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ORIGINAL RESEARCH

RETRACTED ARTICLE: Circular RNA ZNF609 Promoted Hepatocellular Carcinoma Progression by Upregulating PAP2C Expression via Sponging miR-342-3p

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Background: Emerging evidence has revealed that circular RNAs (circRNAs) participated in hepatocellular carcinoma (HCC) development. However, the roles of most circRNAs have not been studied.

Methods: CircZNF609, miR-342-3p and RAP2C expressions were assessed by qPCR or Western blot. Loss-of-function experiments were performed using si-circZNF609 transfection, followed by CCK-8 assay, flow cytometry, wound healing assay and transwell assay. Informatic tools and rescue experiments were carried out to investigate the underlying mechanisms.

Results: We showed that circZNF609 was overexpressed in HCC tissues and cells, as well as associated with poor clinical characteristics. Depletion of circZNF609 restrained HCC cell viability, migration and invasion while enhanced cell apoptosis. As to mechanism, miR-342-3p was sponged by circZNF609, and RAP2C was targeted by miR-342-3p. The effects on HCC cells induced by si-circZNF609 could be reversed by miR-342-3p inhibitor or RAP2C. In vivo, circZNF609 knockdown inhibited tumorigenesis of HCC mice, confirming the findings in vitro.

Conclusion: CircZNF609 was highly expressed in HCC tissues and driven HCC progression by sponging miR-342-3p and upregulating RAP2C. This study may provide new potential therapeutic targets for HCC treatment.

Keywords: hepatocellular carcinoma, circular RNA, circZNF609, miR-324-3p, PAP2C

Introduction

Hepatocellular carcinoma (HCC) is a common malignant, ranking third in the death rate caused by cancer.[1] HCC is featured with metastasis, which in resistant to standard clinical therapies.[2] The 5-year survival rate is disappointing, which is under 10%.[3] Therefore, it is needed to elucidate the pathology of HCC development, which would prolong the life of HCC patients.

Recently, circular RNA (circRNA) has been attracting more and more attention, due to its regulating functions in various biological processes, including caner progression.[4] circRNAs are a subclass of non-coding RNAs, which was initially identified as unimportant "noise".[5] However, recent studies have gradually confirmed the dysregulation of circRNAs in a lot of diseases and the important roles they played in multiple physiological processes, including caner progression.[6] For example, circ-CEP128 sponged miR-145-5p to upregulate SOX11, thereby enhancing bladder cancer.[7] CircFOXO3 aggravated prostate cancer development via

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sequestering miR-29-3p.[8] Circ-LDLRAD3 knockdown inhibited pancreatic cancer by interacting with miR-137-3p and modulating PTN.[9] In this study, we found that circular RNA ZNF609 (circZNF609) was upregulated and induced oncogenic phenotypes in HCC. A common way for circRNAs to function is to sponge miRNAs, thereby upregulating downstream target genes.[10] CircZNF609 is located in human chromosome 15. 64q. CircZNF609 is derived from ZNF609 gene sequences. ZNF609 belongs to zinc finger family, which participates in regulation of gene expression in DNA, RNA and protein levels.[11] CircZNF609 was first reported to be essential in the development of the central nervous system. Recent studies revealed that circZNF609 is highly expressed not only in neurons, but also in various types of cancer cells, such as breast cancer,[12] colorectal cancer,[13] prostate cancer,[14] and renal carcinoma.[15] Nevertheless, the function of circZNF609 in HCC was still unclear. Thus, we designed experiments to investigate the expression files of circZNF609 in HCC, and the effects on HCC cell proliferation, apoptosis and metastasis. To further elucidate the possible mechanisms, informatic tools and rescue experiments were carried out. Taken together, we showed that circZNF609 expression higher in HCC tissues and cells, and positively associated with HCC cell proliferation, migration and invasion. The underlying mechanism may be circZNF609/miR-34[illegible]-3p/RAP2C pathway.

Methods

Patient Samples

The study was approved by the Ethics Committee of Guizhou Medical University. Every patient involved in this study signed informed consent. HCC tissues and nearby normal tissues were collected from 45 patients undergone surgical resections from 2017 to 2019 in Guizhou Medical University. Tissues were stored in liquid nitrogen immediately.

Cell Culture and Transfection

Chinese Academy of Sciences Cell Bank (Shanghai, China) provided human normal liver cell line (LO2) AND HCC cell lines (LM3, Hep-3B, Huh7 and HepG2). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium plus with 10% FBS. Si-circFNZ609, circ-FNZ609 overexpression plasmids (circZNF609 OE), miR-324-3p mimics and miR-324-3p inhibitor were transfected into cells using Lipo2000 (Invitrogen, USA), in line with the protocol.

RNA Isolation and qRT-PCR

Trizol (Invitrogen, USA) was utilized to isolate total RNA from tissues or cells, according to the manufacturer's instruments. RNA was then reverse transcribed into cDNA using MMLV (Promega, Nanjing, China) to be the templates of RT-qPCR. RT-qPCR was performed with KAPA SYBR Fast 1step Uni (KAPABIOSYSTEMS, Beijing, China). GAPDH and U6 were used as internal controls for mRNA/circRNA and miRNA, respectively. The calculation of relative gene expression level was analyzed with $2^{-\Delta\Delta Ct}$ method.

CCK-8 Assay

Transfected cells were seeded into 96-well plates with the density of 1*10[illegible] cells/well. At the time point of every 24 h, 10μL CCK-8 reagent (Beyotime, Hangzhou, China) was added to each well for an incubation of 2 h at 37°C. The absorbance at 450nm was tested by Absorbance Microplate Reader ELx808 (EnSight, USA).

Flow Cytometry

Annexin V-FICT/PI Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China). $2*10^5$ cells were collected with PBS and centrifuged at 1000rpm at 4°C for 5 min. 100 μL Binding Buffer was added to suspend cells. Thereafter, 5 μL Annexin V-FITC and 5 μL PI Staining Solution were added and incubated for 10 min in dark at room temperature. 400 μL Binding Buffer was added and the absorbance at 450 nm was detected on a flow cytometry (FACScan, BD Biosciences).

Scratch Test

Transfected cells were placed into 6-well plates and incubated to 80% density. Scratches were made by a 100μL pipette tip. Images were photographed by IX71 inverted microscope (Olympus, Japan) at 0 h and 24 h for analysis of cell migration ability.

Transwell Assay

Cell invasion assay was performed with transwell chambers (Costar Technology, Beijing, China). Cells were placed into the upper chamber coated with matrigel and incubated in FBS-free medium. The bottom chamber was added with medium plus with 10% FBS. After 24 h, 4% paraformaldehyde and 0.5% crystal violet were added to

fix and stain cells. Invaded cells were photographed by IX71 inverted microscope (Olympus, Japan).

Dual-Luciferase Reporter Experiment

The wild type (wt) or mutant (mut) fragments of circZNF609 and PAP2C were subcloned into pGL3 Luciferase Reporter Vectors (Promega, Beijing, China). The plasmids were then co-transfected with miR-324-3p mimics or its negative controls (miR-NC mimics). After 48 hours, Dual-luciferase Reporter Assay System (Promega, Beijing, China) was applied to assess the luciferase reporter activities.

RNA Immunoprecipitation (RIP)

RIP was carried out using EZ-Magna RNA Immunoprecipitation Kit (Merck-millipore, Germany) according to the manufacturer's instruments. In brief, cell lysates were incubated with beads supplemented with Ago2 or IgG antibodies at 4 for 12 hours. The beads were washed and purified for Western blot and qPCR.

Western Blot

RIPA buffer plus (Solabio, Beijing, China) with protease inhibitor (Promega, Beijing, China) was utilized for total protein isolation from tissues or cells. Loading samples were prepared with equal amounts by Bicinchoninic Acid Kit (Sigma-Aldrich, USA). After separation with 8% SDS-PAGE and transcription to PVDF membranes (Roche), sil milk was used to block the cyst signals. Thereaf primary antibodies were prepared for incubation overnight: PAP2C (1:800, Sigma, USA) and GAPDH (1:2000, Tiangen Bio, China). Secondary antibodies conjugated with HRP (Sigma, USA) were prepared for h incubation of membranes. ECL Western blotting Detection Reagents (GE Healthcare, USA) were utilized to develop the signals.

RNA in situ Hybridization (RNA ISH)

CircZNF609 expression in HCC tissues was detected by ISH. Formalin fixed paraffin-embedded (FFPE) tissue sections were incubated with biotin-labeled circZNF609 probes for 18 hours at 40 °C. Then DAB substrate was used to detect the probes. Promega (Wuhan, China) provided the Biotin-labeled probes with sequence as follow: 5′-CTGCACTTCCATCATACTGACCAC-3′.

Immunohistochemistry Assay (IHC)

Tissues were sliced into 4-μm sections. Primary antibodies were prepared for incubation overnight: rabbit polyclonal antibody against PAP2C (SAB1303858, 1:500, Sigma).

Tumor Xenografts

Female BALB/c nude mice were (about 6-week-old) purchased from Shanghai Laboratory Animal Center. Mice were divided into two groups randomly, with 6 mice/group. For experimental group, mice were injected with HepG2 cells ($2*10^6$) which stably expressing sh-circZNF609; for control group, mice were injected with HepG2 cells ($2*10^6$) which stably expressing sh-negative control (sh-NC). The tumor volume was recorded weekly. The tumor volumes were measured by a caliper every three days and calculated with the following formula: tumor volume = (length × width^2)/2. Five weeks later, mice were sacrificed and tumors were collected for following assays. This animal study was authorized by Ethics Committee of Guizhou Medical University and carried out in accordance with the Guidelines for Animal Care in the National Institutes of Health.

Statistical Analysis

Data were processed by Prism Graphpad 7 and presented as Mean±SD of three independent experiments. Differences between two groups were analyzed by Student's *t*-test, while differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). Correlation was determined using Pearson analysis. $P<0.05$ was considered to be statistically significant.

Results

CircZNF609 Expressions in HCC Tissues and Cells

To explore the role of circZNF609 in HCC progression, qPCR was performed to determine the expression profile of circZNF609 in tumor tissues and matched nearby normal tissues in HCC patients. Results revealed that the expression levels of circZNF609 in tumor tissues were markedly higher than those in normal ones (Figure 1A, P=0.004). ISH was further carried out to show the expression of circZNF609 in HCC tissues and normal tissues (Figure 1B), which was consistent with qPCR results. Also, in vitro we found that circZNF609 was upregulated in HCC cell lines, in comparison with that in normal liver cells (Figure 1C). Additionally, high circZNF609 expression was associated with shorter overall survival (Figure 1D) and poorer clinical

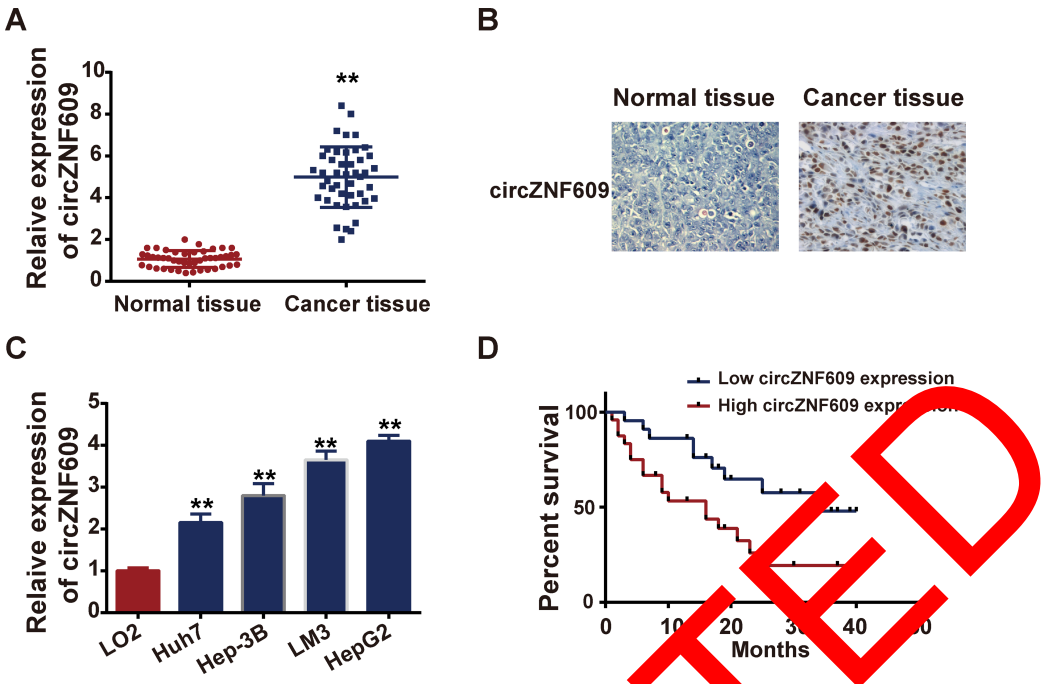

Figure 1 CircZNF609 was overexpressed in HCC.
Notes: (**A**) Expression files of circZNF609 in HCC patient tissue samples and matched nearby tissues detected by qPCR. (**B**) IHS was performed to assess the circZNF609 expressions in tissues. (**C**) Expression of circZNF609 in normal liver cell line (LO2) and HCC cell lines (Hep3B, Huh7, LM3 and HepG2). (**D**) Survival curves of HCC patients associated with circZNF609 expressions. **P<0.05.

outcomes (Table 1). All these primary exploration results indicated that circZNF609 might exerted oncogenic roles in HCC.

Table 1 The Association Between CircZNF609 Expression and Clinicopathological Characteristics of HCC Patients

Characteristics Total	N 45	EWSAT1 Expression		P value
		High (n=30)	Low (n=15)	
Age				
≥50		26	8	0.589
<50	11			
Tumor stage				
T2	18	10	8	0.029*
T3-T4	27		7	
Lymph-node metastasis				
Yes	21	17	4	0.042*
No	24	16	8	
Tumor size (cm)				
>2.5	24	18	6	0.165
≤2.5	21	12	7	
Multiple lesions				
Positive	25	16	9	0.718
Negative	20	15	5	

Note: *P < 0.05 represents statistical differences.

Silencing of CircZNF609 Inhibited HCC Development in vitro

To verify the oncogenic role of circZNF609 in HCC, si-circZNF609 was transfected into HCC cells to perform loss-of-function experiments. Evident knockdown efficiency of si-circZNF609 was showed in Figure 2A. After si-circZNF609 transfection, cell viability was monitored weekly, and results demonstrated that cells in circZNF609 knockdown group exerted lower viability, compared with those in control group (Figure 2B). Consistently, silencing of circZNF609 aggravated cell apoptosis, in comparison with control group (Figure 2C). As metastasis is a characteristic feature of cancer cells, wound healing assay and transwell assay were carried out to detect the abilities of cell migration and invasion. As results shown in Figure 2D–E, circZNF609 knockdown downregulated HCC cell migration and invasion. These data confirmed the tumor promoter role of circZNF609.

circZNF609 Served as a Sponge of miR-324-3p

In order to investigate the mechanism of circZNF609 regulated HCC, we predicted the miRNA targets with the help of CircBank (Table S1). We identified

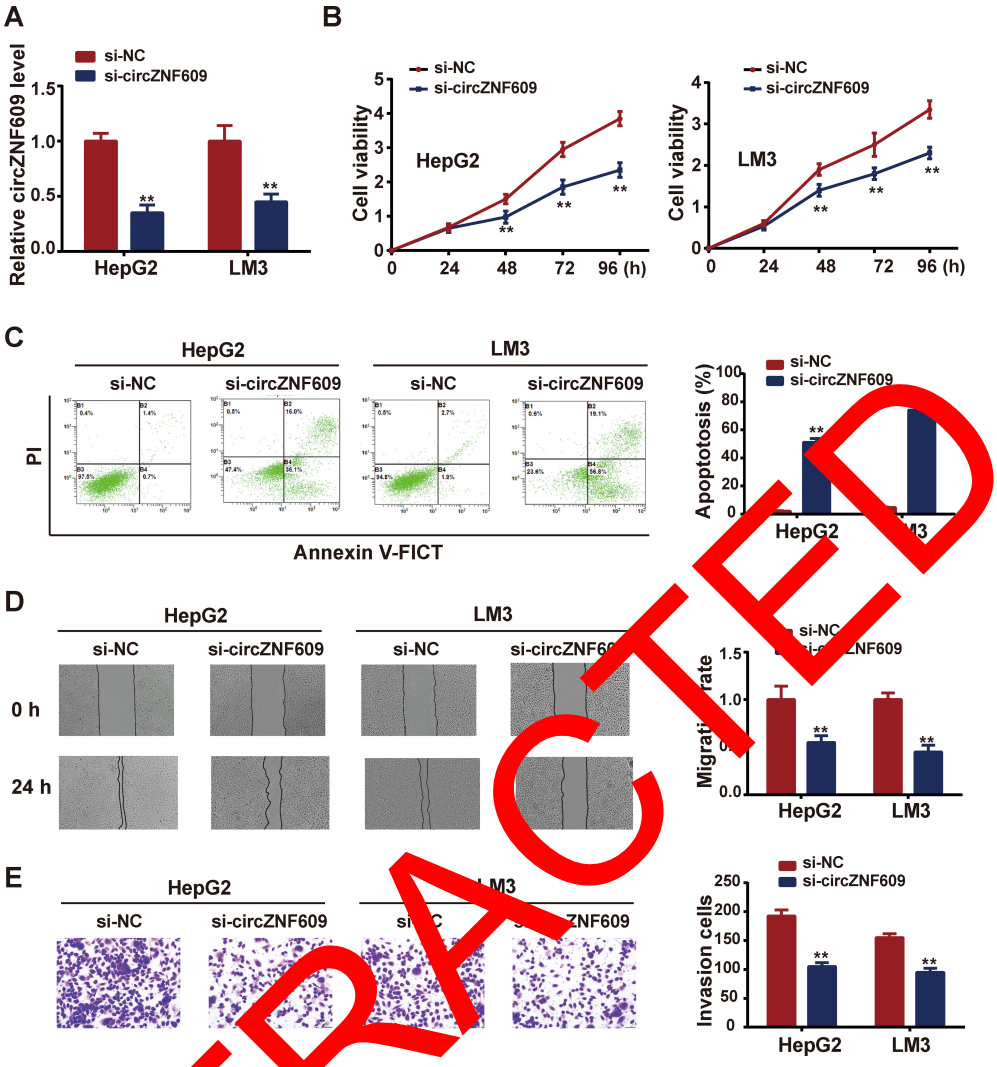

Figure 2 Silencing of circZNF609 restrain[ed] HCC cell viabilit[y], [m]igration and invasion, while enhanced cell apoptosis.
Notes: After si-circZNF609 transfectio[n], (**A**) [e]xpression of circ[Z]NF609 after transfection with si-circZNF609; (**B**) cell viability was assessed by CCK-8 assay; (**C**) cell apoptosis was detected by flow cytometry; (**D**) c[e]ll migration was determined using wound healing assay; (**E**) cell invasion was tested via transwell assay. **P<0.05.

miR-324-3p as [t]he poten[t]ial interacting miRNA of circZNF609 (Figure [3]A). Lucifer[a]se reporter experiment was follow[e]d [t]o confirm [t]he prediction (Figure 3B). Next, [w]e knock[e]d down circZNF609 expression by transfect[i]on o[f] si-circ[Z]NF609, and observed upregulation of mi[R]-324-3p (Figure 3C). Moreover, anti-Ago2 RIP assay demonstrated that TTN-AS1 and miR-376a were directly interacted in Ago2 complex (Figure 3D). Additionally, miR-324-3p expression file in patient tissues was examined by qPCR. As shown in Figure 3E, miR-324-3p was lowly expressed in HCC tissues, in comparison with that in matched nearby normal tissues. Pearson analysis revealed that there existed a negative correlation between circZNF609 and miR-324-3p (Figure 3F).

CircZNF609 Upregulated RAP2C via miR-342-3p

As it is known that the most common way for miRNA to exert biological function is to repress target gene expression by binding to its 3′UTR. We searched the potential target genes of miR-324-3p with the help of TargetScan. Among the potential targets, we focused on RAP2C, which was reported to be involved in tumorigenesis previously. The predicted binding sites between PAP2C and miR-324-3p were shown in Figure 4A. Luciferase reporter experiment was performed, showing that miR-324-3p mimics could repress the luciferase reporter activity in RAP2C wild type (wt) group, but not in mutant (mut) group, confirming the predicted interacting sites (Figure 4B). Next, wondering whether

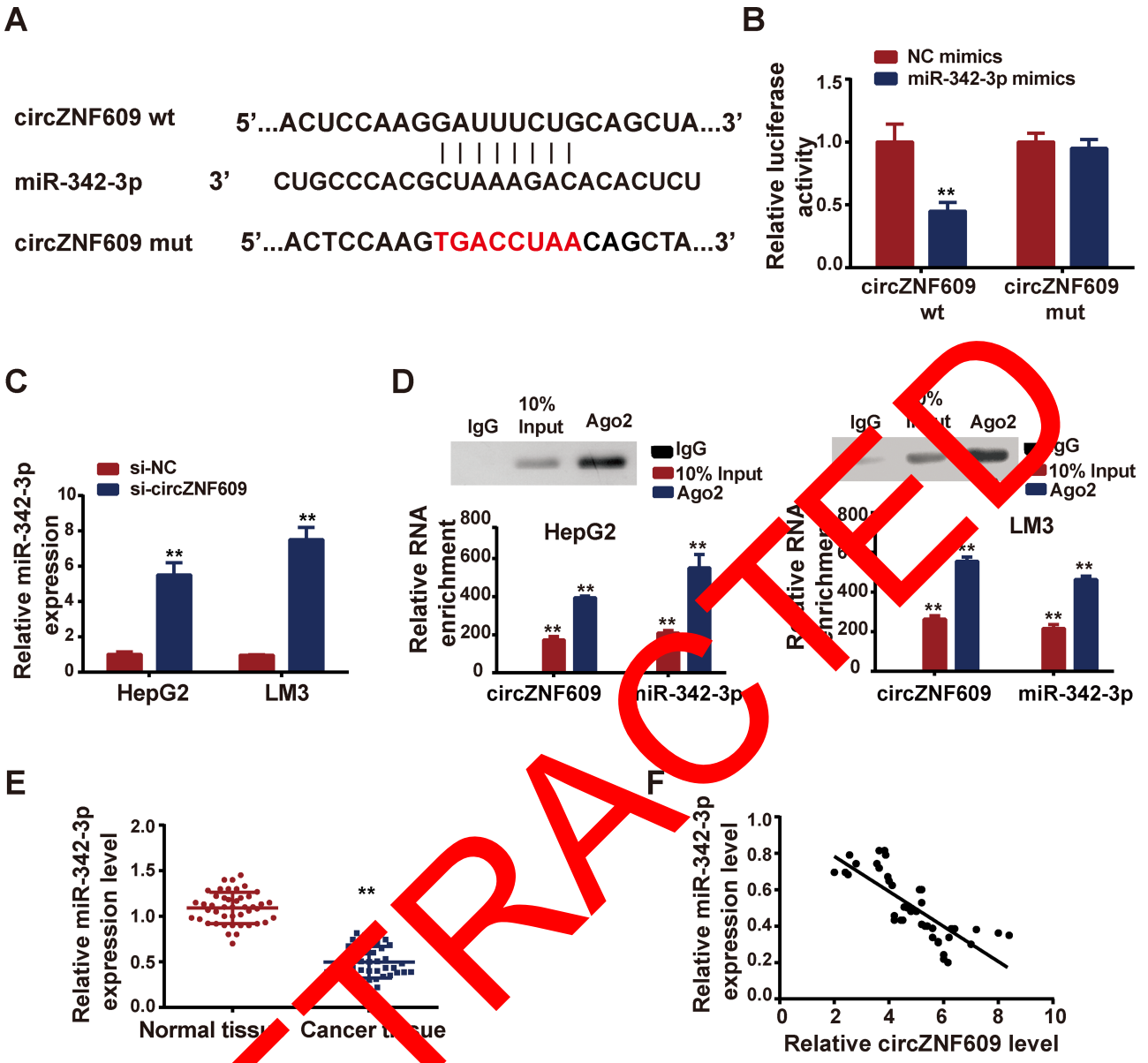

Figure 3 CircZNF609 interacts with miR-342-3p.

Notes: (**A**) CircBase 2.0 predicted interacting sequences between circZNF609 and miR-342-3p; (**B**) miR-342-3p mimics were co-transfected with circZNF609 wild type (wt) or circZNF609 mutant (mut), and luciferase reporter activity was measured. (**C**) Si-circZNF609 was transfected, and miR-342-3p expression was detected by qPCR. (**D**) Anti-Ago2 RIP was carried out to detected the direct interactions between TTN-AS1 and miR-376a. (**E**) Expressions of miR-342-3p in HCC patient tissue samples and matched nearby tissues tested by qPCR. (**F**) Correlation between miR-342-3p and circZNF609 analyzed by Spearman. **P<0.05.

circZNF609 could regulate RAP2C via miR-324-3p, we co-transfected miR-324-3p mimics with/without circZNF609 overexpression plasmids (circZNF609 OE), and the mRNA and protein expressions of RAP2C were determined by qPCR and Western blot. Results demonstrated that RAP2C expressions were inhibited by miR-324-3p mimics, while circZNF609 overexpression reversed the inhibition both in mRNA and protein levels (Figure 4C–D).

miR-324-3p Silencing or RAP2C Knockdown Reversed the Si-circZNF609-Induced Suppression on HCC Development

To confirm that circZNF609 exhibit oncogenic effects on HCC progression through modulating miR-324-3p/RAP2C axis, miR-324-3p inhibitor or RAP2C overexpression plasmid (RAP2C OE) was co-transfected with si-circZNF609.

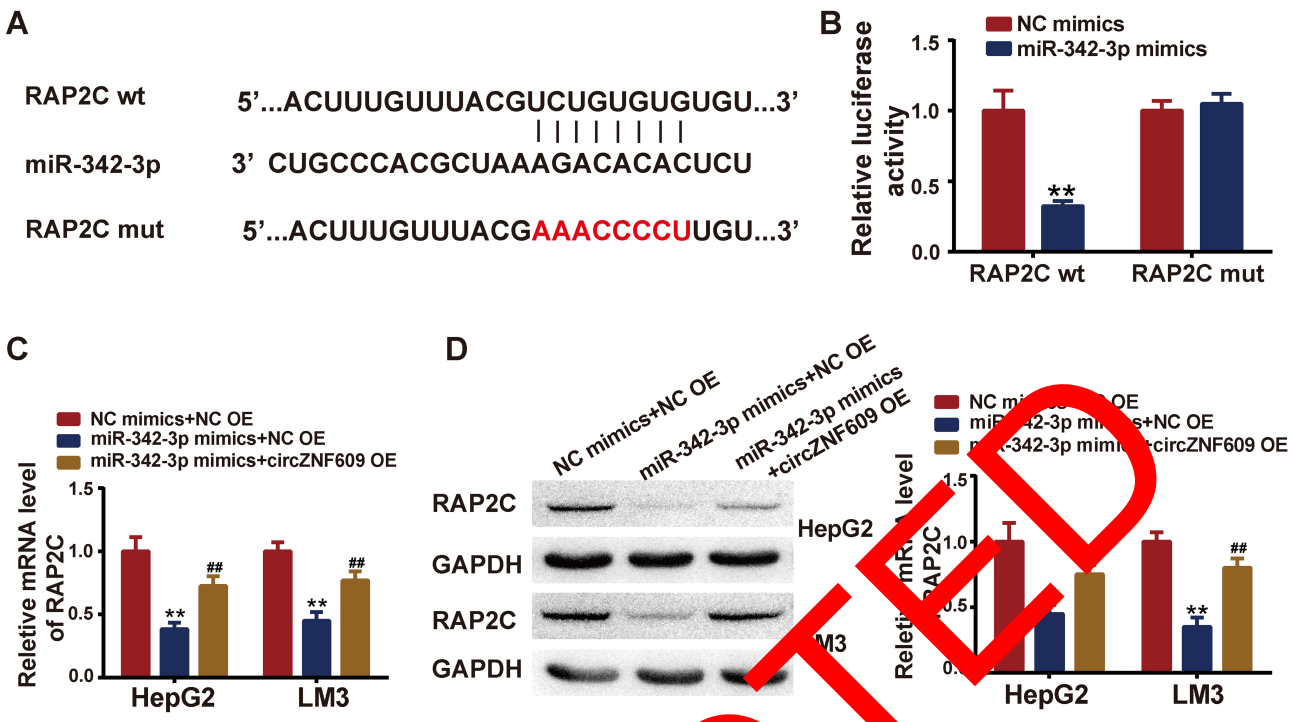

Figure 4 RAP2C was targeted by miR-342-3p.

Notes: (**A**) TargetScan predicted binding sites between miR-342-3p and RAP2C; (**B**) miR-342-3p mimics were co-transfected with RAP2C wild type (wt) or RAP2C mutant (mut), and luciferase reporter activity was measured. (**C–D**) MiR-342-3p mimics were co-transfected with circZNF609 overexpression plasmids (circZNF609 OE) or negative control, qPCR and Western blot were performed to test the RAP2C expression levels. **P<0.05 vs miR-NC+NC OE; ##P<0.05, vs miR-342-3p mimics+NC OE.

CCK-8 assay revealed that si-circZNF609 inhibited HCC cell viability, while miR-324-3p inhibitor or RAP2C OE reversed the inhibition (Figure 5A). Moreover, miR-342-3p inhibitor or RAP2C OE could mitigate the si-circZNF609-induced promotion on the apoptosis (Figure 5B). Further, si-circZNF609-induced suppression on cell migration and invasion were also reversed by miR-324-3p inhibitor or RAP2C OE (Figure 5C–D). Figure 5E presents the quantitative analysis.

sh-circZNF609 Holds Tumorigenesis in HCC Mice

In vivo experiments were carried out to verify the in vitro results. HepG2 cells expressing sh-circZNF609 or sh-NC (negative control) stably were injected into mice. Tumor sizes were monitored every week, and results showed that circ-ZNF609 knockdown repressed tumor growth (Figure 6A). After 5 weeks, mice were sacrificed and tumors were collected for weight measurement and subsequent assays. Figure 6B displays that circ-ZNF609 depletion suppressed tumor weight. Figure 6C demonstrates that in circ-ZNF609 knockdown mice, circ-ZNF609 and PARP2 expressions were downregulated, while miR-324-3p was upregulated. The protein expression levels of PARP2C were decreased in circZNF609-depletion mice, compared with those in negative control mice (Figure 6D). Further, Ki-67 (a marker of cell proliferation) was expressed lowly in circ-ZNF609 knockdown mice (Figure 6E).

Discussion

Emergency reports have revealed that circRNAs were involved in multiple biological processes, and participate in diverse diseases, including cancer.[16] There reported different circRNAs that played as anti-tumor or tumor-promoting roles in HCC progression, thus providing new potential therapeutic targets for HCC treating. For example, circ-CDR1, circ0067934 and circ100338 were reported highly expressed in HCC patients and promoted cancer progression.[17–19] CircZNF609, a newly discovered circRNA homologous to ZNF609 mRNA, was revealed exhibiting cancer-promoting effects on the progressions of a series of cancers. For instance, Jin et al elucidated that knockdown of circZNF609 restrained tumorigenesis, migration and invasion.[20] Wang et al discovered the upregulation of circZNF609 in breast cancer and identified it as an oncogene in promoting breast cancer progression.[12] In

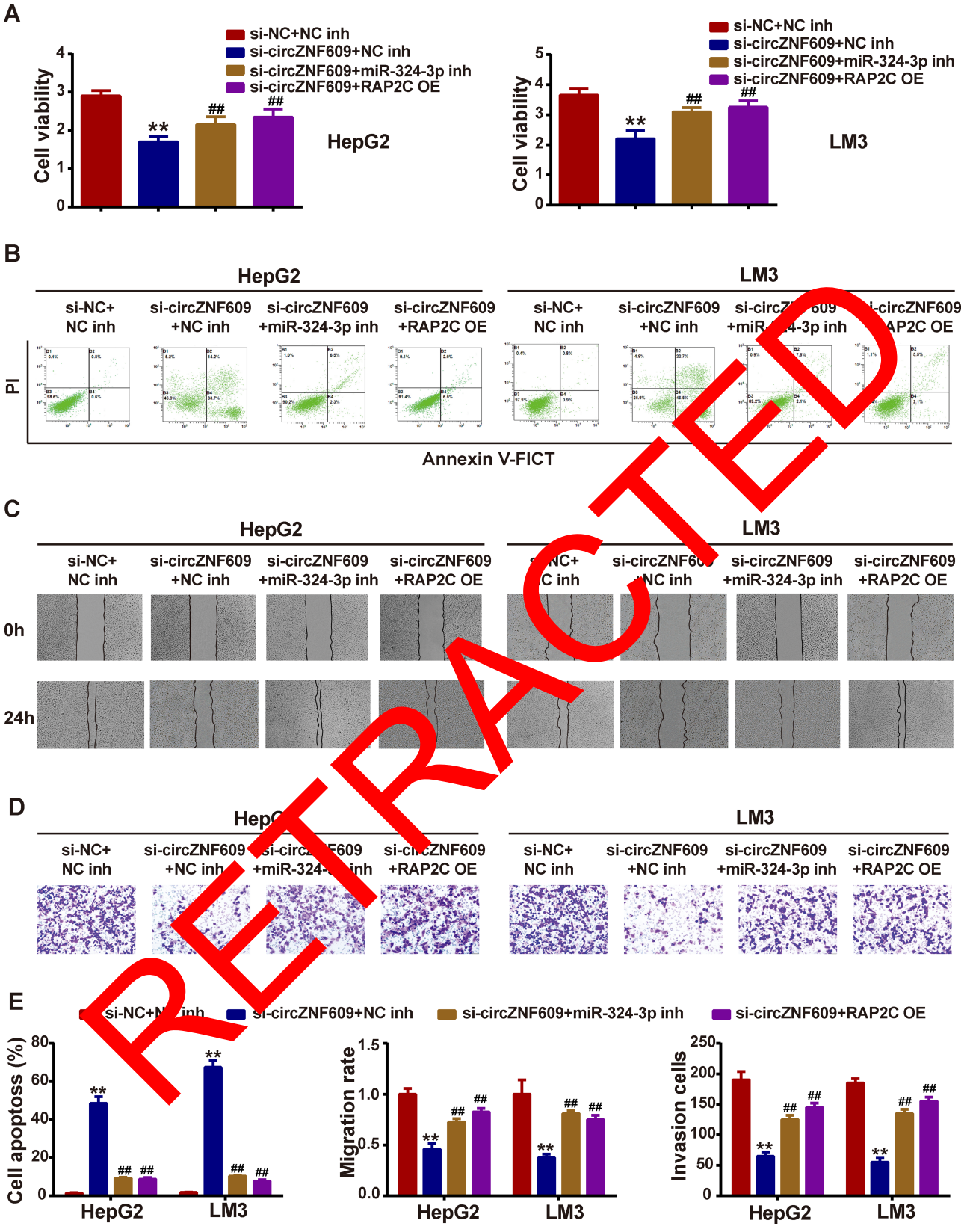

Figure 5 MiR-342-3p inhibitor or RAP2C overexpression reversed si-circZNF609-induced phenotypes in HCC cells.
Notes: MiR-342-3p inhibitor or RAP2C overexpression plasmids (RAP2C OE) was co-transfected with si-circZNF609, (**A**) cell viability was assessed by CCK-8 assay; (**B**) cell apoptosis was detected by flow cytometry; (**C**) cell migration was determined using wound healing assay; (**D**) cell invasion was tested via transwell assay; (**E**) statistical analysis show in bar graphs. **P<0.05, vs si-NC; ##P<0.05, vs Si-circZNF609.

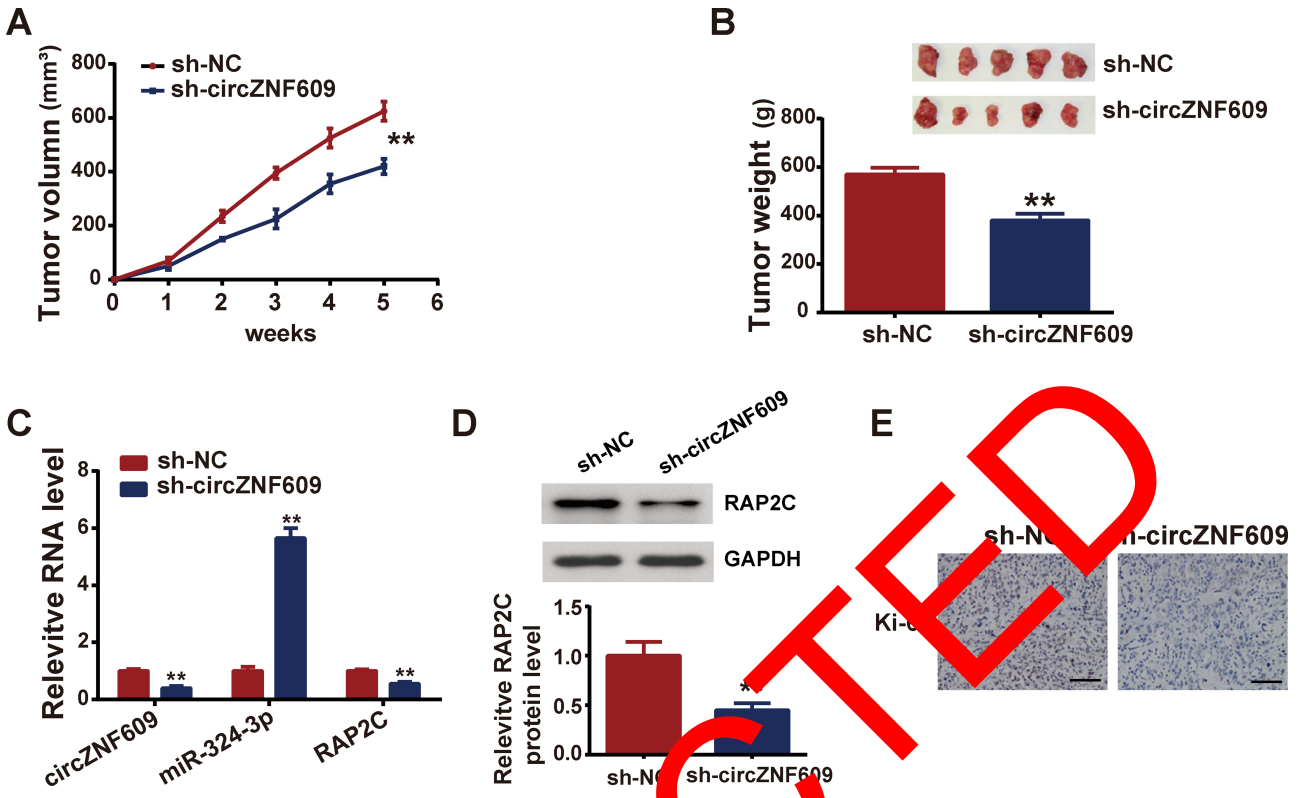

Figure 6 Depletion of circZNF609 restrained tumorigenesis in vivo.

Notes: HepG2 cells expressing sh-circZNF609 were injected into mice, forming sh-circZNF609 mice group, and HepG2 cells expressing sh-NC were also injected into mice, forming negative control group. (**A**) Tumors were measured weekly to record the size; tumors were removed weighed 35 days later; (**C**) expressions of circZNF609, miR-342-3p and RAP2C were determined by qPCR; (**D**) PAP2C protein level was measured by Western blot; (**E**) ICH staining was utilized to assess the Ki67 expression. Scale bar represents 200μm. **P<0.05.

our study, we first found that circZNF609 was overexpressed in HCC patient tissue samples and positively associated with poor prognosis, in line with the reports mentioned above. In vitro, functional experiments demonstrated that depletion of circZNF609 restrained HCC cell viability, migration and invasion, as well as aggravated apoptosis. These results indicated that circZNF609 can accelerate the progression of HCC. It should be noted that we further overexpressed the circZNF609 by transfecting circZNF609 overexpression plasmids, and detected the effects on HCC. However, the results demonstrated that there was no obvious difference in HCC cell viability, apoptosis, migration and invasion induced by circZNF609 overexpression. We suspected that the reason might be that circZNF609 is highly expressed in HCC cells, thus when we continue to overexpress it, there is no significant change in cell phenotypes.

CircRNA can exert functions through different ways. Acting as ceRNAs to sponge miRNAs, thereby modulating downstream target genes is one of the most common ways.[21] CircZNF609 has also been reported to regulate cancer progression via modulating miRNA/mRNA pathway.[10] For example, circZNF609 sequestered miR-134-5p to upregulating BTG-2 to elevate proliferation of glioma cells.[22] CircZNF609 enhanced gastric cancer migration and invasion through modulating CDK6 via sponging miR-483-3p.[23] In this study, we predicted miRNA targets of circZNF609 by CircBank informatic tool. Among the potential targets, we chose miR-342-3p for further study due to its anti-tumor features reported previously. For example, Li et al reported that miR-342-3p yielded cervical cancer cell colony ability and metastasis via downregulating FOXM1.[24] Cheng et al elucidated that miR-342-3p was sponged by lncRNA SNHG7 thereby elevating ID4 to aggravate pancreatic cancer progression.[25] Wang et al also showed that lncRNA H19 regulated miR-342-3p and FOXM1.[26] Gao et al demonstrated the downregulation of miR-342-3p in HCC and depicted its prognostic significance.[27] Further, we predicted target genes of miR-342-3p by TargetScan tool. Among the potential target genes, we were interested in RAP2C for its reported oncogenic roles in many cancer types. For instance, RAP2C

inhibited osteosarcoma cell apoptosis and enhanced metastasis in vitro.[28] RAP2C was upregulated in breast cancer, and regulated MAPK signaling to promote tumorigenesis.[29] In colorectal cancer, RAP2C was suppressed by miR-105 overexpression, thus driving cancer development.[30] In our study, we performed luciferase activity assays to confirm that miR-342-3p was sponged by circZNF609, and RAP2C was targeted by miR-342-3p. Moreover, miR-342-3p was co-transfected with/without circZNF609, and found that RAP2C was suppressed by miR-342-3p, while circZNF609 could reserve this suppression. These results suggested that circZNF609 could upregulate RAP2C via sponging miR-342-3p. Further, we investigated whether miR-342-3p and RAP2C could influence the phenotypes on HCC cells induced by circZNF609. We co-transfected si-circZNF609 with miR-342-3p inhibitor or RAP2C overexpression plasmids (RAP2C OE), and found that the effects on cell viability, migration and invasion induced by si-circZNF609 were attenuated by miR-342-3p inhibitor or RAP2C OE. These data revealed that circZNF609 regulated HCC cell progression via modulating miR-342-3p/RAP2C axis. Additionally, mouse models with circZNF609 knockdown (sh-circZNF609 group) and their negative control models (sh-NC group) were established to carry out in vivo experiments. Results showed that the tumors in sh-circZNF609 mice grew smaller and lighter, in comparison with sh-NC mice. Meanwhile, circZNF609 and RAP2C expressed lower in circZNF609 depletion mice, while miR-342-3p expressed higher, compared with sh-NC mice.

Conclusion

CircZNF609 was highly expressed in HCC tissues and cells, and driven HCC progression by upregulating RAP2C via sponging miR-342-3p. This study may provide new potential therapeutic target for HCC treatment.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Guizhou Medical University. Informed consent was obtained from all individual participants in the study.

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Disclosure

The authors declare that they have no conflicts of interest.

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