

Circular RNA hsa_circ_103809 suppresses hepatocellular carcinoma proliferation and invasion by sponging miR-620

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Abstract. – **OBJECTIVE:** Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide, particularly in China. In recent years, numerous studies have investigated the roles of circular RNAs (circRNAs) in tumour development because circRNAs generally act as microRNA (miRNA) sponges to regulate gene expression. However, whether circRNAs are also involved in HCC progression remains largely unknown.

MATERIALS AND METHODS: In the present study, we identified a novel circRNA (hsa_circ_103809) and determined its expression in HCC tissues and cell lines by qRT-PCR assays. CCK8, colony formation, wound-healing and transwell assays were performed to assess the effects of hsa_circ_103809 and miR-620 on HCC cell proliferation, migration and invasion. Bioinformatics analysis and luciferase reporter assays were used to explore the correlation between hsa_circ_103809 and miR-620 in HCC cells.

RESULTS: The results showed that hsa_circ_103809 expression was significantly down-regulated in HCC tissues and cell lines. The ectopic expression of hsa_circ_103809 inhibited HCC cell proliferation, migration and invasion. In addition, we found that miR-620 expression was significantly up-regulated in HCC tissues and was negatively correlated with hsa_circ_103809 expression in HCC tissues. Furthermore, we found that hsa_circ_103809 could bind to miR-620 and that hsa_circ_103809 negatively regulates miR-620 expression. We also showed that hsa_circ_103809 inhibited the proliferation and invasion abilities of HCC cells by sponging miR-620.

CONCLUSIONS: Hsa_circ_103809 acts by binding to miR-620 and inhibiting the tumorigenicity of HCC. Thus, this circRNA may serve as a potential biomarker and novel therapeutic target of HCC.

Key Words:

hsa_circ_103809, Hepatocellular carcinoma, miRNA-620, Proliferation, Invasion.

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide, particularly in China¹. Conventional chemotherapy remains ineffective for treating HCC, but surgery is possible for few patients and curative for only a small percentage. Prevention and early diagnosis remain the best approaches for treating HCC². The identification of new efficient biomarkers for HCC is needed.

In the past decades, numerous studies have determined that noncoding RNAs (ncRNAs), such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), are deregulated in HCC tissues and play critical regulatory roles in HCC progression, raising the possibility of diagnostic and therapeutic applications in clinical practice³⁻⁸. However, the roles of circular RNAs (circRNAs), as well as their functions and relevant mechanism in HCC, need to be identified.

CircRNAs are a novel class of noncoding RNAs that play critical regulatory roles in eukaryotic gene expression⁹. The existence of circRNAs in eukaryotic cells was first observed by electron microscopy nearly 40 years ago¹⁰. However, most circRNAs have been identified in the past decade since the advent of high-throughput sequencing and bioinformatics analysis. CircRNAs arise from both protein-coding and noncoding regions of the genome in multiple cell lines and across various species^{11,12}. They are characterized by a covalent and canonical linkage formed between the 3' and 5' splice sites, the so-called "back splice" sites^{12,13}, and are ubiquitously expressed in eukaryotic genes^{14,15}. CircRNAs are now regarded as important regulators of coding sequences¹⁶, which may be the basis for their roles in the genesis and development of tumours. MiRNAs are small non-

coding RNAs of approximately 22 nucleotides in length that evolutionarily conserved in animal genomes¹⁷. In the past few decades, studies have confirmed the extensive regulatory functions of miRNAs in the human genome. MiRNAs regulate gene expression generally through binding to the 3'-untranslated region (3'-UTR) of mRNA target genes, thereby preventing their translation or causing mRNA transcript degradation¹⁸. MiR-620 was first observed to be upregulated in lung adenocarcinoma and to regulate the growth, migration and invasion of human lung adenocarcinoma cells by targeting glypican 5 (GPC5)¹⁹. Another report showed that miR-620 promoted tumour radio-resistance by targeting hydroxyprostaglandin dehydrogenase/15-nicotinamide adenine dinucleotide (HPGD/15-PGDH)²⁰. However, the expression profile and functional role of miR-620 in HCC need to be explored. In the present study, we analysed the expression profile of circRNAs in HCC tissues and identified a novel circRNA, hsa_circ_103809, that was decreased in HCC tissues compared to normal tissues. We investigated the roles of hsa_circ_103809 in HCC cell progression *in vitro*. We found that hsa_circ_103809 may function by binding to miR-620 to suppress HCC proliferation, migration and invasion. Therefore, hsa_circ_103809 may serve as a potential biomarker and therapeutic target for HCC patients.

Materials and Methods

Clinical Specimens and Cell Culture

Liver cancer specimens in this study were acquired from HCC patients who underwent surgical resection at the Central Hospital of Wuhan between May 2016 and Feb 2018. These HCC cases included 18 male patients and 15 female patients with a mean age of 65.6 years (range 37-86 years). None of the patients had received preoperative treatments, such as radiotherapy or chemotherapy. Normal liver tissues were taken at a distance of at least 5 cm from the tumour mass, and all samples were verified histologically. The specimens were stored in liquid nitrogen until analysis. The collection and analysis of clinical specimens were approved by the local Ethics Committee of the Central Hospital of Wuhan. Written informed consent was obtained from all participants prior to participation.

Cell Culture

Human HCC cell lines (HepG2, Huh-7, Hep-3B and QGY-77) and immortalized liver cells

(LO2) were purchased from the Cell Bank of the China Academy of Sciences (Shanghai, China). All the HCC cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA), while the LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA). Both mediums were supplemented with 10% foetal bovine serum (FBS, Cat. 10082-147; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Cat. No. 15140-12; Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37°C in a 5% CO₂ humidified incubator.

Vector Construction and Transduction

Full-length hsa_circ_103809 cDNA was amplified from the mRNAs of Hep-3B cells by RT-PCR using PrimerSTAR Max DNA Polymerase Mix (TaKaRa, Otsu, Shiga, Japan) and specific primers. The PCR products were inserted into a pcDNA3.0 vector (Invitrogen, Carlsbad, CA, USA). A total of 5×10^4 HCC cells (Hep-3B and QGY-77) were then transduced with the vectors (hsa_circ_103809-expressing vector or empty vector control) with Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Oligonucleotide Transfection

hsa_circ_103809 siRNA (siRNA1) and negative control (NC) oligonucleotides and miR-620 mimics and scrambled construct were synthesized by GenePharma (Shanghai, China). All the transfection experiments used Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Hep-3B and QGY-77 cells were seeded in 6-well plates and transiently transfected with hsa_circ_103809 or NC and miR-620 mimics or scrambled construct for 48 hrs using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR Assay)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Moloney Murine Leukaemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA) and oligo dT 15 primers (Thermo Fisher Scientific, Waltham, MA, USA) were used to synthesize cDNA. miR-620 reverse transcription (RT) reactions were performed with a TaqMan MicroRNA Reverse Transcrip-

tion Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Hsa_circ_103809 and miR-620 were amplified using SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA were utilized for hsa_circ_103809 and miR-620 expression normalization, respectively. The relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method²¹. The primers are shown in Table I.

RNase R Digestion

Total RNA (5 μ g) was incubated for 15 mins at 37°C with or without 3 U/ μ g of RNase R (Epicentre Biotechnologies). The RNA was purified by phenol-chloroform extraction and identified by qRT-PCR assays. The RNase R digestion reaction was performed twice according to previously published procedures.

Dual-Luciferase Reporter Assay

The binding sites of hsa_circ_103809, including hsa_circ_103809-Wild and hsa_circ_103809-Mut, were inserted into a pGL3 promoter vector (Realgene, Nanjing, China) to generate reporter plasmids. Briefly, cells were seeded into 24-well plates and transfected with the corresponding reporter plasmids using Lipofectamine 2000 (Invitrogen, Shanghai, China). The cells were collected 48 hrs later and analyzed with a dual-luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8) (Dojindo Co. Ltd., Kumamoto, Japan) assays were used to monitor cell viability. Briefly, cells (5000/100 μ l) were seeded into 96-well plates. Subsequently, 10

μ l of CCK-8 solution were added to each well at the indicated timepoints and incubated for 2 hrs at 37°C. After incubation, a microplate reader was used to measure the corresponding absorbance at 450 nm. Each condition was determined in quintuplicate in three independent experiments.

Colony Formation Assay

Briefly, 500 cells/well were seeded in 6-well plates with the corresponding cell growth medium. After incubation at 37°C for 14 days, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The number of colonies containing at least 50 cells (established by microscopy) was counted. All experiments were repeated at least 3 times.

Wound Healing Assay

Wound healing assays were performed to determine cell motility. HCC cells were seeded into 6-well plates and cultured at 37°C for 16 hrs. After reaching 90% confluence, the cells were scraped with a sterile 200- μ l pipette tip. Then, the cells were incubated for another 24 hrs until analysis. The width of the wound area was measured using Zeiss LSM Image Browser software (version 3.1). The experiments were performed in duplicate at least three times.

Cell Migration and Invasion Assays

Cell migration and invasion abilities were evaluated using Transwell cell culture chambers (Corning Costar Corp, Corning, NY, USA) according to the manufacturer's instructions. Briefly, HCC cells were resuspended in serum-free medium to achieve a final density of 1×10^6 cells/mL. Aliquots of 200 μ l of the cell suspension were added to the transwell filter membrane chambers, and the bottom chambers were filled with 600 μ l of complete medium supplemented with 10% fetal bovine serum (FBS) as a chemoattractant. For the invasion assays,

Table I. The primer sequences in qRT-PCR assay.

Gene	Primer sequences
GAPDH	Forward: 5'-TATGATGATATCAAGAGGGTAGT-3' Reverse: 5'-TGTATCCAACTCATTGTCATAC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACATA-3' Reverse: 5'-AACGATTACGAATTTGCGT-3'
Has_circ_103809	Forward: 5'-AAAGAGTTTTTGAAGACGTC-3' Reverse: 5'-TGGTGGCATGTTTTGTCATT-3'
MiR-620	Forward: 5'-ACACTCCAGCTGGGATGGAGATAGATAT-3' Reverse: 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGATT
TCTAT-3'	

transwell membranes were pre-coated with 10 μ l of Matrigel (diluted 1:3; BD Biosciences, Franklin Lakes, NJ, USA). After incubation for 24 hrs at 37°C, the invasive cells on the bottom surface were fixed in 4% paraformaldehyde and stained with a 0.1% crystal violet solution. The cells remaining on the upper membrane surface were removed. Five randomly selected fields in each membrane were counted using an inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 20 $\times$ magnification.

Statistical Analysis

Statistical analyses were performed using SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Statistically significant differences were calculated using independent sample *t*-test, chi-square test, or one-way ANOVA followed by Tukey's post-hoc analysis of variance. $p < 0.05$ was considered statistically significant.

Results

circRNA Deregulation and hsa-circ-103809 Down-Regulation in HCC Tissues

To evaluate circRNA expression profile in HCC tissues, we analyzed the differentially expressed circRNAs in HCC tissues and adjacent non-cancerous tissues from the GEO database. The 3 circRNA GeneChips used were GSE94508, GSE97322 and

GSE78520 (Figure 1A). The differentially expressed circRNAs from GSE94508 (Table SI), GSE97322 (Table SII) and GSE78520 (Table SIII) are listed. Through fold enrichment sorting in HCC tumour and non-tumour groups, the 250 most up- and down-regulated circRNAs in HCC tissues compared to those in matched non-tumour tissues are shown in Figure 1A. We created a Venn diagram for the circRNAs shown in Figure 1B. Three circles represented the circRNAs from three different studies (blue for GSE78520, yellow for GSE94508 and green for GSE97322). A total of five genes were identified to be in common among the three GEO studies; of these, hsa-circ-103809 had the highest expression fold change (Figure 1B). Subsequently, we detected the expression of these 5 circRNAs in HCC tissues and non-tumour tissues from 10 patients by qRT-PCR assay to confirm their expression. We found that all 5 of the circRNAs were lower in HCC tumour tissues than in the matched controls. Among them, hsa-circ-103809 expression was changed most significantly (Figure 1C). We examined hsa-circ-103809 expression levels in another 33 HCC patients, and the results obtained were the same. Thus, the expression of hsa-circ-103809 was down-regulated in HCC tissues ($p < 0.001$, Figure 1D). In addition, the results indicated that hsa-circ-103809 expression was related to tumour size (cm) ($p = 0.0050$), invasion ($p = 0.0060$), lymph node metastasis ($p = 0.0161$), distal metastasis ($p = 0.0127$) and TNM stage ($p = 0.0122$) (Table II).

Table II. Correlation between has_circ_103089 expression and clinical parameters in HCC (dCt).

Characteristics	No. of patients	Mean $\pm$ SD	<i>p</i> -value
Total no. of patients	33		
Age (year)			
> 50	19 (43.8%)	10.42 $\pm$ 1.95	0.4353
$\leq$ 50	14 (56.2%)	9.94 $\pm$ 1.35	
Gender			
Male	18 (39.6%)	10.04 $\pm$ 2.14	0.6069
Female	15 (60.4%)	10.37 $\pm$ 1.32	
Tumor size (cm)			
< 3	21 (54.2%)	10.32 $\pm$ 1.39	0.0050
$\geq$ 3	12 (45.8%)	11.94 $\pm$ 1.63	
Invasion			
T0-T2	23 (64.6%)	9.26 $\pm$ 1.26	0.0060
T3-T4	10 (35.4%)	10.84 $\pm$ 1.73	
Lymphatic metastasis			
N0	24 (70.8%)	10.02 $\pm$ 1.43	0.0161
N1-N3	9 (29.2%)	11.43 $\pm$ 1.38	
Distal metastasis			
M0	26 (83.3%)	10.29 $\pm$ 1.42	0.0127
M1	7 (16.7%)	11.86 $\pm$ 1.28	
TNM stage			
0, I, II	22 (66.7%)	10.37 $\pm$ 1.04	0.0122
III, IV	11 (33.3%)	11.47 $\pm$ 1.27	

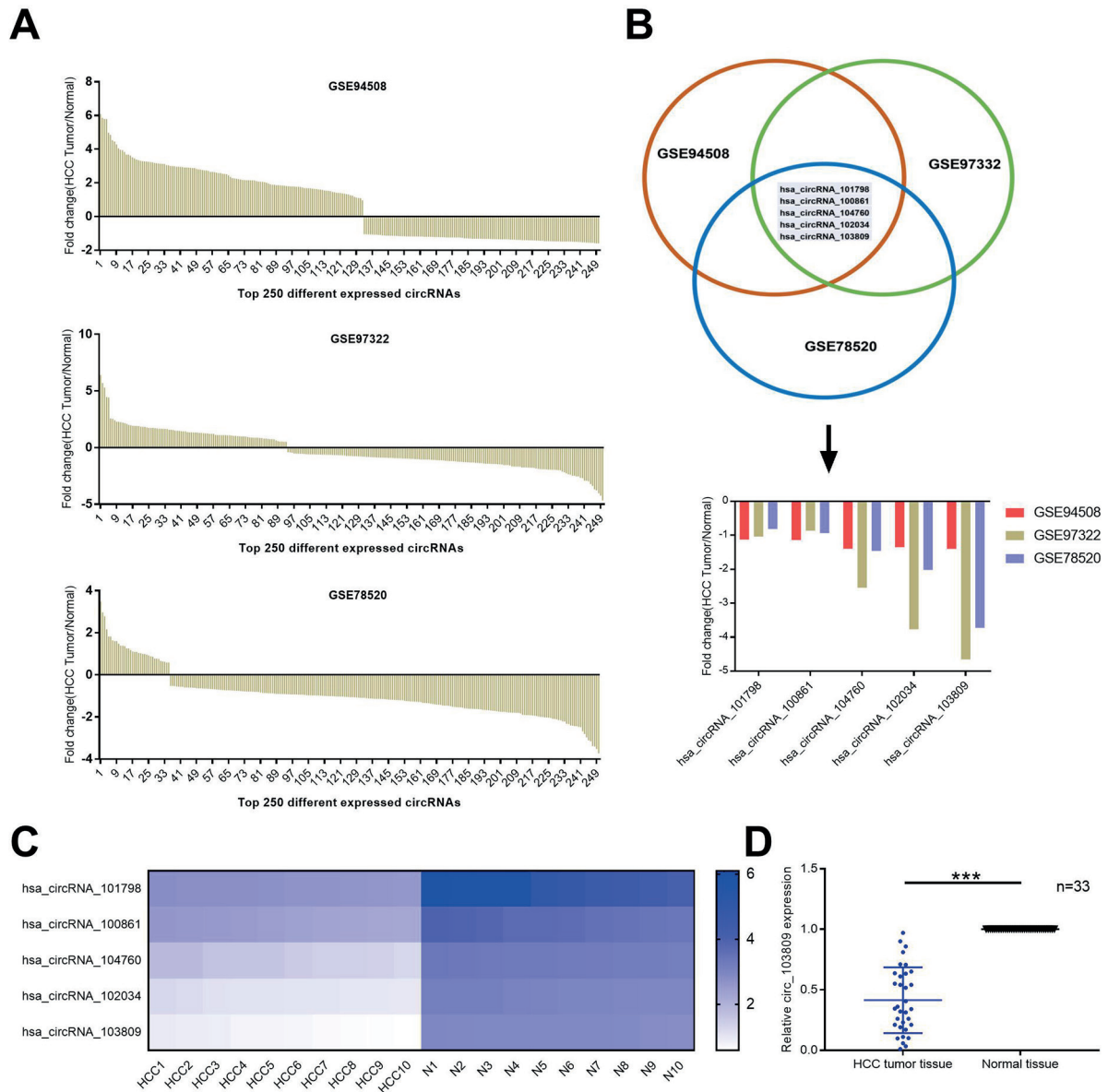


Figure 1. circRNA deregulation and hsa-circ-103809 down-regulation in HCC tissues. **(A)** Histograms were created to illustrate the top 250 circRNAs differentially expressed in normal tissues and HCC tissues for each study from the GEO. **(B)** Venn diagram for the circRNAs in HCC. The three circles represent the circRNAs from the three different studies (blue for GSE78520, yellow for GSE94508 and green for GSE97322). A total of five circRNAs were identified to be in common among the three GEO studies; of these, hsa-circ-103809 had the highest expression fold change. **(C)** Expression levels of the five circRNAs in paired HCC tissues and normal liver tissues in 10 patients. **(D)** hsa-circ-103809 expression levels detected by qRT-PCR assays in HCC tissues were compared to those in normal tissues ($n = 33$, $***p < 0.001$). GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma.

The Identification and Characterization of hsa_circ_103809

We obtained the details of this gene in the UCSC genome database and analyzed the associated information about the gene transcript. The UCSC database transcription information showed that the transcript, called ZFR, was

located on chromosome 5, and was located between the base sites 32,376,829 and 32,391,170. Hsa_circ_103809 is a 693 bp transcript derived from exon 13 to exon 17 of the ZFR gene (Figure 2A). qRT-PCR assays indicated that both cDNA and genomic DNA (gDNA) could be produced using convergent primers; however,

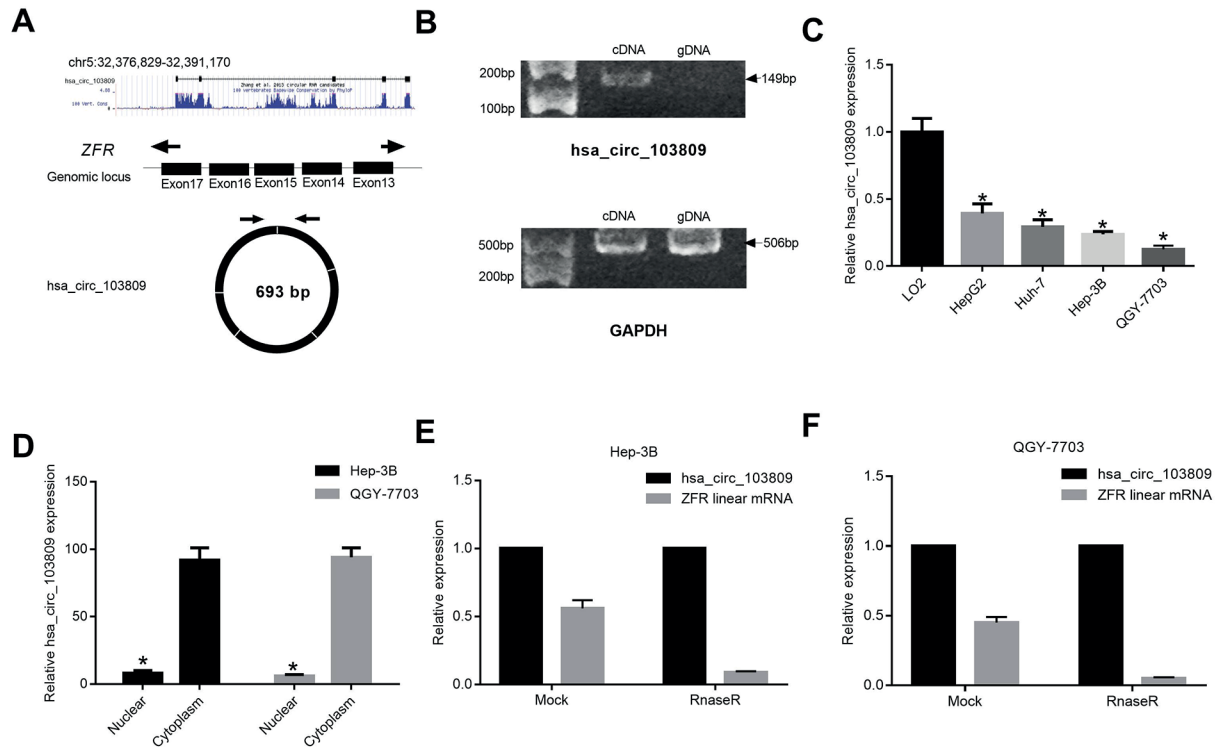


Figure 2. Identification and characterization of hsa_circ_103809. (A) Position and associated information for hsa_circ_103809 based on the UCSC Human Genome Database (<http://genome.ucsc.edu>). (B) Verification that hsa_circ_103809 is a circRNA using divergent and convergent primers. Both cDNA and genomic DNA (gDNA) could be produced using convergent primers, but only cDNA could be amplified using divergent primers. (C) The results of qRT-PCR assays showed that the expression levels of hsa_circ_103809 were down-regulated in HCC cell lines compared with that in normal liver cells ($*p < 0.05$). (D) Cellular distribution of hsa_circ_103809 in Hep-3B and QGY-77 cells. Hsa_circ_103809 is distributed mainly in the cytoplasm ($*p < 0.05$). (E-F) Real-time PCR showing the resistance of hsa_circ_103809 to RNase R digestion in HCC cell lines. (E) Hep-3B and (F) QGY-77 cells. gDNA, genomic DNA; UCSC, University of California Santa Cruz Human genome database (<http://genome.ucsc.edu>).

only cDNA could be amplified using divergent primers (Figure 2B). This finding suggested that hsa_circ_103809 is circular. We detected the expression levels of hsa_circ_103809 in four types of HCC cell lines and normal liver cells using qRT-PCR assays and found that the expression levels of hsa_circ_103809 were down-regulated in HCC cell lines, especially in Hep-3B and QGY-7703 cell lines, compared with those in normal liver cells ($p < 0.05$, Figure 2C). Furthermore, the cellular distribution of hsa_circ_103809 in Hep-3B and QGY-77 cells was determined by qRT-PCR assays. As shown in Figure 2D, Hsa_circ_103809 is distributed mainly in the cytoplasm ($p < 0.05$, Figure 2D). We performed RNase R digestion and qRT-PCR assays to further confirm that hsa_circ_103809 was circular (Figure 2E-F).

Hsa_circ_103809 Suppresses HCC Cellular Proliferation and Colony Formation

To investigate the biological significance of hsa_circ_103809 in HCC progression, Hep-3B and QGY-77 cells were transduced with hsa_circ_103809-overexpressing vectors and an empty vector control. Real-time PCR was used to confirm the transfection efficiency ($p < 0.05$, Figure 3A-B). Subsequently, we performed CCK8 assays to measure the proliferation of HCC cells and found that the overexpression of hsa_circ_103809 reduced cell proliferation compared with that in control cells after 72 hrs ($p < 0.05$, Figure 3C-D). Next, we performed colony formation assays to determine whether hsa_circ_103809 could affect the clonogenic capacity of HCC cells. The results are shown in Figure 3E-F. HCC cells overexpressing

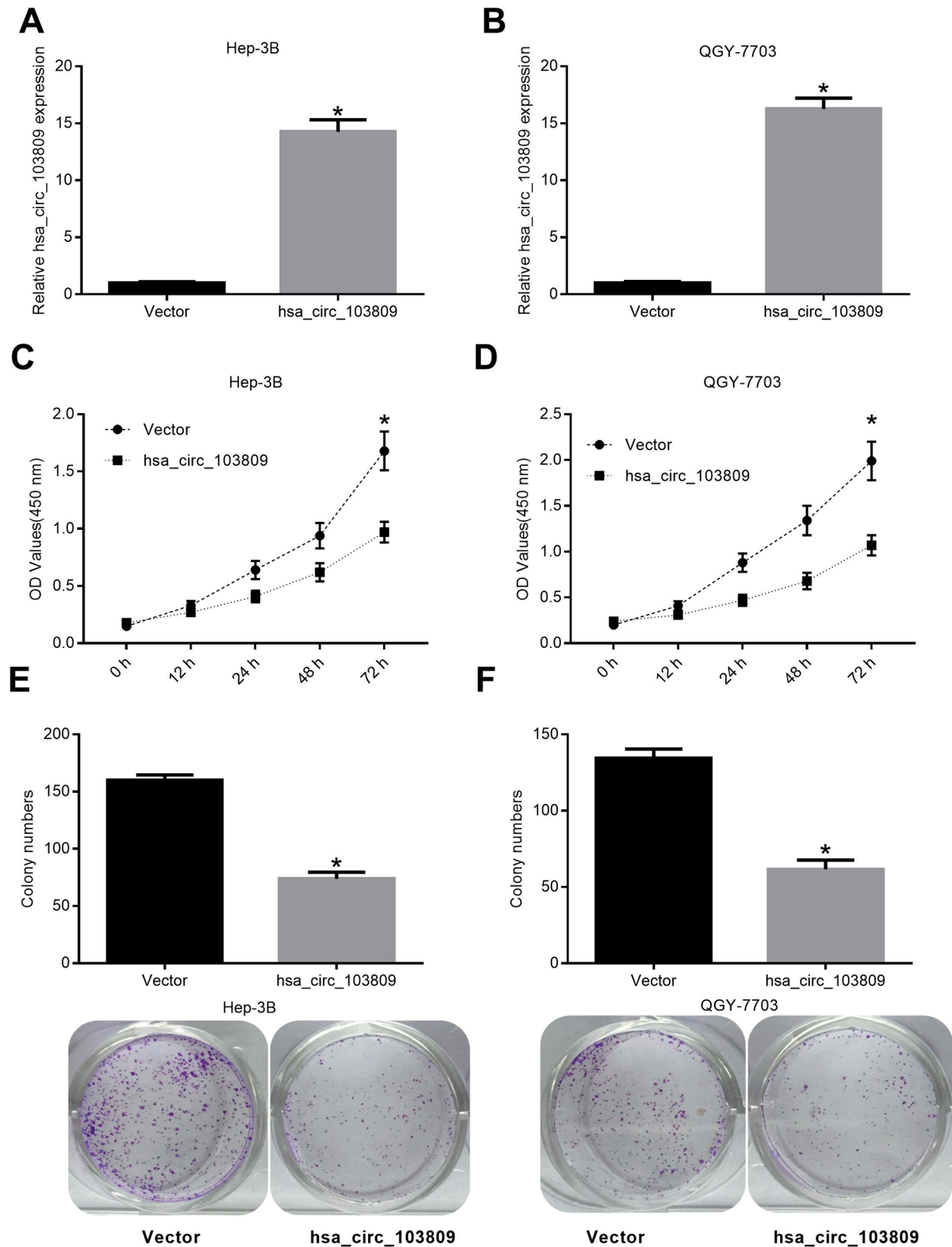


Figure 3. Hsa_circ_103809 suppressed HCC cellular proliferation and colony formation. (A-B) Real-time PCR was used to detect hsa_circ_103809 expression in Hep-3B and QGY-77 cells transduced with the hsa_circ_103809-overexpressing plasmid or empty vector control (* $p < 0.05$). (C-D) CCK8 assays were used to detect the cell proliferation ability of Hep-3B and QGY-77 cells (* $p < 0.05$). (E-F) Colony formation assays were performed to detect the clonogenic capacity of Hep-3B and QGY-77 cells (* $p < 0.05$).

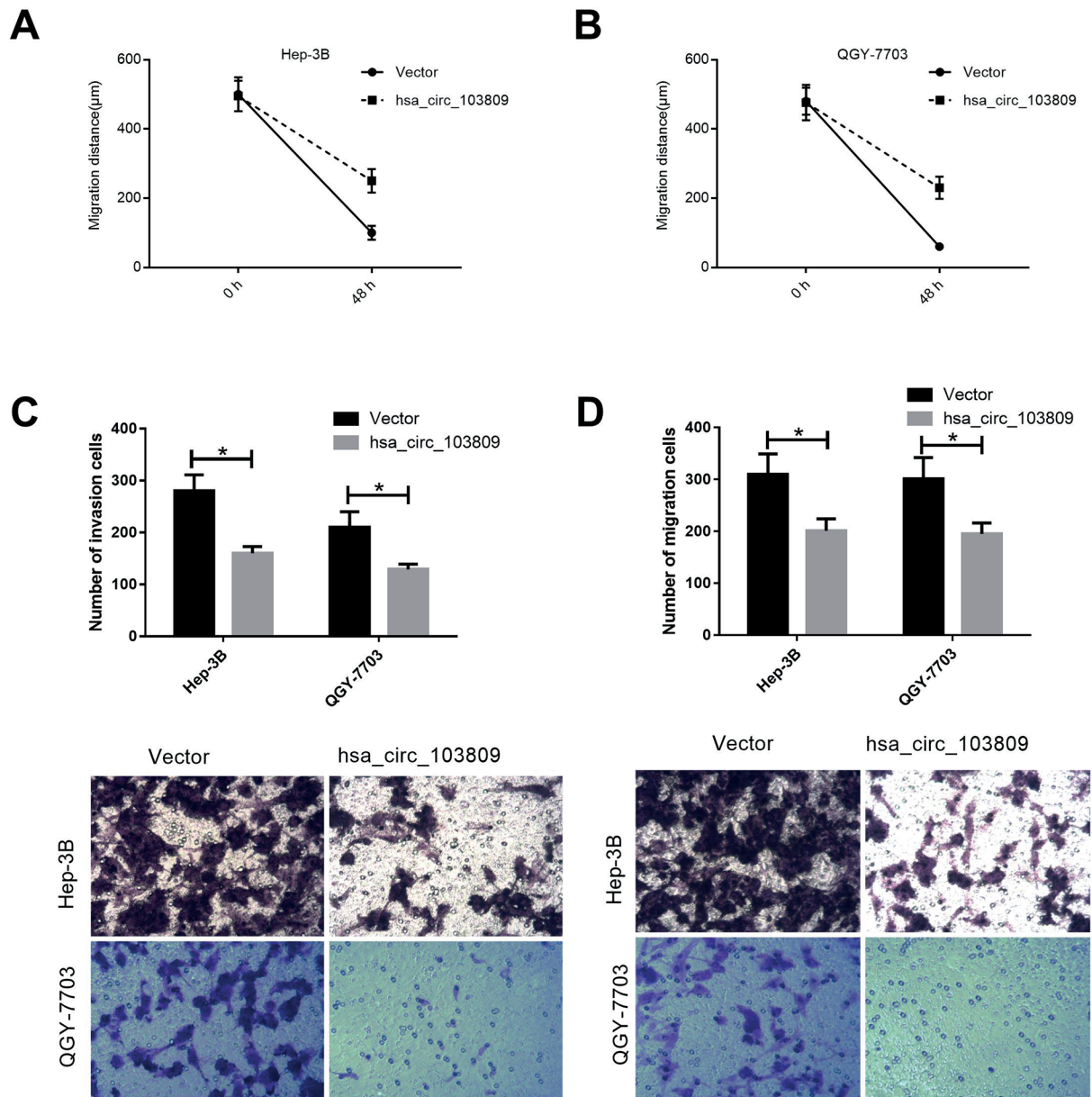


Figure 4. Hsa_circ_103809 inhibited HCC cellular motor ability, migration and invasion. (A-B) Wound healing assays were used to detect the motor ability of Hep-3B and QGY-77 cells (magnification $\times 200$; scale bars, 10 μm). (C) Cell invasion ability was measured by transwell assays in HCC cells (magnification $\times 200$; scale bars, 10 μm , $*p < 0.05$). (D) Transwell assays were used to detect the migration of HCC cells (magnification $\times 200$; scale bars, 10 μm , $*p < 0.05$).

hsa_circ_103809 exhibited fewer colony foci than the control cells ($p < 0.05$, Figure 3E-F). These results suggest that hsa_circ_103809 could inhibit proliferation and colony formation in HCC cells.

Hsa_circ_103809 Inhibits HCC Cellular Migration and Invasion

It had been shown that cirRNAs are involved in tumour metastases²², one of the major character-

istics of malignancy. We performed wound healing assays to detect the effect of hsa_circ_103809 on HCC cell motility. The data are shown in Figure 4A-B. Compared with the controls, HCC cells transfected with the hsa_circ_103809-over-expressing plasmid showed a wider wound area 48 hrs after cell propagation ($p < 0.05$, Figure 4A-B). We further investigated the cell migration and invasion capabilities using Transwell assays. As

shown in Figure 4C-D, HCC cells overexpressing hsa_circ_103809 had a significant decrease in the number of migrated cells compared with the control cells ($p < 0.05$, Figure 4D). Consistent with the migration assay results, HCC cells overexpressing hsa_circ_103809 exhibited a significant increase in cell invasion ability compared with the control cells ($p < 0.05$, Figure 4C). Collectively, these results indicate that hsa_circ_103809 can prevent cell motility, migration and invasion in Hep-3B and QGY-77 cells.

Hsa_circ_103809 Serves as a Sponge for miR-620

Because hsa_circ_103809 was down-regulated in HCC tissues and cells and could suppress cell proliferation and invasion in HCC cell lines, we considered the mechanism through which it participates in the pathogenesis of HCC. Accumulating evidence has suggested that circRNAs could function as miRNA sponges. TargetScan (<http://www.targetscan.org>) analysis predicted that hsa_circ_103809 may recognize binding sites on miR-620 (Figure 5A). To demonstrate the interaction of hsa_circ_103809 with miR-620, a fragment of hsa_circ_103809 including the predicted

target site or the mutant target site was ligated downstream of the firefly luciferase gene (named hsa_circ_103809-Wild and hsa_circ_103809-Mut, respectively) (Figure 5A). The constructs were co-transfected with miR-620 mimics or negative control (NC) into Hep-3B and QGY-77 cells. The miR-620 mimics induced a reduction in the relative luciferase expression in the hsa_circ_103809-Wild group compared with the negative control group. In contrast, no difference was detected in the luciferase activity of hsa_circ_103809-Mut between the miR-620 mimic and control groups (Figure 5B-C). These data demonstrate that miR-620 directly targets hsa_circ_103809 *in vitro*. Moreover, the results from the qRT-PCR assays showed hsa_circ_103809 could down-regulate miR-620 expression levels in HCC cells ($p < 0.05$, Figure 5D).

hsa_circ_103809 Prevents the Proliferation and Invasion of HCC Cells by Down-Regulating miR-620

To investigate the functional relationship between hsa_circ_103809 and miR-620 in HCC cell lines, three siRNA sequences were designed for the splice site of hsa_circ_103809 (Figure 5E). After verification, siRNA1 was found to be

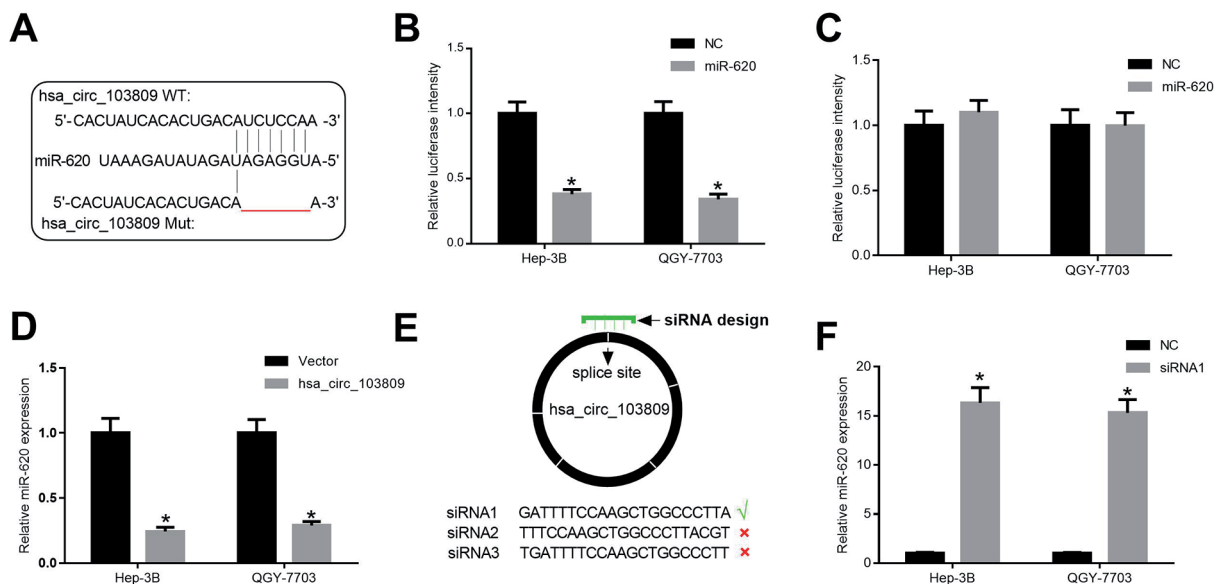


Figure 5. Hsa_circ_103809 acts as a sponge for miR-620. (A) Sequence alignment of human miR-620 with hsa_circ_103809. Bottom: mutations in the hsa_circ_103809 sequence were used to create mutant luciferase reporter constructs. (B) Activities of a luciferase reporter containing the wild-type hsa_circ_103809 3'UTR in Hep-3B and QGY-77 cells after transfection with negative control or miR-620 mimics. (C) Activities of a luciferase reporter containing the mutated hsa_circ_103809 3'UTR in HCC cells after transfection with miR-620 mimics or negative control. (D) Real-time PCR was used to detect miR-620 expression in HCC cells transfected with the hsa_circ_103809-overexpressing plasmid and empty vector control (* $p < 0.05$). (E) Three siRNA sequences were designed for the splice site of hsa_circ_103809, but only siRNA1 was found to be effective. (F) Expression levels of miR-620 in HCC cells transfected with hsa_circ_103809 siRNA or negative control (* $p < 0.05$).

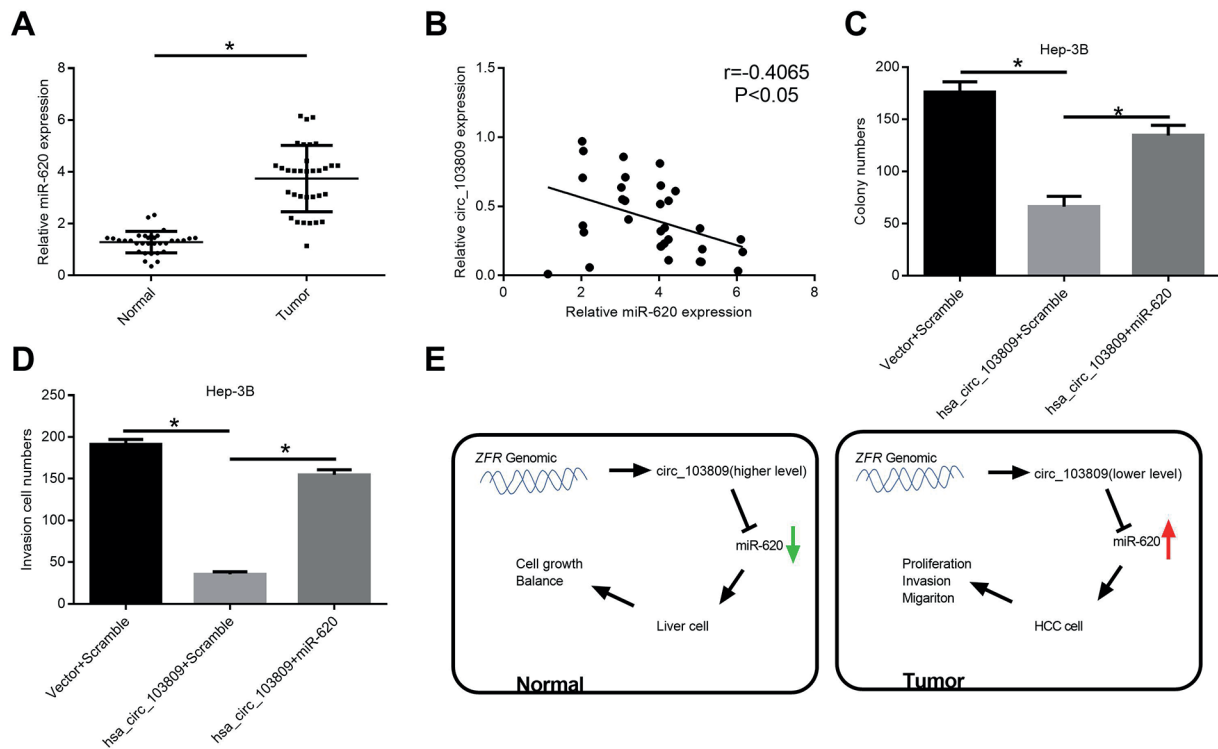


Figure 6. Hsa_circ_103809 prevents the proliferation and invasion of HCC cells by down-regulating miR-620. **(A)** Dot plots of miR-620 levels detected by qRT-PCR assays in HCC tissues and adjacent non-cancerous tissues ($n = 33$, $*p < 0.05$). **(B)** Correlation analysis of the relationship between hsa_circ_103809 and miR-620 expression levels in HCC tissues ($r = -0.4065$, $p < 0.05$). **(C)** Clonogenic capacity of Hep-3B cell overexpressing hsa_circ_103809 transfected with or without miR-620 mimics ($*p < 0.05$). **(D)** Invasion capacity of Hep-3B cells overexpressing hsa_circ_103809 transfected with or without miR-620 mimics ($*p < 0.05$). **(E)** Regulatory mechanism of hsa_circ_103809 in HCC and normal liver cells.

effective, whereas both siRNA 2 and siRNA 3 were invalid (data not shown). qRT-PCR assays were used to confirm the transfection effects ($p < 0.05$, Figure 5F). We performed qRT-PCR assays to measure miR-620 expression levels in paired HCC tissues and non-tumour tissues in 33 patients. The result showed that miR-620 was down-regulated in HCC tissues compared with that in non-tumour tissues ($p < 0.05$, Figure 6A). Moreover, hsa_circ_103809 was found to be negatively correlated with miR-620 ($r = -0.4065$, $p < 0.05$, Figure 6B). We have previously shown that hsa_circ_103809 could prevent the colony formation and invasion of HCC cells. The data in Figure 6C-D show that the overexpression of miR-620 by miR-620 mimics could attenuate the inhibitory effects induced by hsa_circ_103809 in Hep-3B cells ($p < 0.05$, Figure 6C-D), suggesting that hsa_circ_103809 could interfere with the proliferation and invasion of HCC cells by down-regulating miR-620.

Discussion

CircRNAs are a class of covalently closed RNA molecules that typically comprise single or multiple exon sequences, as well as introns^{14,23}. Compared with miRNAs and long noncoding RNAs, the abundance of circRNAs is relatively low. Nevertheless, with the development of high-throughput sequencing and bioinformatics analysis, increasing numbers of novel circRNAs are being identified; these circRNAs have been shown to be involved in cancer occurrence and progression and may be valuable prognostic biomarkers²⁴⁻²⁶. However, the expression profile and function of circRNAs in HCC are not entirely clear. In the present study, we first identified hsa-circ-103809, derived from exon 17 to exon 13 of the ZFR gene, to be decreased in HCC tissues compared with matched non-tumour tissues. The overexpression of hsa_circ_103809 could suppress the proliferation, colony formation, migration and invasion abilities of Hep-3B

and QGY-77 cells. Many circRNAs are known to play critical roles in cancer, but their exact mechanisms of action remain unclear. Kristensen et al²⁴ summarized the potential functions of circRNAs, which may account for their interference with cancer. The most common hypothesis is as follows. First, many circRNAs can function as miRNA sponges or decoys, thereby regulating target mRNA translation^{24,27,28}. Moreover, several circRNAs with multiple binding sites for a single or multiple RNA-binding proteins could function as protein sponges or decoys^{24,29,30}. In addition, circRNAs can function as protein scaffolds to connect two or more proteins^{24,31}. Most circRNAs are located in the cytoplasm and play the roles described above. However, a handful of circRNAs retained in the nucleus may be involved in transcription and splicing^{24,32,33}. To explore the mechanism of action of hsa_circ_103809, we performed a bioinformatics analysis using TargetScan and found that miR-620 contained the complementary binding region with hsa_circ_103809, which was confirmed by dual-luciferase reporter assays. Rescue experiments revealed that miR-620 could reverse the inhibitory effect of hsa_circ_103809 on Hep-3B cell colony formation and invasion. All of these findings suggest that hsa_circ_103809 can act as a miR-620 sponge to suppress HCC proliferation and invasion. There are currently few reports about miR-620. Increased miR-620 levels have been reported to induce radio-resistance, promote cell proliferation and deregulate the G2/M checkpoint following irradiation by targeting hydroxyprostaglandin dehydrogenase/15-nicotinamide adenine dinucleotide (HPGD/15-PGDH)²⁰. Here, we demonstrate that hsa_circ_103809 directly targets miR-620 and decreases its expression in HCC cells. However, the exact functions and mechanism of action of miR-620 in HCC cell progression remain unknown. Moreover, our study revealed that hsa_circ_103809 could act as a miR-620 sponge to exert an anti-cancer role in HCC progression. However, we could not exclude the possibility that other important mechanisms may be involved, and we will continue to focus on this issue in our future studies.

Conclusions

We showed that hsa_circ_103809 expression is down-regulated in HCC tissues and in HCC cell lines. The overexpression of hsa_circ_103809 can inhibit HCC cell growth and invasion by binding

to miR-620, which increases the possibility that hsa_circ_103809 may serve as a biomarker in HCC patients. These findings provide novel insight into the functional mechanisms of noncoding RNAs in HCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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