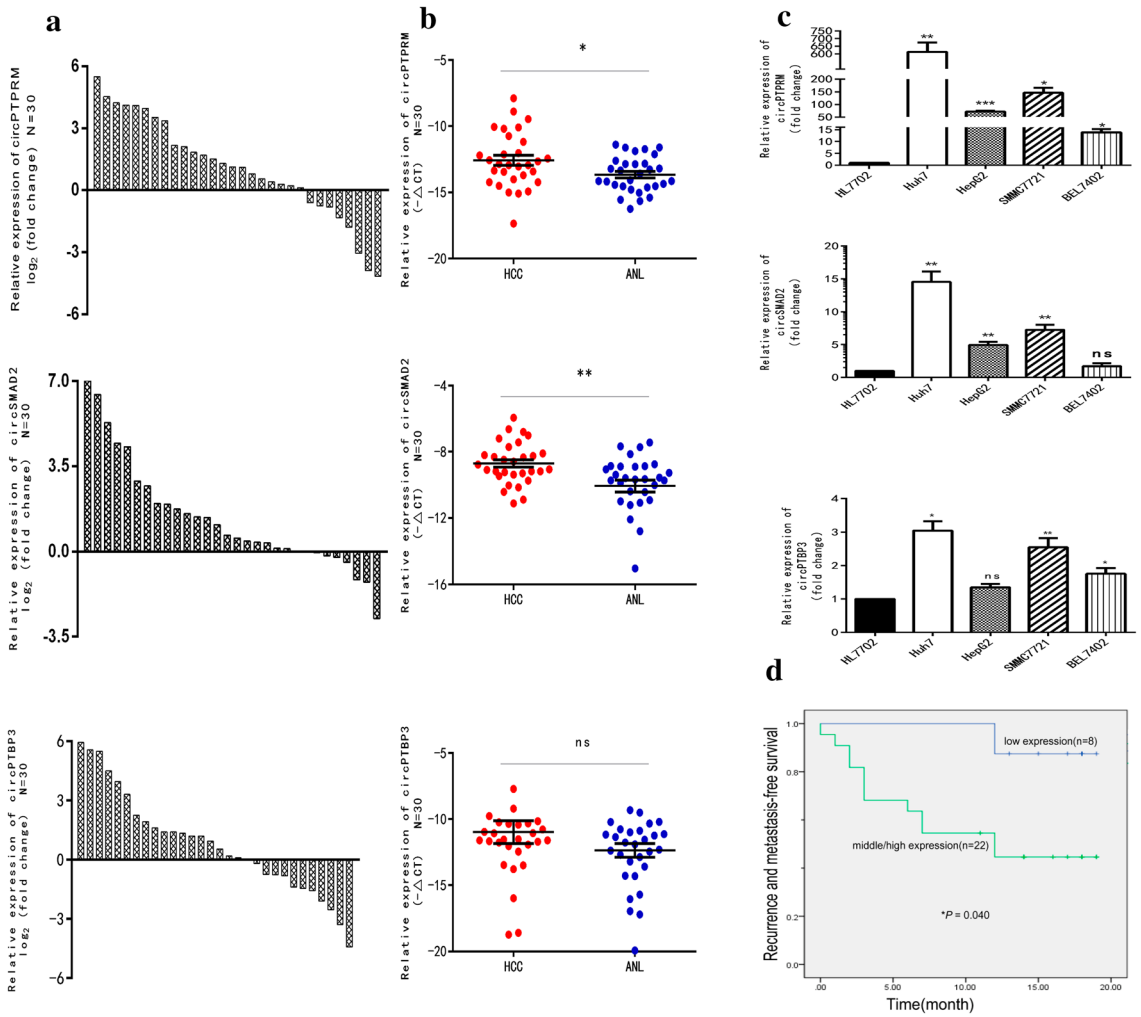

Fig. 2 CircPTPRM, circSMAD2 and circPTBP3 expression in HCC tissues and cell lines. **a** qRT-PCR analysis of circPTPRM, circSMAD2 and circPTBP3 expression in 30 HCC pairs and their ANL tissues. Expression of circRNA was normalized to GAPDH. **b** Relative circPTPRM, circSMAD2 and circPTBP3 expression levels in HCC tissues and ANL; **c** qRT-PCR analysis of circPTPRM, circSMAD2 and circPTBP3 expression in HCC cells (Huh7 HepG2, SMMC-7721,Bel-7402) and normal hepatocytes (HL-7702). **d** KM survival curve relative to circPTPRM expression. $*p<0.05$, $**p<0.01$, $***p<0.001$, ns $p>0.05$. HCC, hepatocellular carcinoma; qRT-PCR, real-time quantitative reverse transcription-polymerase chain reactions; ANL, adjacent non-tumorous liver; KM, Kaplan–Meier

found that CircSMAD2 is downregulated in HCC tissues and cell lines, and is markedly associated with the degree of differentiation in HCC tissue, which contrasted our qRT-PCR analysis. These discrepancies may be related to individual differences and small sample sizes in human HCC tissue, which requires further assessment.

A large number of circRNA studies have focused on exon circRNA, and intron and exon–intron circRNA (exon–intron circular RNA, EIcircRNA). Studies have confirmed that some EicircRNAs upregulate the expression of their parental genes [40, 41]. CircPTPRM is an EIcircRNA composed of exons and introns, encoded by the PTPRM gene. PTPRM is closely related to tumorigenesis, development and prognosis. Hellberg et al. [32] found that PTPRM regulates PKC signaling through its interaction with RACK1 in prostate cancer cells to restore e-cadherin dependent adhesion. Sun et al. [33] showed that PTPRM regulates the tyrosine phosphorylation of ERK and JNK to coordinate the migration and invasion of breast cancer cells. Laczmanska et al. [34] revealed that PTPRM is highly methylated in sporadic colorectal cancer, and the imbalance of tumor suppressor promoter methylation plays an important role in the molecular pathogenesis of tumor development. We therefore hypothesized that circPTPRM affects HCC cell proliferation, invasion and migration by upregulating the expression of its parental gene PTPRM. However, this hypothesis required further confirmation.

Table 3 circPTPRM, circSMAD2 and circPTBP3 expression and clinicopathological features in HCC patients

Characteristics	Cases	circPTPRM expression		*p* value	circSMAD2 expression		*p* value	circPTBP3 expression		*p* value
		Low	Middle/high		Low	Middle/high		Low	Middle/high	
Age (years)										
≥60	12	2 (16.7%)	10 (83.3%)	0.419	4 (33.3%)	8 (66.7%)	0.678	3 (25.0%)	9 (75.0%)	0.694
<60	18	6 (33.3%)	12 (66.7%)		4 (22.2%)	14 (77.8%)		7 (38.9%)	11 (61.1%0	
Gender										
Male	25	8 (32.0%)	17 (68.0 %)	0.287	7 (28.0%)	18 (72.0%)	0.595	10 (40.0%)	15 (60.0%)	0.14
Female	5	0 (0.0%)	5 (100.0%)		1 (20.0%)	4 (80.0%)		0 (0.0%)	5 (100.0%)	
HBsAg status										
Positive	25	7 (28.0%)	18 (72.0%)	0.595	6 (24.0%)	19 (76.0%)	0.589	7 (28.0%)	18 (72.0%)	0.3
Negative	5	1 (20.0%)	4 (80.0%)		2 (40.0%)	3 (60.0%)		3 (60.0%)	2 (40.0%)	
AFP (ng/ml)										
≥20	18	5 (27.8%)	13 (72.2%)	0.604	5 (27.8%)	13 (72.2%)	0.604	5 (27.8%)	13 (72.2%)	0.461
<20	12	3 (25.0%)	9 (75.0%)		3 (25.0%)	9 (75%)		5 (41.7%)	7 (58.3%)	
HCC_history										
Positive	7	1 (14.3%)	6 (85.73%)	0.638	2 (33.3%)	5 (66.7%)	0.623	0 (0.0%)	7 (100.0%)	0.064
Negative	23	7 (30.4%)	16 (69.6%)		6 (26.1%)	17 (73.9%)		10 (41.7%)	13 (58.3%)	
Liver cirrhosis										
Positive	24	6 (25.0%)	18 (75.0%)	0.645	6 (25.0%)	18 (75.0%)	0.645	7 (29.2%)	17 (70.8%)	0.372
Negative	6	2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)		3 (50.0%)	3 (50.0%)	
Tumor number										
>1	6	2 (33.3%)	4 (66.7%)	0.645	1 (16.7%)	5 (83.3%)	0.48	3 (50.0%)	3 (50.0%)	0.372
1	24	6 (25.0%)	18 (75.0%)		7 (29.2%)	17 (70.8%)		7 (29.2%)	17 (70.8%)	
Tumor size (cm)										
≥5	14	3 (21.4%)	11 (78.6%)	0.689	2 (14.3%)	12 (85.7%)	0.226	4 (28.6%)	10 (71.4%)	0.709
<5	16	5 (31.3%)	11 (68.7%)		6 (37.5%)	10 (62.5%)		6 (37.5%)	10 (62.5%)	
Differentiation										
High + moderate	23	7 (30.4%)	16 (69.6%)	0.638	6 (26.1%)	17 (73.9%)	0.623	7 (30.4%)	16 (69.6%)	0.657
Moderate-low + low	7	1 (14.3%)	6 (85.7%)		2 (28.6%)	5 (71.4%)		3 (42.9%)	4 (57.1%)	
Recurrence and metastasis										
Positive	14	1 (7.1%)	13 (92.9%)	0.039*	2 (14.3%)	12 (85.7%)	0.226	1 (7.1%)	13 (92.9%)	0.007*
Negative	16	7 (43.8%)	9 (56.2%)		6 (37.5%)	10 (62.5%)		9 (56.3%)	7 (43.8%)	
TNM stage										
I + II	19	7 (36.8%)	12 (63.2%)	0.199	8 (42.1%)	11 (57.9%)	0.014*	8 (42.1%)	11 (57.9%)	0.246
III + IV	11	1 (9.1%)	10 (90.9%)		0 (0.0%)	11 (100.0%)		2 (18.2%)	9 (81.8%)	

HCC hepatocellular carcinoma, *TNM* tumor node metastasis, *AFP* alpha fetoprotein

*$p < 0.05$

In addition, predicted miRNAs related to circPTPRM using ceRNA analysis with Target Scan software (Fig. 4a). CircPTPRM may affect the expression of FHL1 by acting on hsa-mir-1271-5p, hsa-mir-223-3p, hsa-mir-96-5p, and hsa-mir-208a-5p. Wang et al. [42] preliminarily explored the role of FHL1 as an anticancer gene in HCC. Histone deacetylase 8 (HDAC8), a class I histone deacetylase, remarkably correlated with HCC tumorigenesis [43, 44]. We speculate that circPTPRM affects the expression of HDAC8 through its action on hsa-miR-223-3p, hsa-miR-455-3p, hsa-miR-890, hsa-miR-138-2-3p, hsa-miR-106a-5p and hsa-miR-17-5p. We simultaneously employed topGO software to perform GO (Gene Ontology) analysis and obtained relevant GO items and corresponding genes with significant enrichment. This allowed us to infer the key biological functions of circPTPRM (Fig. 4b), including protein binding, histone H3-K9 acetylation, and neurotrophin TRK receptor signaling.

The major limitation of this study arises from the in vitro nature of the experiments and the biological functions of circPTPRM require validation in vivo. The relationship between PTPRM and potential target miRNAs and circPTPRM also require further investigation in HCC.

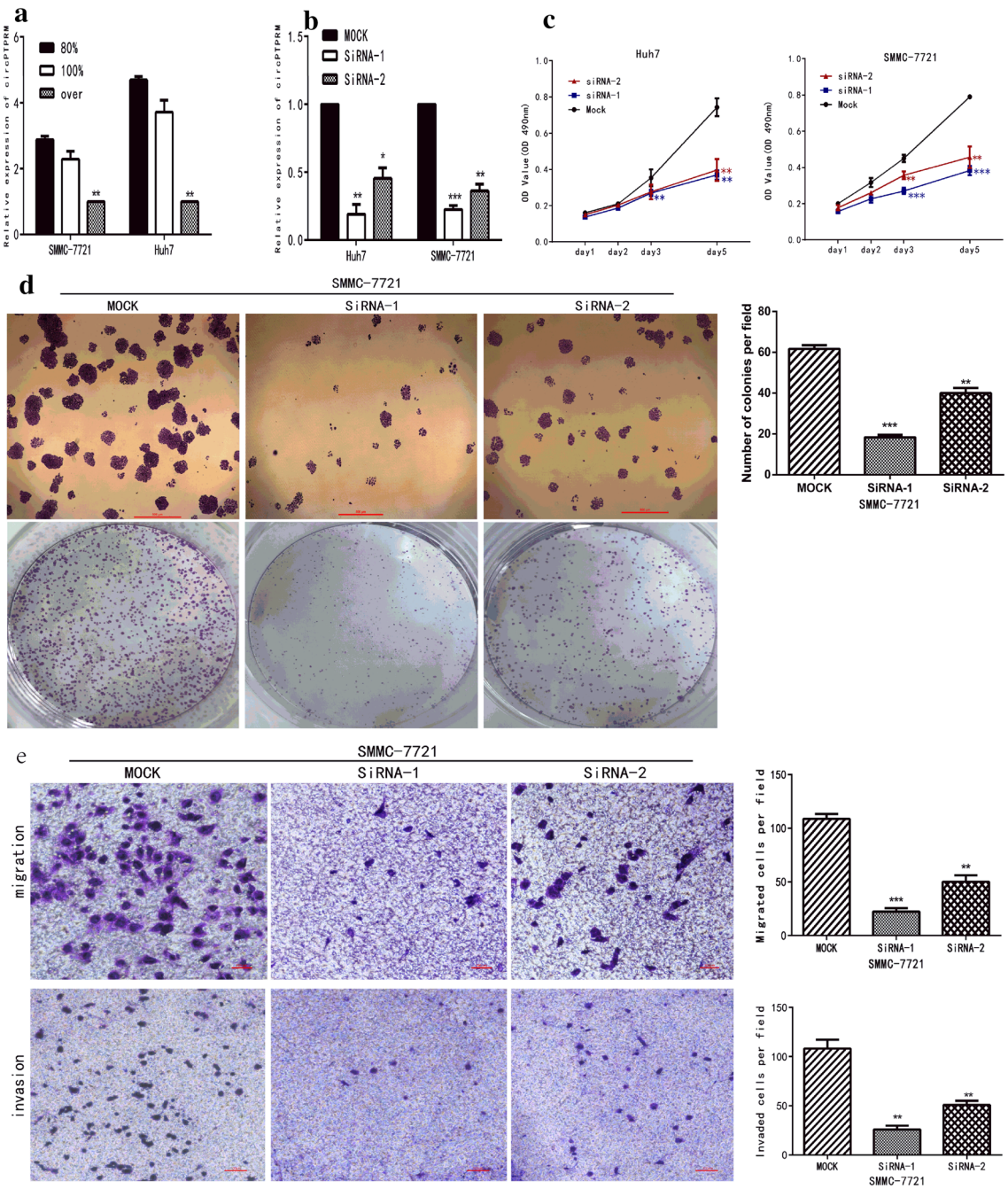

Fig. 3 CircPTPRM silencing suppresses HCC cell proliferation and invasion. **a** qRT-PCR analysis of circPTPRM expression in 80% and 100% confluent cells. **b** qRT-PCR analysis of circPTPRM transfection efficiency in HCC cells. **c** MTT assays to evaluate the proliferation of HCC cells after circPTPRM-siRNA transfection. **d** Colony-formation analysis of HCC cells after treatment with circPTPRM-siRNA. **e** Transwell chamber assays of HCC cells after treatment with circPTPRM-siRNA. Data are mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ VS controls. HCC, hepatocellular carcinoma; qRT-PCR, real-time quantitative reverse transcription-polymerase chain reactions

In conclusion, we demonstrate that circRNA was differentially expressed in HCC tissue. circPTPRM was significantly upregulated in HCC and may function in tumorigenesis and metastasis. This provides the first reported association of circPTPRM with disease, and provides new insight into the molecular pathogenesis of HCC.

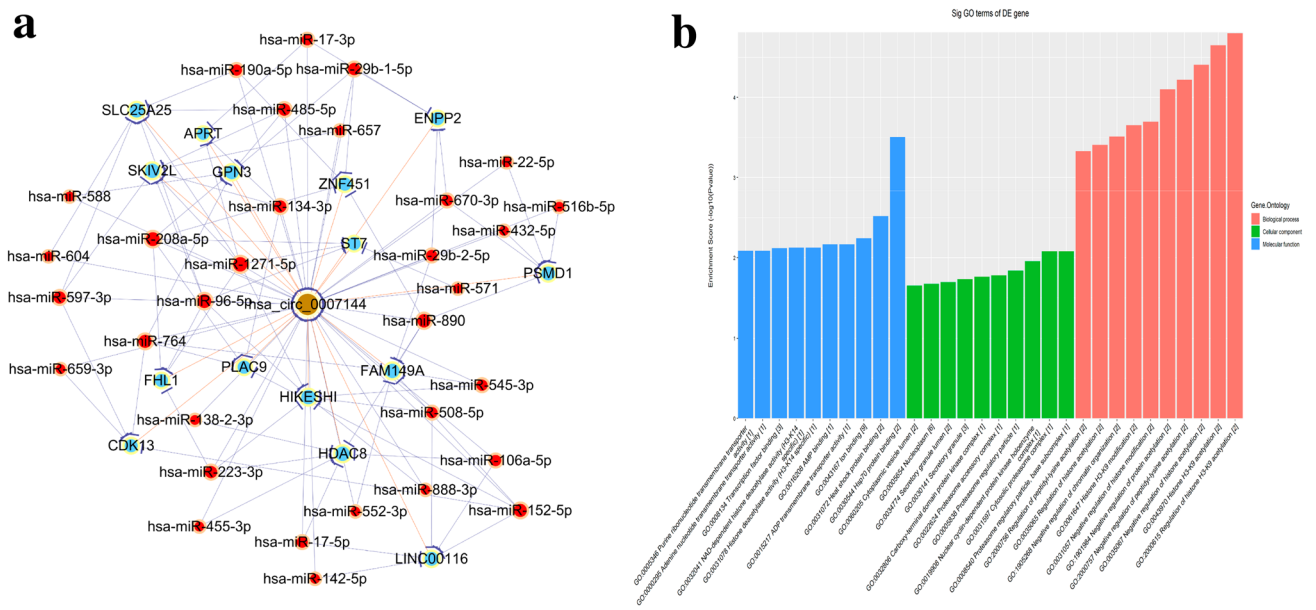

Fig. 4 CeRNA and GO analysis of CircPTPRM. **a** miRNAs related to circPTPRM were assessed using ceRNA analysis. **b** Bar charts of the top 10 GO items. *P* values are ordered from low to high. *Y* axis: *p* values (−log10). GO, gene ontology

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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