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ORIGINAL RESEARCH

Circular RNA circRASSF5 Functions as an Anti-Oncogenic Factor in Hepatocellular Carcinoma by Acting as a Competitive Endogenous RNA Through Sponging miR-331-3p

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Objective: Recently, emerging studies have validated that circular RNAs participate in multiple biological progresses in various human malignant tumors, including hepatocellular carcinoma (HCC). However, until now, the elucidated mechanism of circular RNAs is only the tip of the iceberg. In this study, we firstly identify a novel circular RNA circRASSF5 (the only circular RNA derived from the *RASSF5* gene), and attempt to investigate its biological function and underlying mechanism in HCC.

Methods: qRT-PCR, Western blotting and IHC were applied to detect the expression of related genes. CCK-8 assay, EdU staining, wound healing and transwell assays were used to investigate HCC proliferation, migration and invasion abilities. Animal model studies were included to investigate the function of circRASSF5 in HCC tumorigenesis and metastasis. RNA pull-down assay, luciferase reporter assay and FISH (fluorescence in situ hybridization) assay were performed to explore the potential biological mechanism underlying circRASSF5 function in HCC.

Results: CircRASSF5 is obviously downregulated in both HCC tissues and cell lines. Low level of circRASSF5 is negatively associated with larger tumor size, severe vascular invasion, more portal vein tumor embolus and unfavorable prognosis. Loss-of-function assay reveals that circRASSF5 remarkably impedes the growth and metastasis of HCC cells in vitro and in vivo. Mechanistically, circRASSF5 directly interacts with miR-331-3p as a sponge, and then enhances the expression of PH domain and leucine-rich repeat protein phosphatase (PHLPP), thus restraining the progression of HCC cells.

Conclusion: Altogether, we validate that circRASSF5 is a tumor suppressor in HCC, which competitively sponges with miR-331-3p and then enhances the tumor inhibitory effect of PHLPP, indicating the potential application value of circRASSF5 for HCC diagnosis and clinical treatment.

Keywords: circRASSF5, miR-331-3p, PHLPP, HCC

Introduction

Hepatocellular carcinoma (HCC) is pathologically characterized by high degree of malignance and clinically characterized by later state when diagnosis, high degree of cirrhosis and high recurrence rate, which ranks the fifth cancer-caused death and presents an increasing trend world widely.[1–3] Despite great improvements have been achieved in the remedy strategies (surgery resection, liver transplantation, interventional therapy, chemotherapy, immunotherapy, etc) for HCC, the prognosis is still poor owing to the heterogeneity of HCC.[4–7] Collectively, as is now well known, the microenvironment of HCC is complex and diverse, tumors grow and evolve through a constant crosstalk with the surrounding microenvironment, and emerging evidence indicates that angiogenesis and immunosuppression frequently

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occur simultaneously in response to this crosstalk.[8,9] Accordingly, strategies combining anti-angiogenic therapy and immunotherapy seem to have the potential to tip the balance of the tumor microenvironment and improve treatment response. In addition, HCC is an extremely complicated disease regulated by multiple genes, for example, the unlimited activation and amplification of oncogenes or the abnormal inactivation and deficiency of tumor anti-oncogenes.[10,11] Consequently, there is an active demand for figuring out the molecular mechanism beneath the pathophysiology in depth and developing new early diagnosis biomarkers or effective therapeutic approaches for HCC.

As a sort of novel non-coding RNAs, circular RNAs (circRNAs) are characterized by a covalently closed-loop structure without 5′ cap and 3′ polyadenylation tail.[12,13] Growing data have validated that circRNAs exert multilevel functions in the expression of genes, including gene transcription and translation, RNA–protein interactions and subcellular location of proteins.[14,15] In addition, circRNAs can develop their biological functions by sponging microRNAs, RNA-binding proteins or coding proteins.[16] Furthermore, emerging researchers have revealed that circRNAs participate in tumor genesis and development by controlling the stemness, differentiation, proliferation, invasion and other biological properties of cancer cells.[17] For instance, circular RNA CDR1as inhibits the tumorigenesis of glioma via disrupting the p53/MDM2 complex.[18] Circular RNA circ-ZKSCAN1 depressed the proliferation and metastasis of bladder cancer through miR-1178-3p/p21 axis.[19] circSEPT9 promoted the occurrence and progression of tumor in triple-negative breast cancer, which was regulated by E2F1 and EIF4A3 through circSEPT9/miR-637/LIF axis.[20] In HCC, circular RNA circRHOT1 could intensify the proliferation and metastatic capacity of tumor cells by recruiting TIP60 to the promoter of NR2F6 thus enhancing its transcription.[21] Recently, a few circRNAs have been identified to have a hand in the tumorigenesis and progression of HCC.[22] However, when it comes to the biological process and functions, underlying mechanisms and potential clinical role of circRNAs implicated in HCC, it still remains uncovered.

In our present research, we firstly identified a bran-new circular RNA circRASSF5 (hsa_circ_0016242), which is derived from exons 2 to 5 of *RASSF5* gene, and explored its expression pattern, physiopathological role, underlying mechanism and potential value for clinical applications in HCC. Expression of circRASSF5 was obviously down-regulated in HCC tissues and cell lines, when compared with the adjacent non-tumorous tissues (ANT) and normal liver cell line. The down-regulation of circRASSF5 in HCC tissues was negatively correlated with multiple tumor features, such as larger tumor size, severe vascular invasion, more vital vein tumor embolus and poorer long-term prognosis in HCC patients who underwent hepatectomy. A series of in vitro and in vivo functional experiments validated that circRASSF5 could restrain the proliferation, migration, invasion and metastasis abilities of HCC cells, which indicates that circRASSF5 is a tumor suppressor in HCC. Mechanistically, circRASSF5 was proved to function as a sponge of oncogenic miR-331-3p, and enhance the expression of PH domain and leucine-rich repeat protein phosphatase (PHLPP). In conclusion, circRASSF5 may serve as an anti-oncogenic factor in the development of HCC, and reduced circRASSF5 expression may provide a promising channel for early diagnosis, long-term prognosis prediction and potential therapeutic methods development in clinical HCC treatment.

Materials and Methods

HCC Samples

HCC samples were acquired from 172 pathologically diagnosed patients at Nanjing Drum Tower Hospital, Clinical College of Nanjing Medical University (Nanjing, Jiangsu, China), which were composed of tumor tissues and matched adjacent nontumorous tissues. Each histopathological diagnose of HCC was confirmed by at least three experienced pathologists independently. Before the usage of each tissue, written consent approval was obtained from each patient. This study was approved by the ethics committee of Nanjing Drum Tower Hospital, Clinical College of Nanjing Medical University (Nanjing, Jiangsu, China) and conducted following the ethical principles for medical research involving human subjects of the Helsinki Declaration.

Cell Culture

Human HCC cell lines including HepG2, Huh7, SMMC-772, MHCC-97H and Hep3B, the human normal liver cell line L02 and HEK293T cell line were acquired from KeyGen (Nanjing, Jiangsu, China). In this study, all cells were cultured in DMEM, which was supplemented with 10% FBS (Gibco), and were tested for mycoplasma contamination.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from HCC tumorous and paired adjacent non-tumorous liver tissues mentioned above and cultured cells, and TRIZOL reagent (Invitrogen, CA, USA) was used in accordance with the instructions from manufacturer. Complementary NDA (cDNA) Synthesis SuperMix (TransGen Biotech, China) was applied for reverse transcription, and then SYBR Green real-time PCR kit (Toyobo, Japan) was used for real-time PCR. The primers used in our present research are listed in Table S1. We selected GAPDH as the internal control to detect the mRNA expression of circRASSF5, RASSF5, PHLPP1 and PHLPP2, and U6 as the control for miRNAs expression.

Vector Construction and Cell Transfection

Small hairpin RNAs (shRNAs) of circRASSF5 and circRASSF5-overexpressing plasmids were synthesized by GenePharma (Shanghai, China). circRASSF5-overexpressing plasmids were transfected into Hep3B and SMMC-7721 cells, while circRASSF5 shRNAs were transfected into MHCC-97H and Huh7 cells, and the transfection efficiency was presented in Figure S2D and E. The miRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China), and the transfection efficiency was presented in Figure S3D and E. The sequences of the mimics and inhibitors and control miR-331-3p are listed in Table S2. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfections according to the protocols.

Cell Proliferation, Migration and Invasion Assays

In this study, we used Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) and EdU Assay Kit (Guangzhou RIBOBIO, Guangzhou, China) to examine the growth curve of HCC cells according to the manufacturer's instructions. Two hundred-microliter pipette tips (time 0h) were used to make wounds of cells in the middle of the six-well plates, which were then cultured with serum-free medium. Then, the researchers photographed cell migration after 48h, and measured the distance by normalizing to the 0h control for comparison. Chambers (8 μm pore size, Costar) with Matrigel (BD Science, USA) were utilized for cell invasion assay. Then, 5×10^4 cells were suspended in 200 μL medium which was serum-free, and then added to the upper chambers. DMEM medium (600 μL) which contained 10% FBS was placed into the bottom chambers as a chemoattractant. Then we stained the cells migrated to the other side of the membrane with 0.1% crystal violet solution after incubation at 37 °C for 24 hours, and calculated with the help of a microscope (Olympus, Japan).

Bioinformatics Analysis

For the specificity of circRASFF5, bioinformatics retrieval was conducted in the circBase (http://www.circbase.org), Circular RNA Interactome (https://circinteractome.nia.nih.gov/) and circbank (http://www.circbank.cn). Bioinformatic database CircInteractome and miRanda were used to predict the miRNA-binding sites of circRASSF5. miRanda was used to predict the interaction between mRNA and miRNA. Filtering restrictions were as follows: Context Sore ≥90.

RNA Fluorescence in situ Hybridization (FISH) Assay

We first designed and obtained the Cy5-labeled circRASSF5 probe and Cy3-labeled miR-331-3p probe from GenePharma (Shanghai, China), which were listed in Table S3. Fluorescent in situ hybridization kit (GenePharma, China) was used to detect the signals of the probe in accordance with the manufacturer's protocols. Images were acquired by means of ZEISS LSM800 Confocal Microscope system (Carl Zeiss AG, Germany).

RNA Pull-Down Assay

Biotin-labeled circRASSF5 and control probes were synthesized. After washing in ice-cold phosphate-buffered saline (PBS), cells (about 1×10^7) were lysed in lysis buffer and incubated at room temperature with 3 μg biotinylated probes for 2h. By incubating the streptavidin magnetic beads (Life Technologies, USA) with cell lysates for another 4h, the biotin-coupled RNA complex was pull-downed. Then, we washed the beads with lysis buffer for five times and extracted the bound miRNAs in the pull-down materials with the help of Trizol reagent. qRT-PCR assay was conducted next to analyze the bound miRNAs. The sequences of biotin-labeled circRASSF5 and control probes are presented in Table S3.

Western Blot Assay and Antibodies

For Western blot assay, cells and tissues were lysed with RIPA lysis buffer to obtain protein, which was then separated by SDS-PAGE gels and transferred to PVDF membranes. Then, 5% skim milk in TBST was used to block the membranes, which were incubated with indicated primary antibodies overnight at 4°C, and then secondary horseradish peroxidase (HRP)-conjugated secondary antibodies. We respectively obtained antibodies against PHLPP1 and PHLPP2 from Proteintech (Rosemont, IL, USA) and Bethyl Laboratory (Montgomery, TX, USA), and antibody against GAPDH from Abcam. Chemiluminescent Imaging System (Thermo Scientific, IL, USA) was used to detect protein expression signals. The band intensity was analyzed by Image Gel software. The intensity of bands was shown in the linear range although some bands were overexposed. The whole Western blot images showing all bands and molecular weight markers in our present study are presented in Figure S4.

Luciferase Reporter Assay

The sequences of circRASSF5 and PHLPP-3' UTR and their corresponding mutant versions without miR-331-3p binding sites were synthesized and inserted in to pGL3-Firefly-Renilla vectors, which were obtained by GenePharma Co. (Shanghai, China), termed circRASSF5-WT, circRASSF5-Mut, PHLPP 3'UTR-WT and PHLPP 3'UTR-Mut, respectively. And then the associated vectors and miR-331-3p mimic or circRASSF5 overexpression vector were co-transfected in HEK-293T cells using the Lipofectamine 3000 transfection reagent, respectively. Forty-eight hours after transection, cells were harvested and the luciferase activities were detected by a dual luciferase-reporter system (Promega, USA).

Animal Study

Tumor subcutaneous xenograft model was constructed by using 6-week-old male nude mice with BALB/c background for further examination of the biological function of circRASSF5 on tumor growth in vivo. Mice were subcutaneously injected with approximately 5×10^6 Hep3B cells into the left or the right flanks, which were transfected with indicated vectors in advance. After 4 weeks, mice were sacrificed and the tumors were separated from mice. We then calculated the volume of each tumor (length × width^2 × 0.5). In lung metastasis model, MHCC-97H cells were intravenous injected through the tail after suspending in 200μL PBS. After 6 weeks, researchers sacrificed the mice and stained the lungs with hematoxylin and eosin and calculated the number of metastases. All animal experimental protocols were approved by the Animal Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School and the Animal Ethics Committee of Nanjing Medical University in compliance with the Guideline for Ethical Review of Animal Welfare, China (GB/T 35892–2018).

Statistical Analysis

All data in this research were analyzed with two-tailed Student's *t* test or one-way ANOVA system, followed by post hoc *t* tests. Data are finally exhibited as mean ± SD unless otherwise noted. Significance was determined at $p \leq 0.05$. Statistical analysis was conducted by GraphPad Prism Version 9.0. Kaplan–Meier method was utilized to assess overall survival (OS) and recurrence-free survival (RFS) and the significance was established by Log rank test. A Cox proportional hazards regression model was used to identify independent prognostic factors associated with OS and RFS. *P* value <0.05 was regarded as statistical significance.

Results

Dysregulated RASSF5-Derived circRASSF5 in HCC

Studies have revealed that RASSF5 is down-regulated in various cancers,[23] including HCC,[24,25] and exerts tumor-suppressing role by inhibiting the tumorigenesis and progression of tumors through multilevel cellular biological regulation. The aberrant down-regulation of RASSF5 in HCC was reported to be caused by the hypermethylation of CpG islands in the promoter of RASSF5.[23] Thus, we proposed that circular RNA derived from RASSF5 might also be downregulated in HCC tissues and cell lines, in addition, it might exert tumor-suppressing biological function in HCC. As shown in Figure S1A–C, through bioinformatics retrieval in the circinate databases (circBase: http://www.circbase.org; Circular RNA Interactome: https://circinteractome.nia.nih.gov/; circbank: http://www.circbank.cn), we found that there was only one circular RNA (hsa_circ_0016242) derived from the *RASSF5* gene. Thus, we named hsa_circ_0016242 (circRNA derived from exons 2 and 5 of the RASSF5) as circRASSF5 (Figure 1A) and proposed to explore its expression pattern and potential biological function in HCC. Firstly, we investigated the stability of circRASSF5 in HCC cells, as shown in Figure S1D, RASSF5 mRNA was degraded by RNase R, while circRASSF5 harbors a loop structure with the resistance to the degradation of RNase R. As presented in Figure S1E, Actinomycin D assay was conducted to investigate the stability of circRASSF5, which demonstrated that circRASSF5 has preferable stability than the linear RASSF5 mRNA transcript in MHCC-97H and Hep3B cells. Next, we detected circRASSF5 expression in HCC tissues, and validated that circRASSF5 was remarkably downregulated in HCC tissues compared with adjacent non-tumorous tissues (ANT) (Figure 1B). As presented in Figure 1C, circRASSF5 was remarkedly downregulated in HCC cell lines (HepG2, Huh7, SMMC-772, MHCC-97H and Hep3B) compared with normal liver cell L02. In addition, circRASSF5 level in HCC patients with tumor size >5cm was lower compared with that in patients with tumor size ≤5cm (Figure S2A). Compared with the level of circRASSF5 in patients without vascular invasion, it was further down-regulated in patients with vascular invasion (Figure S2B). Moreover, circRASSF5 level in HCC patients with PVTT (portal vein tumor thrombus) was obviously lower than those without PVTT (Figure S2C). Further clinicopathological feature analysis indicated that lower level of circRASSF5 was negatively related with tumor size (Table 1, $P = 0.003$), vascular invasion (Table 1, $P = 0.019$) and PTVV (Table 1, $P = 0.010$). Furthermore, we also investigated the subcellular location of circRASSF5 in HCC cells by FISH assay, and revealed that circRASSF5

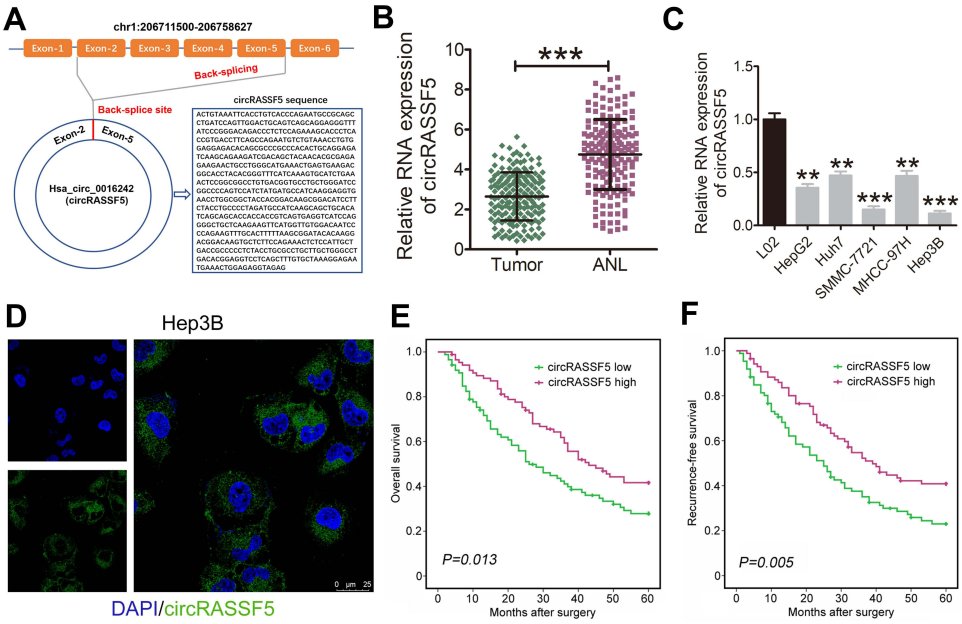

Figure 1 Dysregulated RASSF5-derived circRASSF5 in HCC. (**A**) The scheme illustrating the production of circRASSF5. CircRASSF5 derived from back-splicing of exons 2–5 of *RASSF5* gene and the mature sequence of circRASSF5 is 647 bp. (**B**) qRT-PCR results of the level of circRASSF5 in HCC tissues and adjacent non-tumorous liver tissues. (**C**) qRT-PCR results of the expression of circRASSF5 in HCC cell lines (HepG2, Huh7, SMMC-772, MHCC-97H and Hep3B) and normal liver cell line (L02). (**D**) FISH assay was applied to explore the subcellular location of circRASSF5 in Hep3B cell (original magnification ×400). Green represents circRASSF5 and blue represents cell nucleus. (**E** and **F**) The overall survival (OS) and recurrence-free survival (RFS) rates of 172 HCC patients were analyzed by Kaplan–Meier and Log rank test analyses between low circRASSF5 expression group and high circRASSF5 expression group. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 1 Correlation Between circRASSF5 Expression and HCC Clinicopathologic Features in 172 Patients

Variable	Number	circRASSF5 Expression Level		*P* value
		High Expression	Low Expression	
All cases	172	86	86	
Gender				0.541
Male	143	70	73	
Female	29	16	13	
Age (years)				0.352
<60	102	48	54	
≥60	70	38	32	
HBsAg				0.470
Negative	40	22	18	
Positive	132	64	68	
Liver cirrhosis				0.201
No	60	34	26	
Yes	112	52	60	
Serum AFP (ng/mL)				0.168
≤20	46	27	19	
>20	126	59	67	
Tumor number				0.360
Single	150	77	73	
Multiple	22	9	13	
Tumor size (diameter, cm)				**0.003**
≤5	75	47	28	
>5	97	39	58	
Vascular invasion				**0.019**
No	69	42	27	
Yes	103	44	59	
Portal vein tumor thrombus				**0.010**
No	141	77	64	
Yes	31	9	22	
Edmondson-Steiner grade				0.201
I/II	133	70	63	
III/IV	39	16	23	

Notes: Total data from 172 HCC patients were analyzed. For the expression of circRASSF5 was assayed by qRT-PCR, the median expression level was used as the cutoff. Data were analyzed by chi-squared test. *P*-values in bold indicate statistically significant.

was chiefly distributed in the cytoplasm of HCC cells (Figure 1D). Last but not least, we explored the prognostic value of circRASSF5 in the HCC patients mentioned above. According to the median expression level of circRASSF5, all patients were separated into circRASSF5 high-level group (n = 86) and circRASSF5 low-level group (n = 86), and the results of Kaplan–Meier and Log rank test analyses indicated that lower circRASSF5 expression was remarkable correlated with poorer overall survival (OS, $P = 0.013$) and recurrence-free survival (RFS, $P = 0.005$) in HCC patients (Figure 1E and F). Furthermore, a univariate analysis showed that OS and RFS were obviously related to tumor number, tumor size, vascular invasion, portal vein tumor thrombus and circRASSF5 expression level (Table S4).

CircRASSF5 Inhibits Proliferation, Migration and Invasion of HCC Cells in vitro

To further explore the potential biological role of circRASSF5 in HCC cells, we selected Hep3B and SMMC-7721 cells to construct circRASSF5 stable overexpression cell lines by transfecting with circRASSF5-overexpressing vector, and silenced circRASSF5 expression in MHCC-97H and Huh7 cells by transfecting RNA interference. As shown in

Figure S2D and E, qRT-PCR assay was applied to detect the overexpression and knockdown efficiencies of circRASSF5. In the circRASSF5 knockdown assay, three sh-RNAs were applied to knock down circRASSF5 in MHCC-97H and Huh7 cells, and sh-circ-2 showed the best knockdown efficiency; thus, we selected it for the following experiments. And then, we investigated the proliferation ability of HCC cell by CCK-8 and EdU assays. The results of these two proliferation assays described above proved that overexpression of circRASSF5 obviously repressed the proliferation ability of Hep3B and SMMC-7721 cells, whereas silence of circRASSF5 remarkably elevated the proliferation ability of MHCC-97H and Huh7 cells (Figure 2A and 7B). Next, we conducted wound healing assay to detect the migration ability of HCC cells. Similarly, circRASSF5 overexpression impeded the migration ability of Hep3B and SMMC-7721 cells, while circRASSF5 knockdown enhanced HCC cell migration ability (Figure 2C). Then, transwell assay was carried out to detect the function of circRASSF5 on the invasion ability

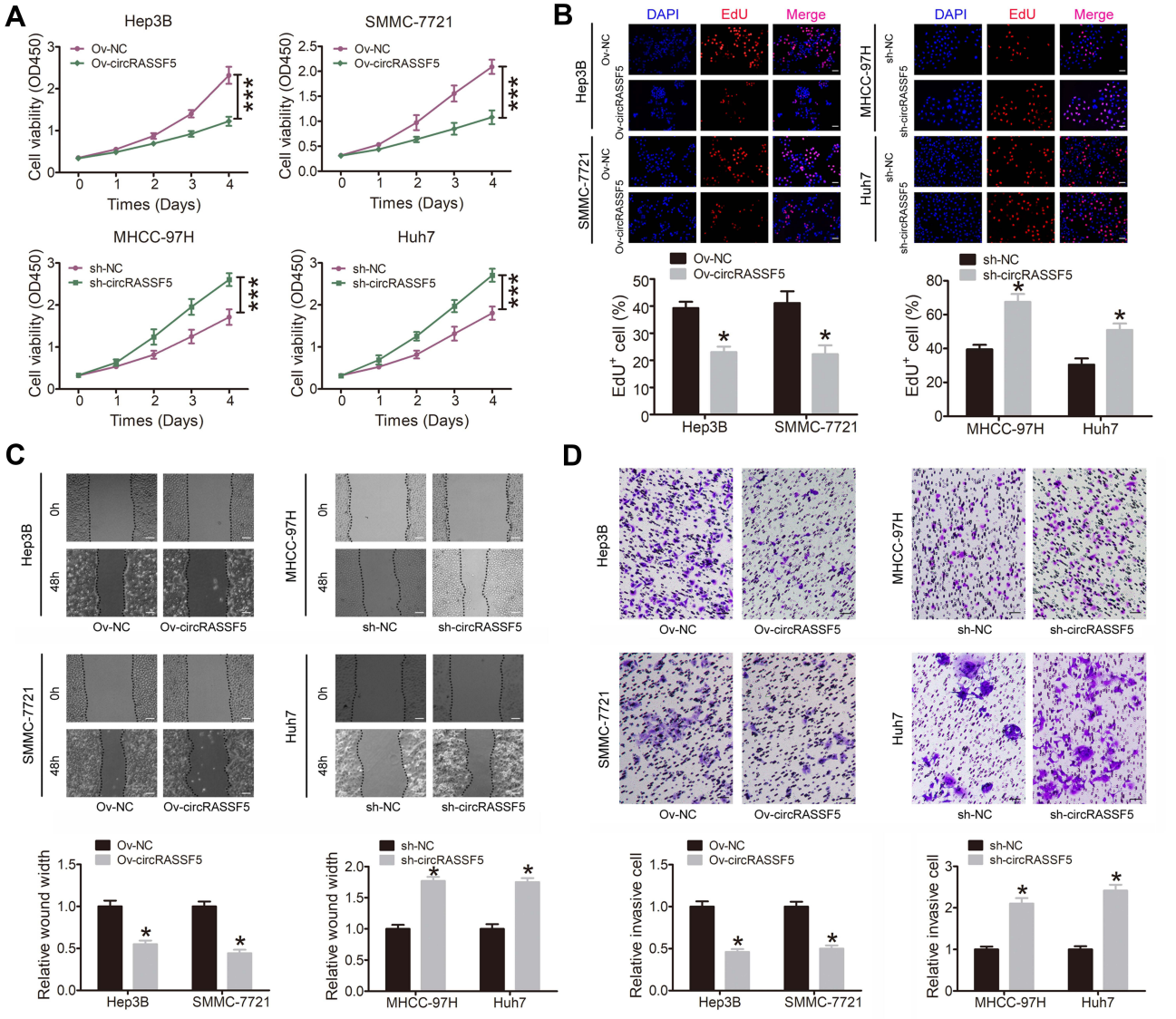

Figure 2 CircRASSF5 suppresses the proliferation, migration and invasion capacities of HCC cells in vitro. (**A**) The proliferation ability of HCC cells was evaluated by CCK-8 assay when circRASSF5 was stably overexpressed or knockdown. (**B**) Proliferation capacity of HCC cells was detected by means of EdU staining assay when circRASSF5 was overexpressed or knocked down (original magnification ×100). (**C**) Wound healing assay was used to assess the migration ability of Hep3B and SMMC-7721 cells after transfected with Ov-circRASSF5 or mock vectors; MHCC-97H and Huh7 cells after transfected with sh-cricRASSF5 or sh-NC vectors (original magnification ×50). (**D**) The invasion ability of Hep3B and SMMC-7721 cells transfected with Ov-circRASSF5 or mock vectors, MHCC-97H and Huh7 cells transfected sh-circRASSF5 or sh-NC vectors were detected via transwell assays (original magnification ×100). Our study performed each experiment in triplicate and exhibited the results as the mean ± SD. *$P < 0.05$; ***$P < 0.001$.

of HCC cells. As presented in Figure 2D, the invasion ability of HCC cells was remarkably restrained when circRASSF5 was overexpressed, whereas circRASSF5 knockdown obviously promoted HCC cells invade. Above all, we suspected that circRASSF5 could exert tumor-suppressing effect on HCC cells by repressing the proliferation, migration and invasion abilities of HCC cells in vitro.

CircRASSF5 Binds to miR-331-3p Directly and Suppresses miR-331-3p Activity

Various researchers have demonstrated that circRNAs could function as miRNAs sponge and then exert vital biological role by regulating their downstream genes.[26] In addition, we have validated that circRASSF5 was primarily distributed in the cytoplasm of HCC cells. Thus, we proposed that the tumor-suppressing effect of circRASSF5 in HCC might be caused by its sponging miRNAs. Here, through bioinformatic prediction by Circinteractome database (https://circinteractome.nia.nih.gov/) and miRanda database (http://mirdb.org/), six candidate target miRNAs (miR-581, miR-331-3p, miR-1299, miR-942, miR-604, miR-516b) were selected as potential targets for the following studies (Figure S3A). To further definite the target miRNAs of circRASSF5 in HCC cells, we constructed biotinylated circRASSF5 probe and the associated RNA pull-down efficiency was validated by qRT-PCR in Hep3B, SMMC-7721, MHCC-97H and Huh7 cells (Figure 3A). Next, the RNA pull-down assay was carried out to further evaluate whether circRASSF5 could directly capture these candidate miRNAs, as shown in Figure 3B, among the six miRNAs, only miR-331-3p was copiously pulled down by circRASSF5 in all four HCC cell lines. To further confirm this sponge interaction, luciferase reporter assays was performed with either the wild-type circRASSF5 sequence (WT) or the mutated predicted binding sites of miR-331-3p (Mut) into the luciferase reporter vector (Figure S3B). After transfection of these vectors in HEK293T cells, we validated that overexpression of miR-331-3p remarkably reduced luciferase reporter activity of WT circRASSF5 reporter but not mutant one (Figure 3C). We also carried out biotin-coupled miRNA capture assay. We transfected biotin-coupled miR-331-3p (biotin-miR-331-3p-WT) or its mut (biotin-miR-331-3p-Mut) into Hep3B and SMMC-7721 cells, which stably overexpressed circRASSF5, and conducted qRT-PCR to examine the level of circRASSF5 which was captured by miR-331-3p. As presented in Figure 3D, results showed that the content of circRASSF5 was obviously higher in biotin-miR-331-3p-WT group compared with that in biotin-miR-331-3p-Mut group, suggesting that miR-331-3p could bind with circRASSF5 as well. Additionally, the result of FISH assays also revealed that the distribution of circRASSF5 and miR-331-3p was nearly coincided in the cytoplasm of HCC cells (Figure 3E). Followed expression detection by qRT-PCR proved that overexpression of circRASSF5 significantly decreased miR-331-3p level, whereas knockdown of circRASSF5 obviously elevated miR-331-3p expression in HCC cells (Figure 3F). However, miR-331-3p failed to influence circRASSF5 expression in HCC cells (Figure 3G). Collectively, we validated that circRASSF5 sponged with miR-331-3p and repressed its level in HCC.

miR-331-3p Exerts an Oncogenic Function in HCC Cells by Targeting PHLPP

Studies have validated that miR-331-3p could exert tumor-promoting function in HCC.[27–29] Similarly, we revealed that miR-331-3p was remarkably overexpressed in HCC cells (Figure S3C). Here, according to the prediction results by the miRanda database (http://mirdb.org/), we found miR-331-3p might target PHLPP 3' UTR with a high score (Figure 4A). Next, we proposed to validate the interaction between miR-331-3p and PHLPP though luciferase report assay. As shown in Figure 4B, we revealed that overexpression of miR-331-3p obviously repressed the activity of luciferase reporter vector carrying the PHLPP 3' UTR-WT sequence but not the Mut group in HEK293T cells. Hence, we proposed that miR-331-3p might participate in the regulation of PHLPP expression in HCC cells. Through qPR-PCR assay, we demonstrated that miR-331-3p down-regulates the mRNA expression of PHLPP1 and PHLPP2 in Hep3B and SMMC-7721 cells (Figure S3F). Meanwhile, the Western blotting assay demonstrated that overexpression of miR-331-3p obviously inhibited the protein level of PHLPP1 and PHLPP2 in HCC cells (Figure 4C). In summary, our research further proved that miR-331-3p could negatively regulate the protein expression of PHLPP in HCC. Additionally, we investigated the influence of miR-331-3p on the biological properties of HCC cells. As shown in Figure S3D and E, we stably constructed miR-331-3p overexpression and down-regulation cell lines in Hep3B and SMMC-7721 cells. The following results of CCK-8 and EdU assays validated that overexpression of miR-331-3p significantly enhanced the proliferation capability of HCC cells (Figure 4D and E). Furthermore, the results of wound

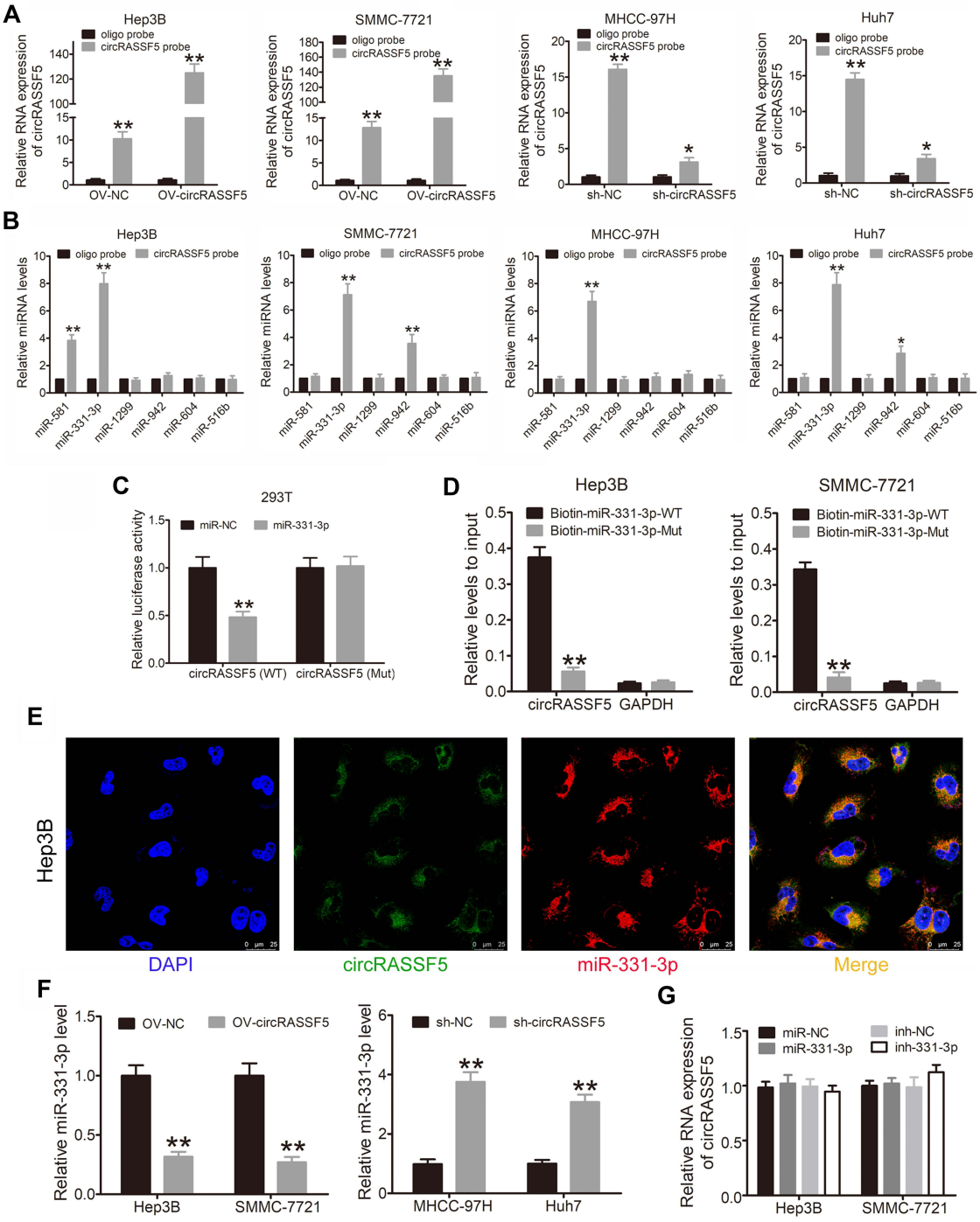

Figure 3 CircRASSF5 directly binds to miR-331-3p and restrains miR-331-3p activity. (**A**) RNA pull-down assay was carried out in Hep3B, SMMC-7721, MHCC-97H and Huh7 cells, and the pull-down efficiency of circRASSF5 probe was verified by qRT-PCR assay. (**B**) The relative expression of 6 potential target miRNAs pull-down by circRASSF5 probe were detected in Hep3B, SMMC-7721, MHCC-97H and Huh7 cells. (**C**) The results of luciferase reporter assay in HEK293T cells after co-transfected with miR-331-3p mimics, circRASSF5 WT or circRASSF5 Mut plasmids. (**D**) Relative levels of circRASSF5 in Hep3B and SMMC-7721 lysates captured by biotinylated wild-type miR-331-3p (Biotin-miR-331-3p-WT) or mutant miR-331-3p (Biotin-miR-331-3p-Mut). (**E**) FISH assay was conducted to investigate the subcellular of circRASSF5 and miR-331-3p in Hep3B cells (original magnification ×400). Nuclei were stained with DAPI. CircRASSF5 was labeled with Cy3 and miR-331-3p was labeled with Cy5. (**F**) The relative miR-331-3p level was investigated by qRT-PCR in HCC cells when circRASSF5 was overexpressed or knockdown. (**G**) QRT-PCR was applied to investigate the levels of circRASSF5 in Hep3B and SMMC-7721 cells when miR-331-3p was overexpressed or knockdown. Our study performed each experiment in triplicate and exhibited the results as the mean ± SD. **P* < 0.05; ***P* < 0.01.

healing and transwell assays indicated that overexpression of miR-331-3p obviously enhanced the migration and invasion abilities of HCC cells (Figure 4F and G). Thus, we suggested that miR-331-3p could target PHLPP and further enhance the progression of HCC partially.

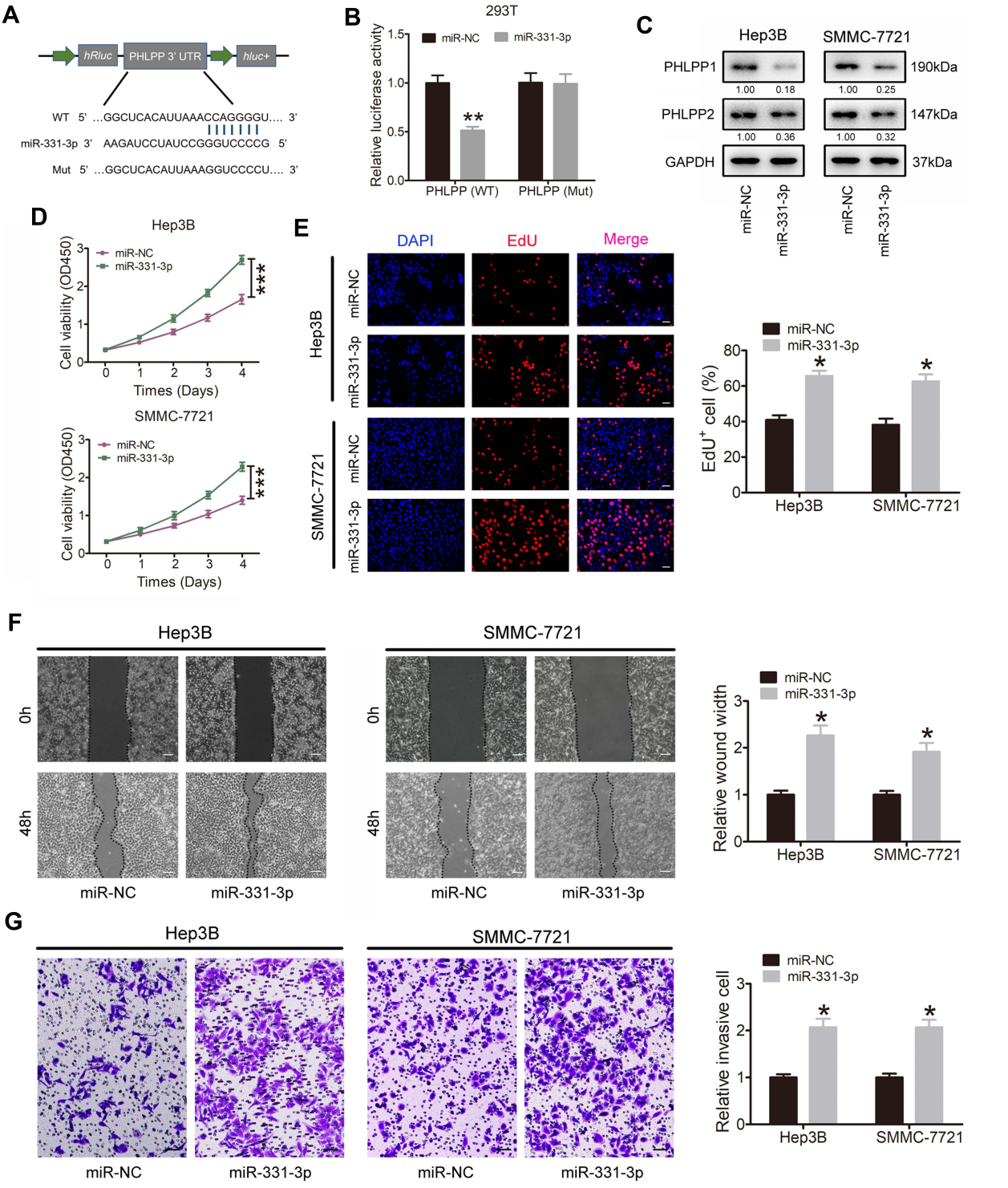

Figure 4 miR-331-3p exerts tumor-promoting effect in HCC cells by targeting PHLPP. (**A**) Schematic of PHLPP 3' UTR WT and Mut luciferase reporter vectors. (**B**) The results of luciferase reporter assay in HEK293T cells after co-transfected with miR-331-3p mimics, PHLPP 3' UTR WT or Mut plasmids. (**C**) Western blot assay was conducted to investigate the expression of PHLPP1 and PHLPP2 in Hep3B and SMMC-7721 cells after transfected with miR-331-3p mimics or control vector. The fold change comparing to miR-NC normalized by GAPDH was listed beneath each band. (**D**) The proliferation ability of HCC cells was assessed by CCK-8 assay when miR-331-3p was stably overexpressed. (**E**) Proliferation capability of HCC cells was detected through EdU staining assay when miR-331-3p was overexpressed (original magnification ×100). (**F**) Wound healing assay was used to investigate cell migration ability in Hep3B and SMMC-7721 cells after transfected with miR-331-3p mimics or mock vectors (original magnification ×50). (**G**) The invasion ability of Hep3B and SMMC-7721 cells transfected with miR-331-3p mimics or mock vectors was detected via transwell assays (original magnification ×100). Our study performed each experiment in triplicate and exhibited the results as the mean ± SD. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

CircRASSF5 Exerts Its Tumor-Suppressing Function Through Regulating miR-331-3p/PHLPP Axis in HCC

In order to further verify how circRASSF5, miR-331-3p and PHLPP interact with each other, luciferase reporter assay was carried out in our study. The experimental results indicated that overexpression of circRASSF5 remarkably enhanced the luciferase reporter activity, whereas co-transfection of circRASSF5 and miR-331-3p eliminated this effect with PHLPP 3' UTR wild-type sequence (WT), which vanished away with mutated binding sites of miR-331-3p (Mut) (Figure 5A). In addition, it was suggested that circRASSF5 could remarkably increase the protein level and mRNA expression of PHLPP1 and PHLPP2, whereas co-transfection of circRASSF5 and miR-331-3p eliminated this effect in Hep3B and SMMC-7721 cells (Figures 5B and S3G). Thus, we proposed that the tumor-suppressing function of circRASSF5 might be achieved through the miR-331-3p-PHLPP pathway. To verify this presumption, a series of rescue experiments were carried out. As shown in Figure 5C, we found that the proliferation ability of HCC cells was repressed when circRASSF5 was overexpressed, whereas overexpression of miR-331-3p could eliminate the suppressing effect caused by circRASSF5. Similarly, up-regulation of miR-331-3p could also eliminated the inhibitory effect on the migration and invasion abilities in Hep3B and SMMC-7721 cells (Figure 5D and E). Collectively, we proposed that the repressing effect of circRASSF5 on the proliferation, migration and invasion abilities of HCC cells was acquired by sponging miR-331-3p and then elevating the expression of PHLPP.

CircRASSF5 Inhibits the Growth and Metastasis of HCC in vivo

In the interest of investigating the biological function of circRASSF5 on HCC, we established in vivo tumor growth and metastasis experiments. As presented in Figure 6A–C, we subcutaneously injected circRASSF5 overexpression Hep3B and the control cells in nude mice, and found that tumor xenografts derived from circRASSF5 over-expressing cells growth slower and gotten less weight compared with the control cells. We also performed Ki67 and PCNA staining and detected the positive rates in the xenograft tumor tissues by immunohistochemistry assay (IHC). The overexpression of cirRASSF5 obviously reduced the proportion of Ki-67 and PCNA positive staining cells in Hep3B cells (Figure 6D). Next, in order to further investigate the suppressing effect of circRASSF5 on the metastasis ability of HCC in vivo, we injected circRASSF5 stably knockdown MHCC-97H cells via mice tail vein to construct mice lung metastasis models. After 6 weeks, we sacrificed the mice to isolated the metastases and then calculated. Obviously, H&E staining assay confirmed that knockdown of circRASSF5 significantly increased the metastatic nodes in the lung of mice (Figure 6E). In summary, we suggest that circRASSF5 inhibits HCC growth and metastasis in vivo.

Discussion

With the great progression of bioinformatics predictive and detection methods, a plethora of circRNAs have been discovered and certified to play vital physiopathological functions during the carcinogenesis and progression of various tumors,[30] including HCC.[31] However, fully explored HCC-related circRNAs are just tip of the iceberg, more efforts need to be made to further identify and clarify bran-new HCC associated circRNAs. Several researchers have pointed out that RASSF5 was a tumor suppressor in various tumors,[23] including HCC.[24,25] In liver cancer, RASSF5 was validated to be epigenetically silenced by the hypermethylation of its promoter.[25] Thus, we proposed circRNAs derived from RASSF5 might also be down-regulated and might exert a tumor-suppressing effect in HCC. Herein, we gradually ascertained the expression patterner of circRASSF5 and its underlying molecular mechanism in HCC through comprehensive functional and molecular biological experiments. So far as we know, our research is the first discovery and exploration of the function of circRASSF5 in HCC. Remarkably, we gotten a tremendous decrease of circRASSF5 level both in HCC tissues and in HCC cell lines, indicating the tumor-suppressing effect of circRASSF5 in HCC. Analysis between clinicopathological features and circRASSF5 expression level in HCC patients revealed that low expression of circRASSF5 was negatively associated with tumor size, vascular invasion and portal vein tumor thrombus. In addition, Kaplan–Meier and Log rank test tests validated the consistency between circRASSF5 expression and survival rates of HCC patients, including overall survival and recurrence-

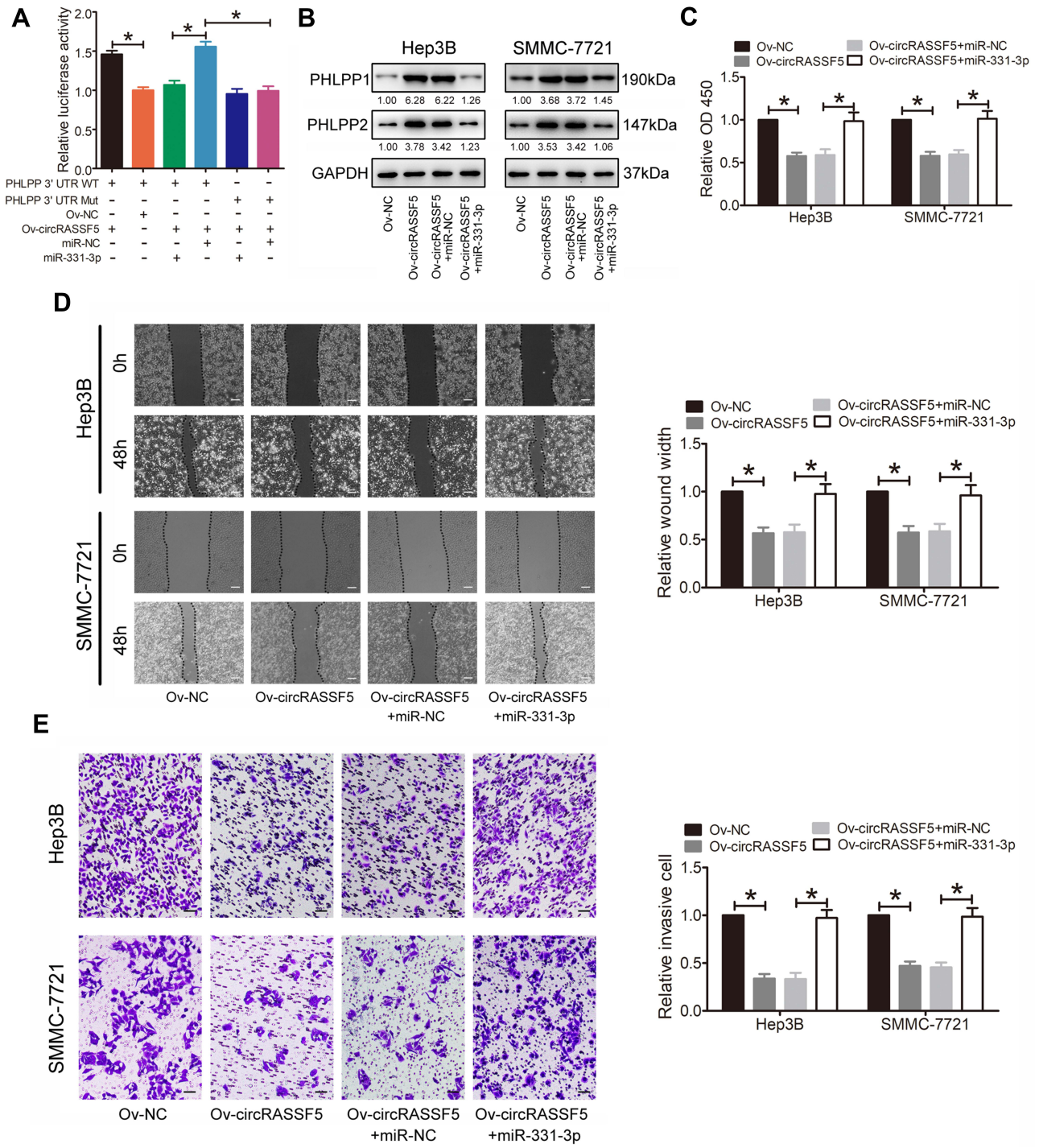

Figure 5 CircRASSF5 exerts its tumor-suppressing function through sponging with miR-331-3p and then strengthening PHLPP expression in HCC cells. (**A**) HEK293T cells were co-transfected with circRASSF5 vector or Mock, miR-331-3p mimics or miR-NC and luciferase reporter vector PHLPP 3' UTR-WT or PHLPP 3' UTR-Mut. And then we detected the relative luciferase activities of each group. (**B**) Western blot assay was applied to evaluate the protein levels of PHLPP1 and PHLPP2 in Hep3B and SMMC-7721 cells which were co-transfected with Ov-circRASSF5 or mock vector and miR-331-3p mimics or miR-NC. The fold change comparing to Ov-NC normalized by GAPDH was listed beneath each band. (**C**) The proliferation ability of HCC cells was detected by CCK-8 assay after co-transfected with Ov-circRASSF5 and miR-331-3p mimics. (**D** and **E**) The migration and invasion abilities of HCC cells were evaluated by wound healing and transwell Matrigel invasion assays after co-transfection of Ov-circRASSF5 and miR-331-3p mimics. Our study performed each experiment in triplicate and exhibited the results as the mean ± SD. *$P < 0.05$.

free survival. In vitro and in vivo functional experiments investigation revealed that circRASSF5 could suppress the proliferation, migration and invasion abilities of HCC cells. All in all, our finding demonstrated the tumor-suppressing function of circRASSF5 in HCC for the first time.

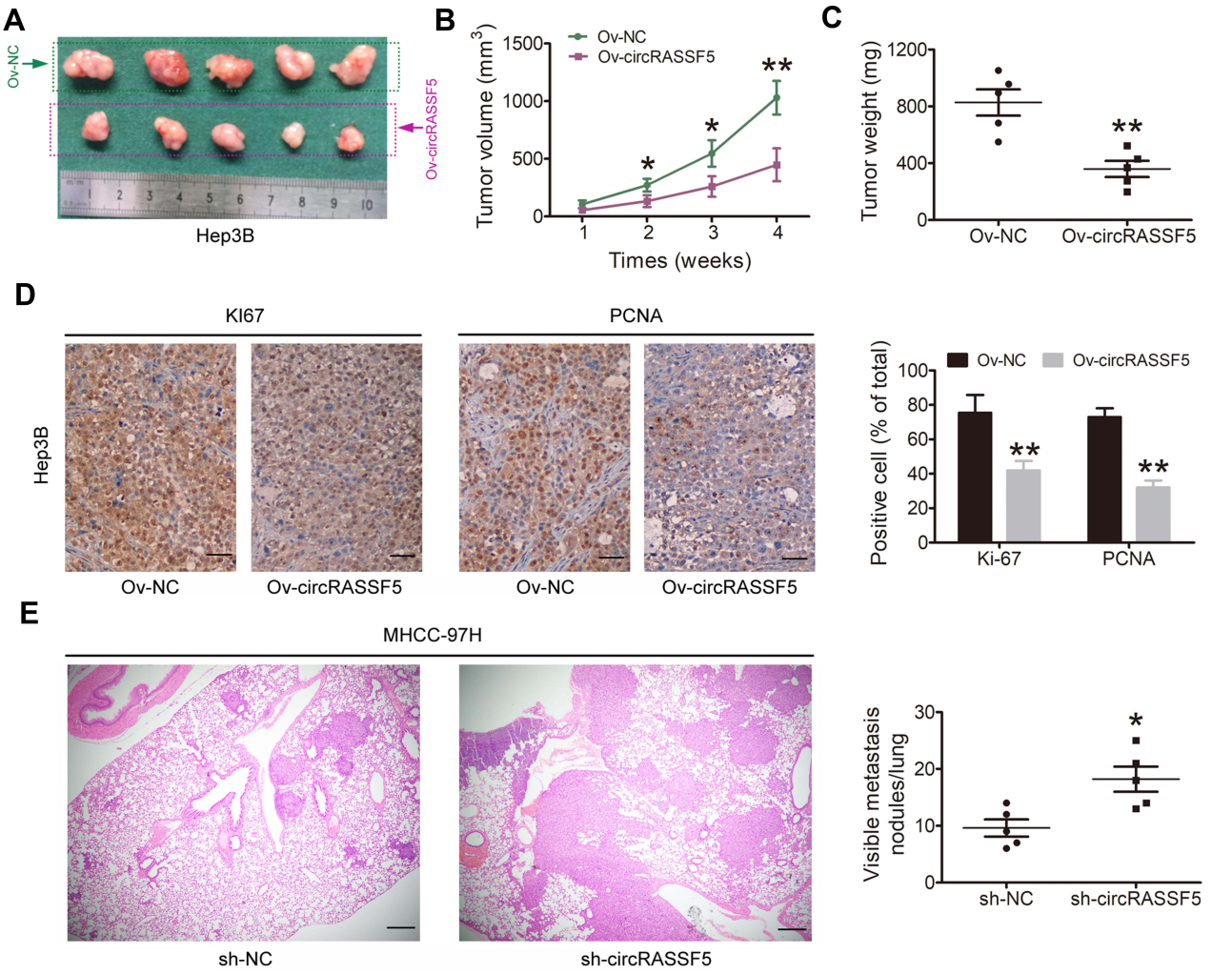

Figure 6 CircRASSF5 inhibits the growth and metastasis of HCC in vivo. (**A**) We applied BALB/c nude mice to construct the subcutaneous xenograft model by subcutaneously injected with circRASSF5 overexpression Hep3B cells and the control cells when mice were 6-week-old (5 mice in each group). Four weeks later, mice were sacrificed and the tumor size was measured. (**B**) We measured the tumor volumes once a week, and the growth curves of them in each group were analyzed and presented. (**C**) The relative weight of each tumor was calculated and analyzed between two groups. (**D**) Immunohistochemistry (IHC) assay was conducted to evaluate the ratio of Ki-67 and PCNA positive cells in the tumor tissues of subcutaneous xenografts (original magnification ×100). (**E**) A lung metastasis model was established by injecting MHCC-97H cells into mice through tail vein. And then the lung colonization was evaluated by the histological examination (H&E) (original magnification ×100). The results are shown as the mean ± SD. *$P < 0.05$; **$P < 0.01$.

Growing evidence have validated that circRNAs may interact with miRNA as sponges, and then participate in the regulation of the biological effect caused by miRNAs.[32] For example, circ-CDYL sponges miR-892a and miR-328-3p and then inhibits the expression of HDGF and HIF1ANN, thus promoting the carcinogenesis of HCC.[33] Circ-ZKSCAN1 impedes the development of bladder cancer by participating in the miR-1178-3p/p21 axis.[19] In lung cancer, circPRKCI could sponge both miR-545 and miR-589 and abolish their suppressive effect on E2F7.[34] CircTP63 was reported to binds with miR-873-3p and then eliminate the suppression of miR-873-3p on FOXM1, thus enhancing the cell cycle progression of lung squamous cell carcinoma.[35] In addition, various researches have indicated that the cytoplasmic location of circRNAs was an important condition for sponging miRNAs. In our present study, we demonstrated that circRASSF5 was chiefly distributed in the cytoplasm of HCC cells by FISH assay. Therefore, we proposed that there might exist miRNAs which could be sponge by circRNASSF5. Here, taken the results of bioinformatics analysis, RNA pull-down, dual-luciferase reporter and FISH assays together, it was revealed that circRASSF5 could bind to miR-331-3p in HCC cells. Studies have validated that miR-331-3p could function as tumor-promoting factor in various human cancers. In our present research, we confirmed the tumor-promoting effect of miR-331-3p in HCC cells. Furthermore, through bioinformatic prediction, dual-

luciferase reporter and Western blotting assays, we validated that miR-331-3p could sponge 3' UTR of PHLPP and then enhanced the protein level of PHLPP in HCC cells. In addition, PHLPP has been identified as a tumor suppressor factor in various cancers,[36–39] especially in HCC.[29,40] On the basis of ceRNA hypothesis, circRNAs might function as a ceRNA and then regulate the expression of miRNA target gene by sponging miRNAs. Here, we observed that overexpression of circRASSF5 significantly upregulated PHLPP protein level. Furthermore, this effect could be abolished by miR-331-3p overexpression. In addition, rescue experiments indicated that miR-331-3p overexpression could eliminate the tumor-suppressing function of circRASSF5, which further validated our hypothesis that circRASSF5 might act as a ceRNA to enhance PHLPP-mediated tumor-suppressing effect via decoying miR-331-3p in HCC.

As the predominant liver malignancy, HCC is always characterized by dysregulation of several crucial cellular signaling pathways such as PI3K/p-Akt/mTOR/NF-κB and VEGF/VEGFR2 pathways.[41] Novel therapies targeting these pathways have been discovered such as regorafenib, which has been examined as novel second-line agents in the treatment of HCC with promising results.[8,9] The PHLPP is a member of the Serine/Threonine protein phosphatase family and consists of two isoforms, PHLPP1 and PHLPP2. PHLPP has been validated to function as negative regulators of PI3K/AKT signal in HCC by acting as a protective factor in the process of tumor metastasis and resistance.[29,40] In our study, we demonstrated that low expression of circRASSF5 was associated with advanced HCC characteristics. Moreover, we also validated that circRASSF5 enhanced the expression of PHLPP. Thus, we proposed that circRASSF5 might function as vital regulator in the treatment efficiency of regorafenib on HCC, which was not yet been investigated in the current study and should thus be addressed in future research.

Conclusions

In summary, our research firstly revealed the aberrant down-regulation of circRASSF5 in HCC tissues and cell lines, validated that lower level of circRASSF5 indicated larger tumor size, more severe vascular invasion, more portal vein tumor thrombus and poorer survival in HCC patients. In addition, we cleared that circRASSF5 sponged miR-331-3p and then strengthen the expression of PHLPP, hence depressed the tumorigenesis and development of HCC. Thus, we proposed that the regulatory network involving circRASSF5/miR-331-3p/PHLPP axis might be a potential therapeutic target for HCC treatment.

Abbreviations

HCC, hepatocellular carcinoma; ANL, adjacent noncancerous liver tissues; circRNAs, circular RNAs; miRNA, microRNA; PHLPP, PH domain and leucine-rich repeat protein phosphatase; CCK-8, Cell Counting Kit-8; FISH, fluorescence in situ hybridization; OS, overall survival; qRT-PCR, quantitative real-time polymerase chain reaction; RFS, recurrence-free survival; RIP, RNA immunoprecipitation.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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