

## Original Article

# Hsa\_circ\_0103809 promotes cell proliferation and inhibits apoptosis in hepatocellular carcinoma by targeting miR-490-5p/SOX2 signaling pathway

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**Abstract:** Background: Circular RNAs (circRNAs) represent a class of non-coding RNAs that are emerging as important regulators during tumorigenesis and provide potential targets for cancer intervention. However, the expression profiles and functions of circRNAs in hepatocellular carcinoma (HCC) have not been completely clarified. Herein, the role of hsa\_circ\_0103809 was investigated in HCC tissues and cell lines. Methods: High-throughput circRNA sequencing was performed to detect the expression profiles of circRNA in HCC tissues. The CCK-8, wound healing and flow cytometry were performed to measure the cell viability, migration and apoptosis in HCC cells. The expression levels of gene and protein in HCC tissues and cell lines were assayed by RT-qPCR and western blotting, respectively. Immunohistochemical staining was used to assess the protein expression of SOX2 in HCC tissues. Results: We discovered that hsa\_circ\_0103809 was significantly increased in HCC tissues and cell lines. Knockdown of hsa\_circ\_0103809 inhibited proliferation and migration and induced apoptosis in HCC cell lines. Investigation to the molecular mechanisms of hsa\_circ\_0103809 in HCC cells had revealed that hsa\_circ\_0103809 directly suppressed miR-490-5p, which targeted to the 3'-UTR of SOX2. Hsa\_circ\_0103809 loss-of-function could increase the expression of miR-490-5p as well as decreased the expression of SOX2. Furthermore, we found that si-0103809 induced growth and migration inhibition and apoptosis could be reversed by transfected with miR-490-5p inhibitors or SOX2 in HCC cells. Conclusion: Our findings suggested that hsa\_circ\_0103809 might facilitate HCC malignant progression, at least partially, by regulating miR-490-5p/SOX2 signaling pathway.

**Keywords:** Hepatocellular carcinoma, circRNA, hsa\_circ\_0103809, miR-490-5p, SOX2

## Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the second leading cause of global cancer-associated mortality with a 1-year survival rate less than 50%, and 50% of the cases and deaths occurred in China [1, 2]. Cancer statistics in China have shown that approximately 466,100 cases are newly diagnosed with liver cancer, and approximately 422,100 deaths occur annually [3]. With the rapid development of diagnostic and therapeutic strategies, patients with HCC can be detected in the early stages and have a favorable prognosis with radical surgeries [4]. However, approximately 70% of HCC patients are diagnosed the most advanced stages and miss the best surgical opportunities

[4]. Therefore, it is imperative to identify potential biomarkers for clinical diagnosis and find new therapeutic targets to design a more powerful therapeutic approach.

In recent years, the rise of the functional study of circular RNAs (circRNAs) in the field of biology elicit a potentially role of circRNAs in the development and progression of cancers [5, 6]. CircRNAs represent a new class of non-coding RNAs, which are characterized by high stability, covalently closed continuous loop, without 5' to 3' polarity and polyadenylated tail [7]. CircRNAs are widely expressed in mammals with cell-type, tissue and developmental phase specific manner, suggesting that circRNAs may be involved in many physiological and pathological processes [8]. Concerning their function, cir-

circRNAs can serve as competing endogenous RNAs (ceRNAs) or miRNA sponges, interact with RNA binding proteins, modulate the stability of mRNAs and regulate gene transcription and translating proteins [9-11]. Recent studies have demonstrated that circRNAs perform their function mainly by acting as miRNA sponges [9, 12]. It is well known that circRNA ciRS-7 can act as a miRNA-7 sponge and contains more than 70 selectively conserved miR-7 target sites [12]. Moreover, circ-HIPK3 [13], circ-ITCH [14] and circMTO1 [8] are able to act as miRNA sponges. CircRNAs have been recently verified to be involved in the development of cancers, including oral cancer [6], gastric cancer [15], breast cancer [16] and HCC [8]. These observations suggest that circRNAs may be a new kind of potential biomarkers and therapeutic targets for cancer. However, differentially expressed circRNAs and their functions have not been completely clarified in the progression of HCC.

In our study, high-throughput sequencing was carried out to investigate the expression profile of circRNAs in HCC tissues and adjacent non-tumorous tissues. We first found that the expression of hsa\_circ\_0103809 was increased in HCC tissues and cell lines. In addition, knockout of hsa\_circ\_0103809 resulted in the inhibition of growth and migration and the induction of apoptosis in HCC cell lines. Furthermore, our findings demonstrated that hsa\_circ\_0103809 could serve as a sponge for miR-490-5p, which directly targeted to the 3'-untranslated region (3'-UTR) of sex-determining region Y-box 2 (SOX2) mRNA and inhibited the expression of SOX2. In collection, these findings indicate that hsa\_circ\_0103809 may be an oncogene in the progression of HCC, and hsa\_circ\_0103809/miR-490-5p/SOX2 signaling pathway may be a promising therapeutic target for HCC treatment.

## Material and methods

### *Patients and specimens*

Seventy-eight pairs of HCC tissues and corresponding adjacent normal tissues were acquired from patients underwent surgical operation in the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between Jan 2010 and June 2015. Samples were rapidly stored in liquid nitrogen for high-throughput RNA sequencing, RT-qPCR and western blotting

assay. Written informed consent was obtained from all of the participants prior to samples collection. The study was approved by the Ethics Committee of the the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

### *Cell culture*

Five human liver cancer cell lines (MHCC97H, HepG2, Huh7, SMMC7721 and SK-Hep1) and a normal liver cell line (HL-7702) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Thermo Scientific HyClone, Beijing, China), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified incubator (Thermo, USA), 5% CO<sub>2</sub>, 95% air atmosphere.

### *Cell transfection and plasmid constructs*

The small interfering RNA (si-RNA) was utilized for cell transfection and was synthesized by RiboBio (Guangzhou, China). The targeted sequence of the functional si-0103809 was 5'-AAGACTTGGTGGTTTTGATGA-3', and si-circRNA-NC was 5'-CCATGGTGAAGTCGTGAAGTT-3'. HepG2 and Huh7 cells were transfected with si-0103809 or si-circRNA-NC for 48 h at 37°C using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The miR-490-5p mimics (5'-CCAUGGAUACUCCCCAGGCGGGU-3'), anti-miR-490-5p (5'-ACCGCCUGGGGAGUAUCCAUGG-3') or miR-NC (5'-GGUCGACCGUGAGGUCGCGAGAG-3') were synthesized by RiboBio (Guangzhou, China) and transfected into HepG2 and Huh7 cells using the Lipofectamine™ RNAiMAX (Life Technologies, USA) according to the manufacturer's instructions. The wild-type (WT) or mutant-type (MUT) 3'-UTR of SOX2 were inserted into the multiple cloning sites of the luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.). The sequence of human SOX2 was obtained from reverse transcription (RT) of total RNA, which was extracted from HCC tissues. pcDNA3.1-SOX2 was constructed by inserting a NheI-XbaI fragment of SOX2 encoding sequence. Primers used for plasmid construction are listed as follows: forward primer 5'-GGAGACCCAAGCTGGCTAGCATGTACAACATGATGGAGACGG-3' and reverse primer 5'-GTTTAAACGGGCCCTCTAGATCACATGTGTGAGAGG-

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GGC-3'. pcDNA3.1-SOX2 were transfected into HepG2 and Huh7 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

### *Total RNA extraction and high-throughput sequencing*

RNA extraction from tumor tissues, non-tumorous tissues and cell lines was performed using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and preserved at -80°C until use. RNA concentration was measured using NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (approximately 4 µg) from each sample was subjected to the RiboMinus Eukaryote Kit (Qiagen) to eliminate ribosomal RNA. Purified RNAs were treated with RNase R (Epicenter, 40 U, 37°C, 3 h). Subsequently, using the NEBNext<sup>®</sup> Ultra<sup>™</sup> RNA Library Prep Kit, RNA-seq libraries were prepared and subjected to deep sequencing with an Illumina HiSeq 3000 by RiboBio (RiboBio, Co. Ltd., Guangzhou, China). Differentially expressed circRNAs were selected by *P* value less than 0.01 and  $|\text{Log}_2 \text{fold change}| \geq 1$ , and the analyze methods were performed as previously described [17, 18].

### *Cell viability detection by CCK-8*

The CCK-8 assay was performed as previously described [19], human HCC cells ( $1 \times 10^4$ ) were seeded in the 96-well plate for 24 hours, 48 hours and 72 hours with different treatment. Cell ( $1 \times 10^4$ ) proliferation was measured using CCK-8 Cell Proliferation/Viability Assay Kit (Dojindo Japan). Absorbance was recorded at 450 nm using Elx800 Reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

### *Wound healing assay*

HepG2 and Huh7 cells ( $2 \times 10^5$ ) were trypsinized and reseeded in each well of a new 6-well plate. With incubation 24 h, the confluent cells monolayers were scratched with a 10 µL sterile pipette tip. Then the non-adherent cells were washed off with sterilized PBS and serum-free medium was added into the wells. The gap area caused by the scratch was monitored by the inverted microscope (Olympus, Japan). Three random non-overlapping areas in each well were pictured at 12 h post-scratch. Scratch width between the two linear regions was quan-

titated for assessing capacity of cells migration.

### *Flow cytometry for apoptosis*

The cell apoptosis assay was determined as previously described [20]. Annexin V-FITC apoptosis detection kit was purchased from Invitrogen (Carlsbad, Calif, USA). The samples were analyzed using flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

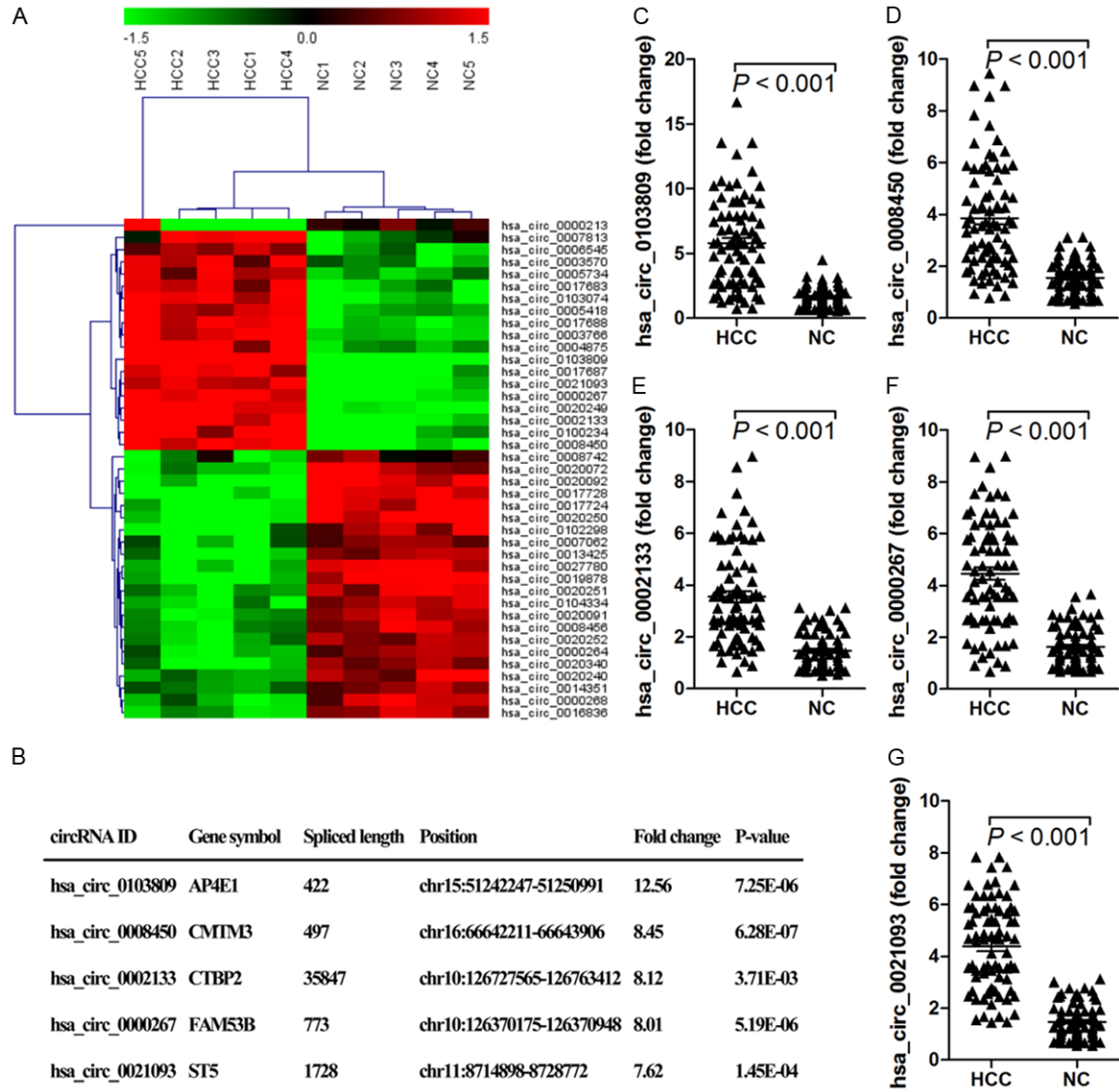
### *Luciferase reporter assay*

HepG2 and Huh7 cells ( $1 \times 10^5$ ) were seeded into 24-wells and co-transfected with luciferase reporter vectors containing the WT or MUT of SOX2 (0.5 µg) or hsa\_circ\_0103809 (0.5 µg) and miR-145 mimics or negative control (50 nM) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The luciferase activity was measured using a dual luciferase reporter assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

### *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Total RNAs were extracted using TRIzol (Invitrogen). Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA) was used to synthesize cDNA. Divergent primers were designed to amplify the head-to-tail splicing of circRNA using ABI7300 System (Applied Biosystems, Foster City, CA, USA) with SYBR Select Master Mix (Applied Biosystems). The PCR primers were as follows: hsa\_circ\_0103809: forward primer 5'-TGCTCCAGTTGACAGGAGT-3' and reverse primer 5'-TCTCTGTGGATAGAACACATTTTCC-3'; hsa\_circ\_0008450: forward primer 5'-GGACTCTGCAGATGAGACCA-3' and reverse primer 5'-AAGTACAAGGCCAGCAGGAAC-3'; hsa\_circ\_0002133: forward primer 5'-CCATAAAGAATGGCCCTTGTGG-3' and reverse primer 5'-ACTTGGACCCACACATCAC-3'; hsa\_circ\_000267: forward primer 5'-AACGACAAGAAGGTCGGTGT-3' and reverse primer 5'-TTTTCA-GGCAGGCATTCCCA-3'; hsa\_circ\_0021093: forward primer 5'-CTCCTTCCCATCTACGGGTG-3' and reverse primer 5'-AAGGGCGACTCCATGAGACTT-3'; miR-490-5p: forward primer 5'-CCATGGATCTCCAGGTGGGT-3' and reverse primer 5'-TGGTGTCTGGAGTCG-3'; SOX2: for-

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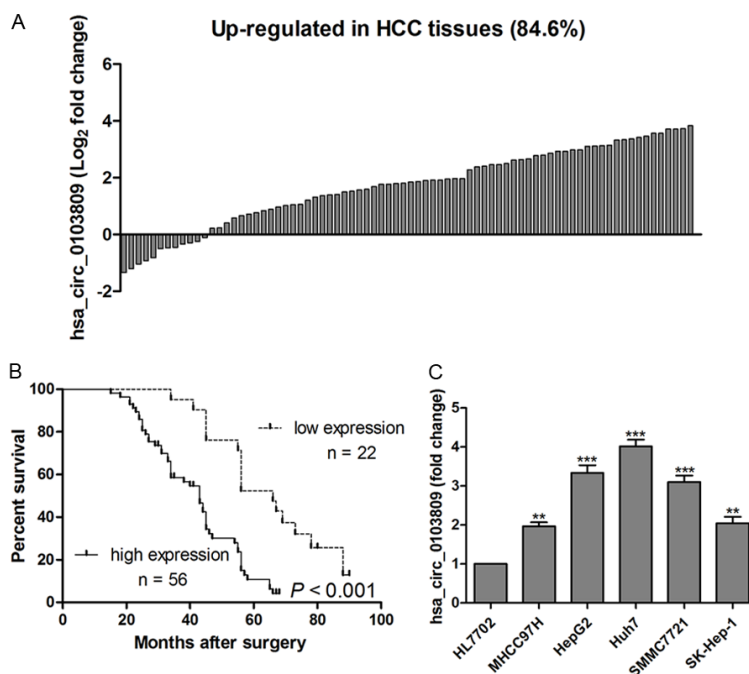
**Figure 1.** Deregulated circRNAs in HCC tissues. The heat map represents significant differentially expressed circRNAs in 5 pairs of HCC tissues and corresponding nontumorous tissues (A). The characteristics of top 5 up-regulated circRNAs (B). The levels of hsa\_circ\_0103809 (C), hsa\_circ\_0008450 (D), hsa\_circ\_0002133 (E), hsa\_circ\_0000267 (F) and hsa\_circ\_0021093 (G) are measured by RT-qPCR assay.

ward primer 5'-AACCCCAAGATGCACAACCTC-3' and reverse primer 5'-CGGGGCCGGTATTTA-TAATC-3'; GAPDH: forward primer 5'-GCAC-CGTCAGCTGAGAAC-3' and reverse primer 5'-TGGTGAAGACGCCAGTGA-3'; U6: forward primer 5'-CTCGCTCGGCAGCACATATACT-3' and reverse primer 5'-ACGCTTCACGAATTTGCGT-GTC-3'. The relative expression levels of circRNAs, miR-490-5p and SOX2 were calculated using the  $2^{-\Delta\Delta Ct}$  method [21] and normalized to the internal control U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were synthesized by Sangon Biotech (Shanghai, China).

### Western blotting

Proteins extraction and separation were performed as previously described [22]. The primary antibody of SOX2 was purchased from Santa Cruz Biotechnology (Cat.no. sc-365964; dilution: 1:1,000; Santa Cruz, CA, USA). Subsequently, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cat.no: sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology) at room temperature for 2 h and visualized by chemiluminescence (Thermo Fisher Scientific, Inc.). Signals were analyzed with Quantity One®

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**Figure 2.** Hsa\_circ\_0103809 is up-regulated in HCC tissues and cell lines and associates with poor prognosis in HCC patients. The fold change of hsa\_circ\_0103809 expression in 78 pairs of HCC tissues and matched adjacent nontumorous tissues was calculated based on RT-qPCR assay (A). Kaplan-Meier survival curve was used to evaluate whether hsa\_circ\_0103809 expression level is associated with overall survival rate in HCC patients (B). The levels of hsa\_circ\_0103809 in five human liver cancer cell lines (MHCC97H, HepG2, Huh7, SMMC7721 and SK-Hep1) and a normal liver cell line (HL7702) is detected by RT-qPCR assay (C). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA).

### Immunohistochemical staining

The paraffin-embedded tumor tissues were cut into 3  $\mu\text{m}$  sections and mounted on glass slides for staining with immunoperoxidase, and the procedures of immunohistochemical staining of SOX2 (Cat.no. sc-365964; dilution: 1:100; Santa Cruz Biotechnology) were performed as previously described [22]. The pictures were visual under a microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). Image Pro-Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA) was used for the analysis of the integrated optical density in the HCC tissues and corresponding adjacent normal tissues of immunohistochemical positive staining.

### Statistical analysis

Data were presented as mean  $\pm$  SEM. Statistical analysis was performed using GraphPad Prism

Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student  $t$ -test was used to analyze two-group differences. Inter-group differences were analyzed by one-way analysis of variance, followed by Tukey's post hoc analysis. Survival analysis was performed using the Kaplan-Meier method with the log-rank test applied for comparison. Spearman's rank analysis was used to identify the correlation between hsa\_circ\_0103809 and miR-490-50, or hsa\_circ\_0103809 and SOX2, or miR-490-5p and SOX2.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### CircRNA expression profiles in HCC tissues

Firstly, circRNA high-throughput sequencing was used to analyze circRNA expression profiles in 5 pairs HCC tissues and corresponding nontumorous tissues (NC group).  $P$  value

less than 0.01 and  $|\text{Log}_2 \text{fold change}| \geq 1$  were performed as the criteria in filtering significantly differently expressed circRNAs. The results demonstrate that forty-one circRNAs were differentially expressed between HCC and NC groups, among which nineteen circRNAs and twenty-one circRNAs were upregulated and downregulated, respectively (Figure 1A). Next, we selected out top 5 up-regulated circRNAs, the characteristics were shown in Figure 1B, and examined the expression of these 5 mostly changed circRNAs in HCC tissues and nontumor tissue samples from 78 patients to confirm their expression by RT-qPCR. Consistent with high-throughput sequencing results, the expression of hsa\_circ\_0103809 (Figure 1C), 0008450 (Figure 1D), 0002133 (Figure 1E), 0000267 (Figure 1F) and 0021093 (Figure 1G) was significantly increased in HCC tissues compared with corresponding nontumorous tissues from HCC patients. Based on the fold change of circRNAs in HCC tumor to corresponding nontumorous tissues, hsa\_circ\_0103809 was selected to focus on our further study.

RT-qPCR results also found that 66 of these cases (84.6%) exhibited up-regulation of hsa\_circ\_0103809 levels in HCC tissues as compared to corresponding nontumorous tissues (**Figure 2A**). In addition, Kaplan-Meier analysis was performed to evaluate the association of hsa\_circ\_0103809 levels in HCC tissues and prognosis in HCC patients. HCC patients were segregated into hsa\_circ\_0103809 high expression group and low expression group according to  $\text{Log}_2$  fold change  $\geq 1$ . HCC patients with high hsa\_circ\_0103809 levels had a significantly poorer prognosis than those of patients with low hsa\_circ\_0103809 levels (**Figure 2B**). Based on above observations, an analysis of hsa\_circ\_0103809 expression was carried out among 5 different HCC cell lines (MHCC97H, HepG2, Huh7, SMMC7721 and SK-Hep1) and a normal liver cell line HL-7702. These findings suggested that hsa\_circ\_0103809 was dramatically increased in 5 HCC cell lines compared with HL-7702 cells, especially in HepG2 and Huh7 cell lines (**Figure 2C**). Therefore, HepG2 and Huh7 cell lines were selected to study the functions of hsa\_circ\_0103809 *in vitro*.

*Silencing of hsa\_circ\_0103809 inhibits proliferation and migration and induces apoptosis in HCC cell lines*

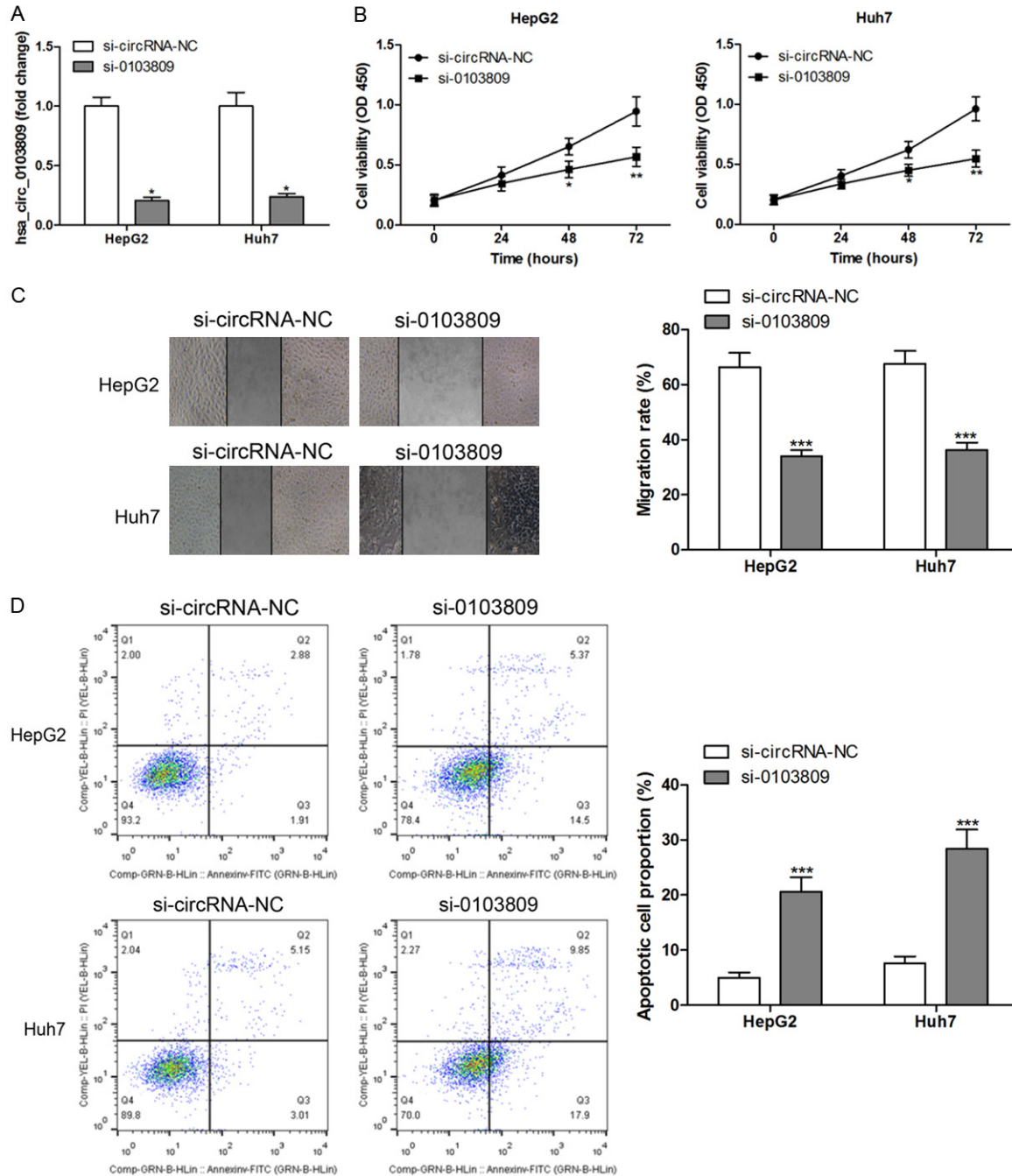
To assess the effects of hsa\_circ\_0103809 on the *in vitro* growth, migration and apoptosis of HCC cell lines, small interfering RNA (siRNA) vector targeting to hsa\_circ\_0103809 (si-0103809) was constructed, and the inhibiting rates of si-0103809 were 79% and 76% in G2 and Huh7 cell lines (**Figure 3A**), respectively. As shown in **Figure 3B**, a significant inhibition of cell proliferation was detected in HepG2 and Huh7 cells at 48 and 72 h after transfected with si-0103809 compared with si-circRNA-NC treated group. In addition, we also found that the migration of HepG2 and Huh7 cells was significantly suppressed in si-0103809 transfected group compared with si-circRNA-NC treated group (**Figure 3C**). We then performed flow cytometry analysis to further evaluate whether hsa\_circ\_0007534 is related to cell apoptosis in HCC cell lines. The results showed that the apoptotic cell proportion was markedly increased in HepG2 and Huh7 cells after transfected with si-0103809 (**Figure 3D**). These results suggest that hsa\_circ\_0103809 depletion can

inhibit proliferation and migration and induces apoptosis in HCC cell lines.

*MiR-490-5p is a potential target for hsa\_circ\_0103809*

Recently, mounting evidence has showed that circRNAs contain the complementary sequences to bind with miRNAs to inhibit their expression [8, 12, 23]. To examine whether hsa\_circ\_0103809 has a similar regulatory mechanism in HCC, miRNAs target sites were predicted by online software miRanda-mirSVR (<http://www.microrna.org>), TargetScan (<http://www.targetscan.org/>) and circinteractome (<https://circinteractome.nia.nih.gov/>). The results indicated that miR-490-5p might be a potential target of hsa\_circ\_0103809. The putative binding site of miR-490-5p on hsa\_circ\_0103809 was highlighted as shown in **Figure 4A**. To confirm this possibility, the luciferase activity reporter assays was performed in HepG2 and Huh7 cells after transfected with the wild type (WT) sequence of hsa\_circ\_0103809 or its mutant type (MUT) sequence and then co-transfected with miR-490-5p mimics or miR-NC. We found that that overexpression of miR-490-5p significantly inhibited the luciferase activity in HepG2 and Huh7 cells transfected with hsa\_circ\_0103809 WT sequence, while mutations in the putative miR-490-5p binding sites in hsa\_circ\_0103809 abolished this effect (**Figure 4A**). We then investigated the expression of miR-490-5p in HCC tissues and HCC cell lines. RT-qPCR showed a significant decrease in miR-490-5p expression in HCC tissues and HCC cell lines (**Figure 4B** and **4C**), which was showed opposite results to hsa\_circ\_0103809 expression (**Figures 1C** and **2C**). Intriguingly, Spearman's rank correlation analysis showed that the expression of miR-490-5p and hsa\_circ\_0103809 was inversely correlated in the 78 human HCC tissues (Spearman's  $R = -0.653$ ;  $P < 0.001$ ; **Figure 4D**). In view of hsa\_circ\_0103809 and miR-490-5p might have the opposite effect during HCC progression, we further investigated whether overexpression of miR-490-5p could inhibit growth and migration in HCC cells. First, HepG2 and Huh7 were transfected with miR-490-5p mimics, and the results showed that up-regulation of miR-490-5p expression with miR-490-5p mimics transfection (**Figure 4E**). Compared with si-miR-NC

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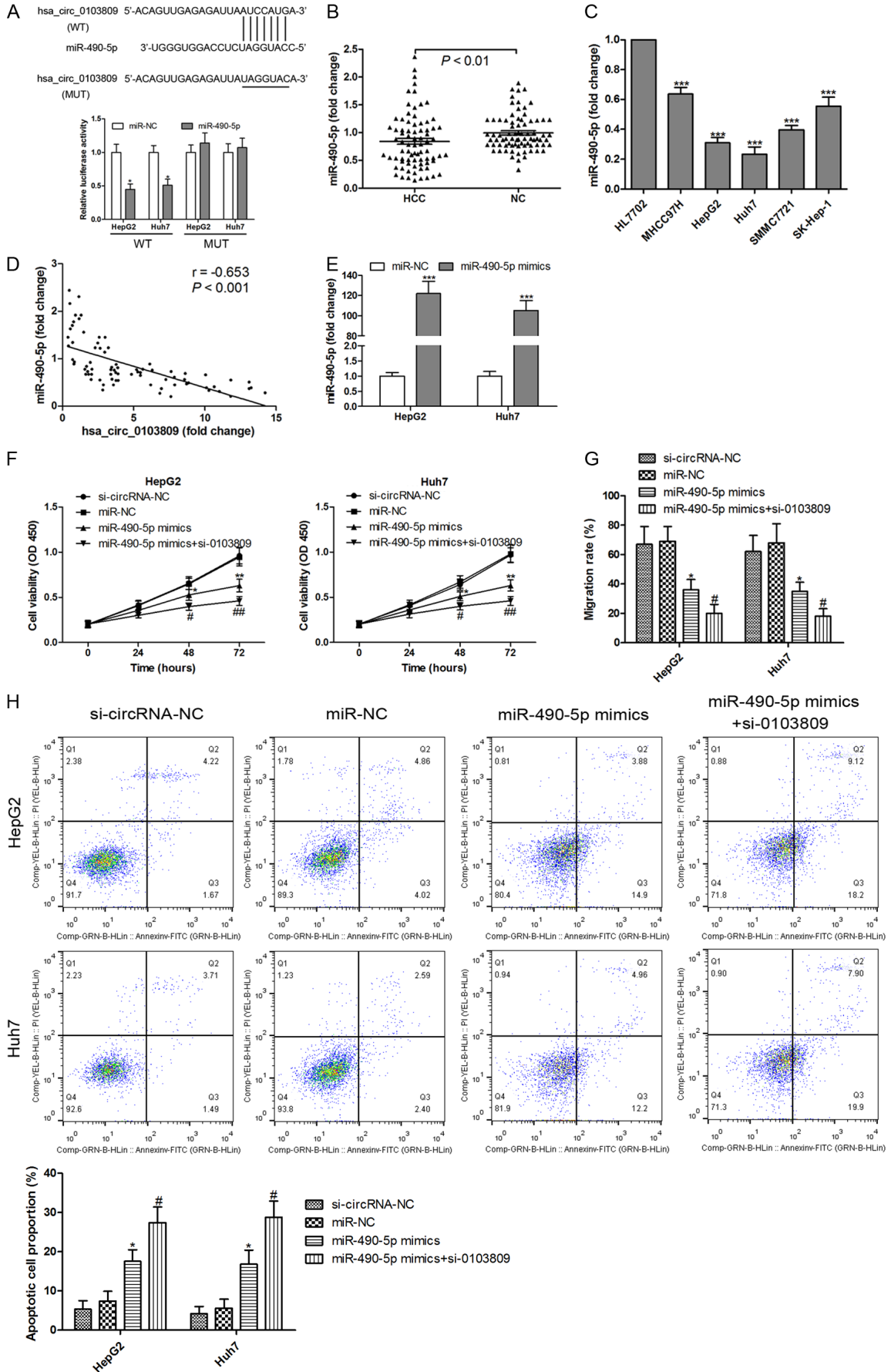


**Figure 3.** Hsa\_circ\_0103809 knockout inhibits proliferation and migration and induces apoptosis in HCC cells. Hsa\_circ\_0103809 expression is significantly reduced in HepG2 and Huh7 cell after transfected with si-hsa\_circ\_0103809 (si-0103809) (A). After transfected with si-0103809 or si-circRNA-NC, the cell viability is monitored by CCK-8 assay (B), cell migration is determined by wound healing assay (C), cell apoptosis is analyzed using flow cytometry (D). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .  $n = 3$  in each group.

group, the cell viability (**Figure 4F**) and migration (**Figure 4G**) were obviously inhibited in miR-490-5p mimics transfected HepG2 and Huh7 cells, as well as with the increase of apoptotic cell proportion (**Figure 4H**). Interestingly, si-01-

03809 co-transfected with miR-490-5p mimics significantly enhanced the effect of miR-490-5p mimics on cell growth, migration and apoptosis in HepG2 and Huh7 cells (**Figure 4F-G**).

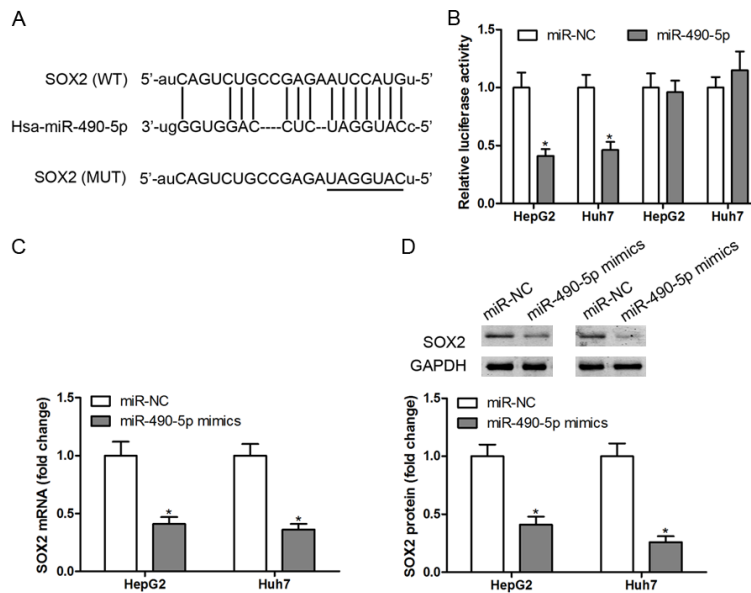
# Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 in HCC





## Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 in HCC

**Figure 4.** MiR-490-5p is a potential target for hsa\_circ\_0103809 and is a tumor suppressor gene in HCC. The putative binding sites of miR-490-5p on hsa\_circ\_0103809 are predicted by online softwares and verified by luciferase activity reporter assays (A). The expression of miR-490-5p is measured by RT-qPCR assay in HCC tissues and cell lines (B and C). The association of miR-490-5p and hsa\_circ\_0103809 is performed by Spearman's rank correlation analysis (D). MiR-490-5p expression is significantly increased in HepG2 and Huh7 cells after transfected with miR-490-5p mimics (E). After treated with si-circRNA-NC, si-miR-NC, miR-490-5p mimics or miR-490-5p mimics+si-0103809, the cell viability is monitored by CCK-8 assay (F), cell migration is determined by wound healing assay (G), cell apoptosis is analyzed using flow cytometry (H). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with miR-NC group; # $P < 0.05$ ; ## $P < 0.01$  compared with miR-490-5p mimics group.  $n = 3$  in each group.



**Figure 5.** SOX2 is a direct target gene of miR-490-5p. The putative binding sites of miR-490-5p on SOX2 are predicted by online softwares and verified by luciferase activity reporter assays (A and B). Overexpression of miR-490-5p significantly inhibits the mRNA and protein expression of SOX2 in HepG2 and Huh7 cells (C and D). \* $P < 0.05$  compared with control group.  $n = 3$  in each group.

### SOX2 is a direct target gene of miR-490-5p

To investigate whether SOX2 was a direct target of miR-490-5p, the online predict softwares miRanda-mirSVR and TargetScan were used for prediction the potential binding sites. As shown in **Figure 5A**, the 3'-untranslated regions (3'-UTRs) of SOX2 contained one conserved binding site of miR-490-5p. To confirm this, the luciferase reporter plasmids containing WT or MUT 3'-UTR of SOX2 were constructed and co-transfected with miR-490-5p mimics or miR-NC. The findings showed that miR-490-5p mimics significantly inhibited the luciferase activity in HepG2 and Huh7 cells transfected with SOX2 WT sequence, while mutations of the putative miR-490-5p binding sites in SOX2 abolished this effect (**Figure 5B**). In addition, we also found that the mRNA (**Figure 5C**) and protein

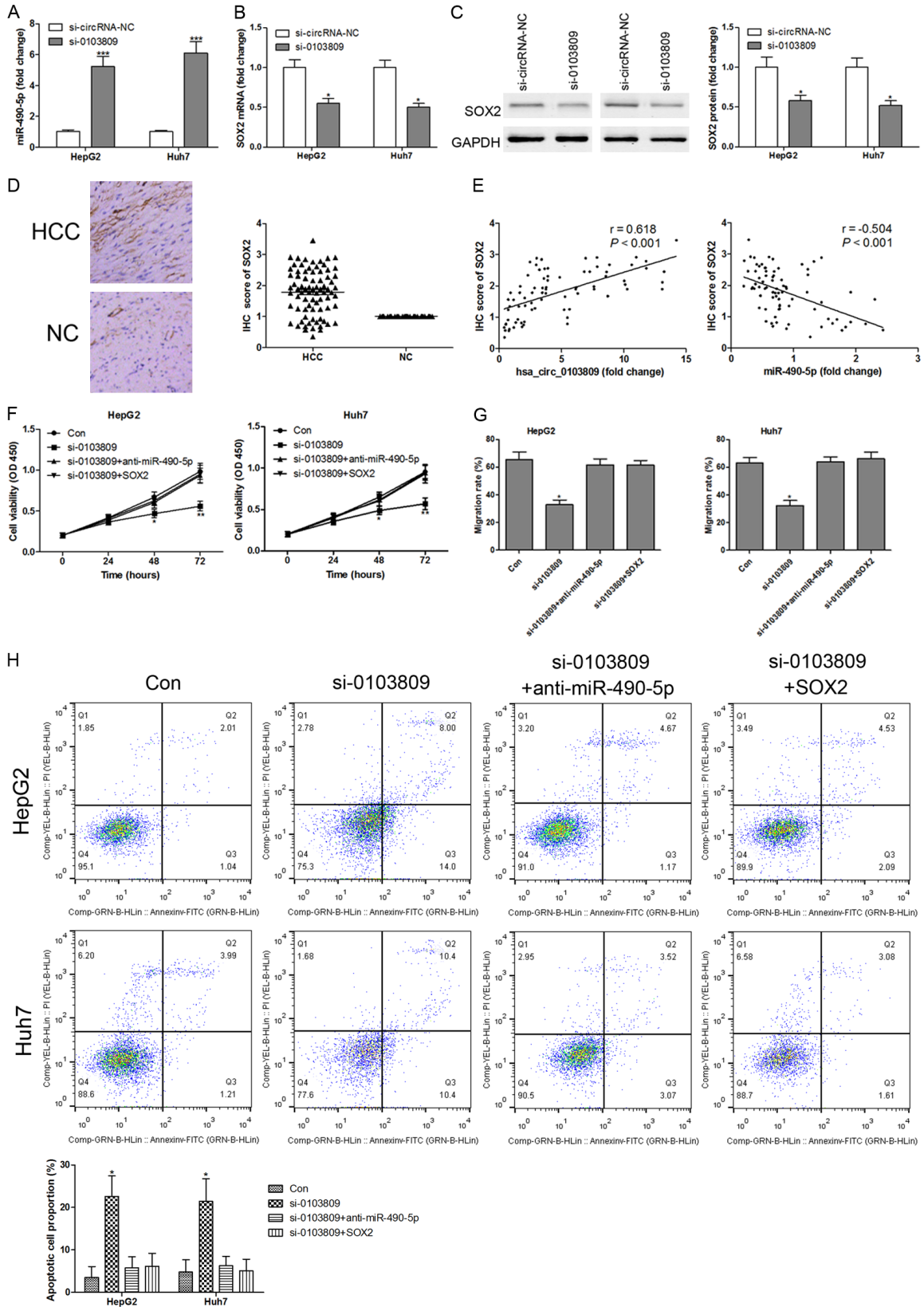
(**Figure 5D**) expression of SOX2 were significantly decreased in miR-490-5p mimics transfected HepG2 and Huh7 cells compared with control group.

### Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 signaling in HCC progression

To further determine whether hsa\_circ\_0103809-mediated effects on HCC depends on miR-490-5p/SOX2 signaling, we checked the expression levels of miR-490-5p and SOX2 in HepG2 and Huh7 cells after transfected with si-0103809 or si-circRNA-NC. As shown in **Figure 6A-C**, up-regulation of miR-490-5p and down-regulation of mRNA and protein expression of SOX2 were observed in hsa\_circ\_0103809-deleted HepG2 and

Huh7 cells. Subsequently, we detected the protein expression of SOX2 in HCC tissues by immunohistochemical staining. As shown in **Figure 6D**, the immunostaining intensity of SOX2 in HCC tissues was obviously higher than that of adjacent nontumourous tissues. Spearman's rank correlation analysis showed that the expression of SOX2 were positively correlated with hsa\_circ\_0103809 (Spearman's  $R = 0.618$ ;  $P < 0.001$ ; **Figure 6E**), as well as inversely correlated with miR-490-5p (Spearman's  $R = -0.504$ ;  $P < 0.001$ ; **Figure 6E**), in the 78 human HCC tissues. Furthermore, we found that inhibition of hsa\_circ\_0103809 significantly inhibited cell growth and migration and induced apoptosis in HepG2 and Huh7 cells (**Figure 6F-H**), which were reversed by transfected with miR-490-5p inhibitors and SOX2 (**Figure 6F-H**).

# Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 in HCC



**Figure 6.** Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 signaling in HCC progression. The expression of miR-490-5p is measured by RT-qPCR assay in HepG2 and Huh7 cells after transfected with si-0103809 (A). The mRNA and protein expression of SOX2 were detected by RT-qPCR and western blotting, respectively, in HepG2 and Huh7 cells

## Hsa\_circ\_0103809 targets to miR-490-5p/SOX2 in HCC

after transfected with si-0103809 (B and C). The protein expression of SOX2 is measured by immunohistochemical staining in HCC tissues (D). The correlation between SOX2 and hsa\_circ\_0103809, or SOX2 and miR-490-5p, is performed by Spearman's rank correlation analysis (E). After treated with Con, si-0103809, si-0103809+miR-490-5p inhibitors or si-0103809+SOX2, the cell viability is monitored by CCK-8 assay (F), cell migration is determined by wound healing assay (G), cell apoptosis is analyzed using flow cytometry (H). \* $P < 0.05$ , \*\* $P < 0.01$  compared with Con, si-0103809+miR-490-5p inhibitors or si-0103809+SOX2 group.  $n = 3$  in each group.

### Discussion

In recent years, the roles of circRNAs in the occurrence and development of cancer are rapidly explored with the development of high-throughput circRNA sequencing techniques, and more than 20,000 circRNAs have been identified in eukaryotes [5]. Meanwhile, functional studies of circRNAs are continuously being extended and deepened in multiple physiological and pathological processes [12]. Increasing evidence suggests that circRNAs can function as competing endogenous RNAs (ceRNAs) or miRNA sponges, which is the classical model of circular RNA function, in the progression of tumours, serving as roles of oncogenes or tumor suppressor genes [6, 8]. CircRNA-miRNA-mRNA axis may function as an extensive regulatory network in gene expression, and their deregulation may cause disease progression including cancer development [8]. For example, circRNA\_100290 as an oncogene plays a role in oral cancer by functioning as a sponge of the miR-29 family and co-expressed with CDK6 [6]. Moreover, circMTO1 acts as the sponge of miR-9 to suppress HCC progression [8]. In our study, we found that hsa\_circ\_0103809 served as a decoy of the miR-490-5p to regulate SOX2 expression in HCC, and our results demonstrated that hsa\_circ\_0103809 might be functioned as an oncogene to promote HCC malignant progression. Thus, we have reason to believe that hsa\_circ\_0103809 may be a novel diagnostic and prognostic biomarker and a potential therapeutic target for HCC.

Hsa\_circ\_0103809 is located in chr15: 5124-2247-51250991, the spliced sequence length is 422 nt, and its associated-gene symbol is AP4E1 (adaptor related protein complex 4 epsilon 1 subunit; circBase database, <http://www.circbase.org/>). In our study, we identified that hsa\_circ\_0103809 was increased in HCC tissues and cell lines and was determined to be correlated with cells proliferation, migration and apoptosis in HepG2 and Huh7 cells. Moreover, the increased hsa\_circ\_0103809

expression in HCC tissues was significantly correlated with poor prognosis of HCC patients. Previous study indicates that hsa\_circ\_0000190 is down-regulated in both gastric cancer (GC) tissues and plasma from patients with GC and can serve as a blood-based non-invasive biomarker for the diagnosis of GC [24]. In addition, hsa\_circ\_0013958 also can be used as a potential non-invasive biomarker for the early detection and screening of lung adenocarcinoma [25]. In HCC, hsa\_circ\_0005075, hsa\_circ\_0001649 and circMTO1 show good diagnostic potential and are considered as the potential novel biomarkers for HCC screening [8, 26, 27]. Our results also provide strong evidence that hsa\_circ\_0103809 can serve as a diagnostic marker for HCC. However, previous studies [8, 26, 27] and our study have not provided evidence that circRNAs can be detected in plasma or serum and are associated with HCC non-invasive examination, which is interesting and important for circRNA functions and requires further investigation.

Molecular mechanisms studies have revealed that circRNAs can promote miRNAs degradation, which is similar to the function of miRNA sponges or antagomirs [10, 12], however, the exact mechanism is still unclear in HCC. Therefore, we determined to explore whether hsa\_circ\_0103809 could regulate miRNA expression in the progression. Based on online predict softwares and luciferase activity reporter assay, we verified that hsa\_circ\_0103809 directly targeted to miR-490-5p in HepG2 and Huh7 cells. miR-490-5p has been reported to be down-regulated in a variety of cancers and cell lines and is considered as a potential tumour suppressor [28-30]. In our study, RT-qPCR assay proved that down-regulation of miR-490-5p level was a frequent event in HCC tissues (74.4%). Moreover, the expression of miR-490-5p was also decreased in HCC cell lines, however, overexpression of miR-490-5p significantly inhibited cell growth and invasion while promoting cell apoptosis in HepG2 and Huh7 cells. miR-490-5p loss-of-function significantly weakened the effects of si-0103809 on growth,

migration and apoptosis in HepG2 and Huh7 cells. These findings suggested the potential tumor suppressive role of miR-490-5p in HCC.

To explore the underlying molecular mechanisms of miR-490-5p in HCC progression, we focused on its post-transcriptional mechanism and proved that SOX2 was a direct target gene of miR-490-5p. SOX2 is a transcription factor functionally important for maintaining stemness in normal stem cells [31]. Meanwhile, SOX2 is a notorious oncogene, which has been implicated in maintaining tumorigenic state in cancer [32]. Numerous of studies show that SOX2 is overexpressed in HCC tissues and correlates with metastasis and low survival rate in HCC patients [33, 34]. In addition, both miR-126 and miR-638 can inhibit migration and invasion in HCC cells by suppressing SOX2 expression [35, 36]. In our study, RT-qPCR and western blotting assay showed that both hsa\_circ\_0103809 knockdown and miR-490-5p overexpression significantly suppressed SOX2 expression in HepG2 and Huh7 cells. Overexpression SOX2 could abolish the growth and migration inhibitory effects of hsa\_circ\_0103809 knockdown. Collectively, we elucidated a novel regulatory network, hsa\_circ\_0103809/miR-490-5p/SOX2, that is hsa\_circ\_0103809 acting as an endogenous sponge to inhibit miR-490-5p expression, resulting in derepression of SOX2 expression in HCC cells.

Taken together, our present results highlight that hsa\_circ\_0103809 acts as an oncogene promoting malignant progression of HCC by regulating miR-490-5p/SOX2 signaling pathway. More importantly, hsa\_circ\_0103809/miR-490-5p/SOX2 axis contributes to the development of a new therapeutic target to HCC treatment. Furthermore, our data also suggest that hsa\_circ\_0103809 may be a potential biomarker for the diagnosis and prognosis of HCC patients.

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#### Disclosure of conflict of interest

None.

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