Thrombosis & Haemostasis: Research

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Research Article

Circular RNA Circrna_0072387 Promotes Hepatoblastoma Progression by Regulating Hsa-Mir-490/Histone Deacetylase 2 Axis

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Received: February 21, 2022; **Accepted:** March 18, 2022; **Published:** March 25, 2022

Abstract

Circular RNAs have been reported to be associated with the occurrence and progress of various cancers. However, its biological roles in Hepatoblastoma (HB) remain undefined. This study intended to explain a possible mechanism of hsa_Circ_0072387 (circ72387) in hepatoblastoma progression. The expression of circ72387, hsa-MIR-490 (miR490) and Histone Deacetylase 2 (HDAC2) in HB tissues and cell lines (HepG2/Huh6) were determined by Quantitative Real-Time Polymerase Chain Reaction (qPCR) or Western blot. Cell Counting Kit-8 (CCK-8) and colony formation assay were used to detect the proliferation of HB cell lines. The interaction between miR490 with circ72387 was predicted by CircInteractome and was verified by Dual-Luciferase reporter assay while the interaction between miR490 with HDAC2 was predicted by Targetscan and was verified by western blotting assay. Results showed that circ72387 was upregulated in HB tissues and cells, and miR490 was downregulated in HB cells. Furthermore, the silence of circ72387 suppressed the proliferation of hepatoblastoma cells. Circ72387 promoted the proliferation of HB cells through competitively binding to HDAC2 against miR490 acting as a Sponge of miR490 in HB. Circ72387 played an essential role in HB development through the circ72387-miR490-HDAC2 regulatory axis, which might become a novel diagnostic marker and potential therapeutic target in HB.

Keywords: Circular RNAs; Hepatoblastoma; Hsa_circ_0072387; Hsa-miR-490; HDAC2

Introduction

Hepatoblastoma (HB), a cancer that originates from stem cells and undifferentiated hepatic progenitor cells, is a rare malignant hepatic neoplasm frequently occurring in infants and toddlers [1]. The annual incidence is fairly constant with 1.0-1.5 cases per million children per year younger than 15 years of age in Western countries [2]. Currently, the mainstream treatment for HB is neoadjuvant chemotherapy combined with hepatectomy. The Overall Survival (OS) rate was 75% if patients could be treated with immediate hepatectomy in an early stage [3]. However, because of its nonspecific symptoms and incipient development, some patients may have already progressed to macrovascular tumor invasion and extrahepatic tumor extension when newly diagnosed [4]. Metastasis was observed in nearly 20% of newly diagnosed HB patients who lost the opportunity of surgery and might result in an unfavorable prognosis [5]. Therefore, more reliable diagnostic biomarkers are required to improve the prognosis of HB patients.

Circular RNAs (circRNAs) are RNAs with continuous covalently closed loops formed by alternative splicing event [6]. Unlike linear RNAs terminated with 5′ caps and 3′ tails, circRNAs are more stable and difficult to be degraded by RNA exonuclease because of their closed loop structures [7,8]. Circular RNAs have been reported to play a role as Competitive Endogenous RNAs (ceRNAs) and regulator of MicroRNA (miRNA) activity by competing for miRNA-binding sites [9]. MiRNAs are short RNA molecules, which can bind to target mRNAs, leading to translational suppression and gene silencing [10]. Therefore, circRNAs combined with miRNA can regulate the expression of target gene and build a circRNAs-miRNA-mRNA axis. CircRNAs have been reported to be associated with the occurrence and progress of many kinds of diseases, including cancers [11,12]. Therefore, figuring out the association between circRNAs and HB may provide a brand-new way for the hepatoblastoma diagnosis and therapy.

However, the roles of circRNAs in HB have rarely been reported, and the functions and regulatory mechanism of hsa_Circ_0072387 (circ72387) remain unknown. In 2018, Liu et al. identified 869 differentially expressed circRNAs in hepatoblastoma samples by circular RNA microarray. They confirmed that circRNAs plays a role in the pathogenesis of hepatoblastoma by bioinformatics analysis [13]. A recent study also pointed out that CDR1as, a highly expressed circRNA in HB cell lines is an oncogene that effects the proliferation and stemness of HB [14]. In conclusion, it is necessary to explore the roles and molecular mechanisms of circRNAs in HB occurrence and progress. CircRNAs may provide a new sight to the precise treatment in HB.

Our research mainly focused on a down-stream target miRNA, hsa-MIR-490 (miR490), which down-regulated in cancers revealed by many other studies. The targets of miR-490 includes a range of

Citation: Gou H, Yu R, Luo J, Liu L, Sun X and Xu C. Circular RNA Circrna_0072387 Promotes Hepatoblastoma Progression by Regulating Hsa-Mir-490/Histone Deacetylase 2 Axis. Thromb Haemost Res. 2022; 6(1): 1074.

cancer-related genes, which involve in proliferation, migration, and invasion, apoptotic cell death, angiogenesis, and so on [15]. More importantly, down-regulation of miR490 is closely related to the development of cancers including Hepatocellular Carcinoma (HCC) [16-26]. It has been proved that miR490 can suppress tumor by repressing the translation of a range of cancer-related genes like SOX2 [16], HDAC2 [18], POU3F2 [19], E2F2 [20], PPM1F [22], AURKA [25], BUB1 [27], CDK1 [28], TNKS2 [29]. These evidences strongly support that miR490 is a potential biomarker in the diagnosis and prognosis of HB.

In this research, we analyzed the function of highly-expressed hsa_circ_0072387 in HB promotion and its mechanism as ceRNAs. hsa_circ_0072387 regulates down-stream mRNA of Histone Deacetylase 2 (HDAC2) by competitively binding with miR490, which finally promotes proliferation of HB cells. We clarified that hsa_circ_0072387 was identified to play an essential role in HB development through the circ72387-miR490-HDAC2 regulatory axis, which might become a novel diagnostic marker and potential therapeutic target in HB.

Materials and Methods

Clinical specimens

22 pairs of human HB and matched tumor-adjacent liver tissues were collected from patients undergoing hepatectomy surgery at the Department of Hepatobiliary Surgery of Shanghai Children's Medical Center (Shanghai, China). The Tissue samples were immediately frozen in liquid nitrogen after resection and were stored at -80°C until use. These patients had not received chemotherapy, radiotherapy, or targeted therapy before admission. This investigation was approved by the Ethics Committee of Shanghai Children's Medical Center and informed consent was obtained from each patient.

HB cell lines

HB cell lines HepG2 and Huh6 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Minimum Eagle's Medium (MEM) and Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with high glucose, 10% Fetal Bovine Serum (FBS), 100 U/ml streptomycin, and 100 U/ml penicillin in 5% CO_2/95%O_2 at 37°C.

Oligonucleotide transfection

SiRNAs for hsa_circ_0072387, siRNA-NC, miR-490 mimics, miR-NC mimics, miR-490 inhibitor, and miR-NC inhibitor were chemically synthesized by Gene Pharma. The sequences of which mentioned above are provided in the supplemental material (Table S1). HepG2 and Huh6 cells were transfected with the oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction and real time-quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from HB specimens and cell lines was extracted using Trizol reagent (Ambion, Texas, America) according to the manufacturer's protocol. cDNA was synthesized by 1μg total RNA with the Prime Script™ RT Reagent Kit No. RR037A (TaKaRa, Dalian, Liaoning, China). RT-qPCR was performed with TaKaRa TB GreenTM Premix Ex TaqTM II No. RR820A (TaKaRa, Dalian, Liaoning, China) on an ABI7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All data were analyzed by the $2^{-\Delta\Delta CT}$ method. Primers used were provided in Table S1. Amplification curve and melting curve were confirmed after finishing all the cycles.

Western blotting assay

Cells and tissues were lysed by RIPA lysis buffer (Beyotime, Shanghai, China) for extracting the total protein. The extracted protein was separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then was transferred onto nitrocellulose membranes (GE Healthcare, Natick, MA, USA). These membranes were blocked using the 5% non-fat milk (Sangon Biotech) before being incubated with primary Antibody against Histone Deacetylase 2 (HDAC2) (1:2000, ab32117, Abcam, Cambridge, UK), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:1000, 97166, Abcam) for 12h at 4°C. Next, membranes were then continuously incubated with secondary antibody (1:4000, ab205718, Abcam). At last, the protein signal was visualized using an Odyssey Instrument (LI-COR Biosciences, Lincoln, NE, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control.

Cell proliferation and colony formation assays

For colony formation assays, cells were seeded into 12-well plates at a density of 500 cells per well. The cells in the plates were incubated for approximately 7-9 days in fresh medium, washed with PBS, fixed with 95% ethanol and stained with 0.1% crystal violet. The numbers of cell colonies representing the colony-forming ability were analyzed by Image J.

For Cell Counting Kit-8 (CCK8) assays, $1x10^3$ HB cells with the indicated transfection were seeded in triplicate into 96-well plates. At the indicated time points, the medium of each well was replaced with 100 μl fresh medium which was supplemented with 10 μl CCK8 reagent (Beyotime, Shanghai, China), and the cells were incubated for 2-4h at 37°C. Finally, the absorbance was determined by a multiplate reader (Bio Tek, Vermont, USA) at a wavelength of 450nm.

Measurement of caspase3 activity

100 μl Caspase-Glo® 3/7 reagent (Promega, G8090) was added into 96-well plates with 100μl blank control, negative control and HB cell lines. 96-well plates were vibrated at 300-500rpm for 30s followed by incubation at Room Temperature (RT) for 3h. Fluorescence intensity was determined by Luminometer.

Dual-luciferase reporter assay

The whole sequence of circ72387 with miR490 binding site was cloned downstream of the Renilla luciferase gene in the dual luciferase plasmid psiCHECKTM-2 Vector (Yeasen, Shanghai, China) to construct the psiCHECKTM-2-circ72387 vector. After seeding 293T cells in 24-well plates 24h prior to transfection, 0.8 μg of psiCHECKTM-2-circ72387 vector or psiCHECKTM-2 vector, with the addition of 20pmol of miRNA mimics or negative control mimics, were co-transfected into cells using Lipofectamine 2000 (Invitrogen). 48h after transfection, firefly and Renilla signals were determined by Luminometer.

Statistical analysis

All data were shown as the mean ± Standard Deviation (SD)

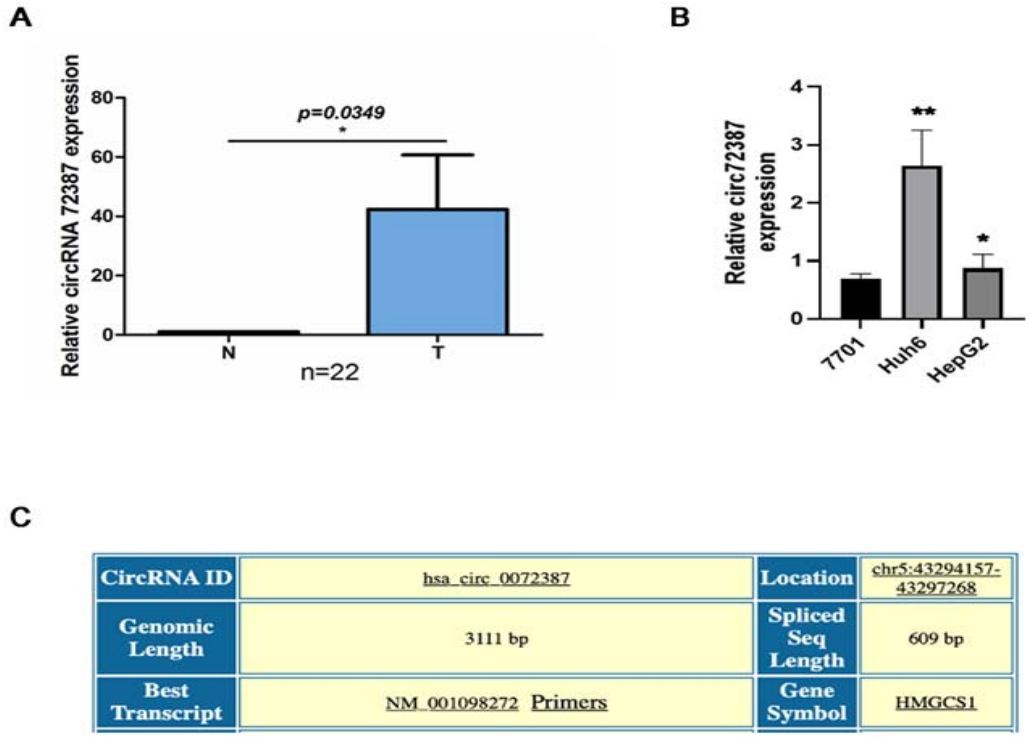

Figure 1: Circ72387 was Upregulated in HB Tissues and Cells. a) Relative expression of circ72387 in HB tissues compared with normal tissues by qRT-PCR. b) Relative expression of circ72387 in HB cell lines (HepG2 and Huh6) compared to normal human hepatocyte (7701) by qRT-PCR. c) Circ72387 gene information offered by CircInteractome.

from at least three independent experiments. Statistical data analysis was performed with SPSS 23.0 and GraphPad Prism 8.0. Student's t-test or a one-way Analysis of Variance (ANOVA) was utilized to analyze significant differences between different groups. Statistical significance was considered when $P< 0.05$.

Results

Circ72387 was upregulated in HB tissues and cells

Based on the previous HB tissue sequencing data, we focused on circ72387 which up-regulated in HB. To further confirm the level of circ72387 in HB tissues and cells, qRT-PCR was performed. The result indicated that circ72387 was remarkably upregulated in HB tissues (n=22) compared with normal tissues (n=22) (Figure 1a). Equally, circ72387 level was significantly increased in HB cell lines (HepG2 and Huh6) compared to normal human hepatocyte (7701) (Figure 1b). We analyzed circ72387 by an online database CircInteractome (https://circinteractome.nia.nih.gov/index.html) that circ72387 with a genomic length of 3111bp was generated from exon 5-8 of HMGCS1 gene, which located on chromosome 5 (Figure 1c). To sum up, the observation supplies a basis for circ72387 to be a circular RNA and a potential prognosis marker.

Circ72387 promotes the proliferation of HB cells

To ascertain the role of the circ72387 in HB cells, we performed loss-of-function experiments by transfection of si-circ72387 in HepG2 and Huh6 cells. SiRNA-3 and SiRNA-6 were selected for the following experiment as qPCR revealed that the level of circ72387 in HepG2 and Huh6 cells was reduced to half of those cells transfected with si-NC after transfected with SiRNA-3 and SiRNA-6 (Figure 2a). CCK8 assays (Figure 2b), and colony formation assays (Figure 2c) demonstrated that circ72387 silencing greatly reduced the proliferation and viability of HB cells. Additionally, the relative caspase-3 activity results revealed that the expression of circ72387 showed no effect on the apoptosis of HB cells (Figure 2d). Collectively, these data demonstrated that circ72387 regulated the proliferations and viability of HB cells.

Circ72387 Serves as a Sponge of MiR490 in HB

Hsa-MIR-490 (miR490) was selected as the downstream target miRNA with the help of CircInteractome and previous studies (Figure 3a). CancerMIRNome, an interactive analysis and visualization database for miRNome profiles of human cancer [30], demonstrated that the level of miR490 was extensively downregulated in many kinds of cancer (Figure 3b) and was significantly decreased in TCGA-LIHC ($p=8.24e-45$) (Figure 3c). We also observed a drop in the expression of miR490 in HB cell lines (Figure 3d) and a sharp increase in the expression of miR490 after silencing circ72387, which indicated that circ72387, might serve as a sponge of miR490 in HB (Figure 3e). Dual luciferase reporters were co-transfected with miR490 mimic into 293T cells. The results demonstrated that the luciferase reporter activity was significantly inhibited by miR490 compared to the miRNA Mimic Negative Control (miR-NC), indicating that circ72387 was strongly combined with miR490 (Figure 3f). Moreover, miR490inhibitor could partly rescue the viability (Figure 3g) and proliferation (Figure 3h) of HB cells induced by the silence of circ72387. This result suggested that other downstream targets might also play roles in the regulation

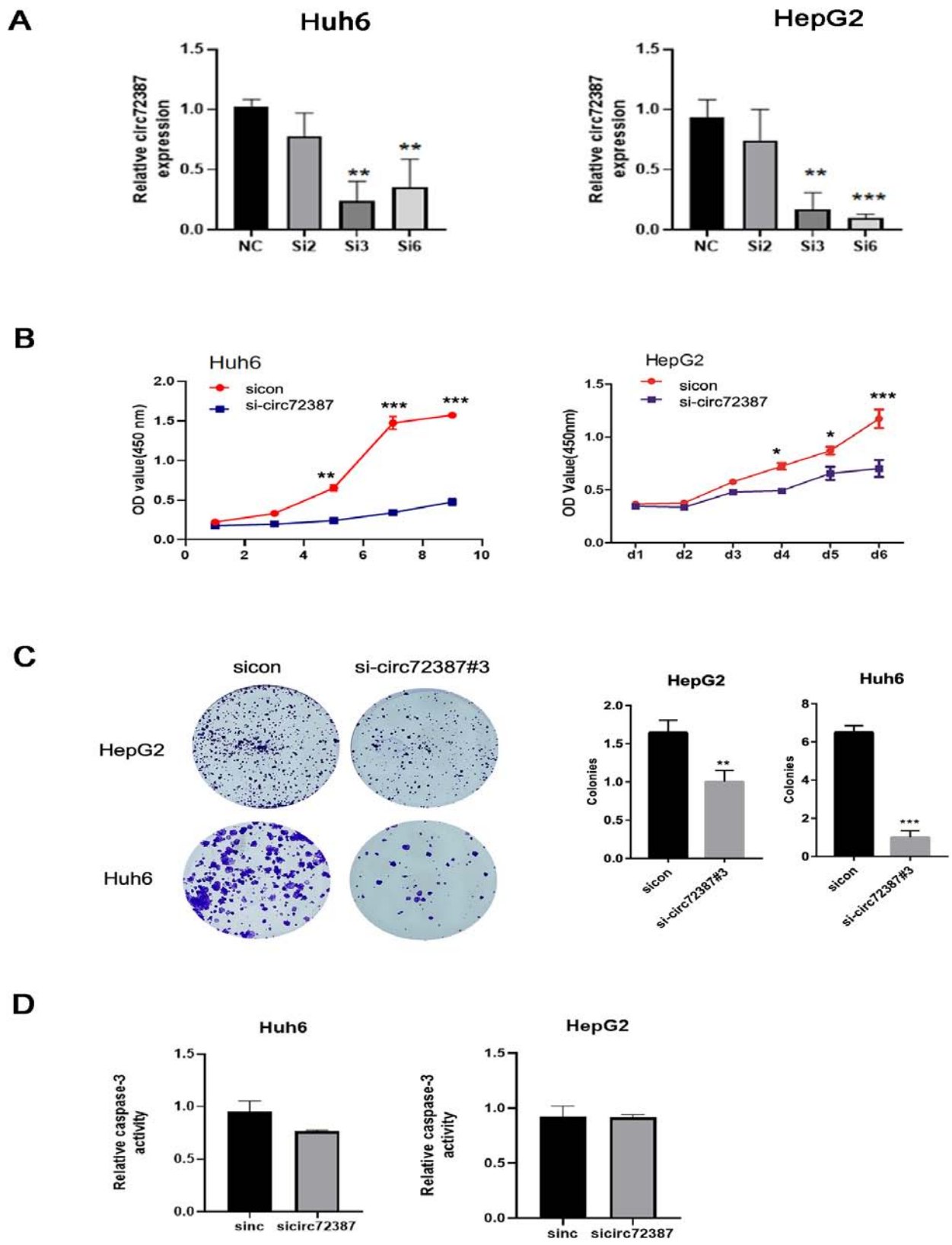

Figure 2: Circ72387 Promotes the Proliferation of HB Cells. a) Relative expression of circ72387 in HB cell lines (HepG2 and Huh6) transfected with si-NC, si-circ72387#2, si-circ72387#3 and si-circ72387#6. b) The viability in treated HB cell detected by Cell Counting kit-8 (CCK8) assays. c) Colony formation assays d. Relative caspase3 activity was measured by Caspase-Glo® 3/7 reagent.

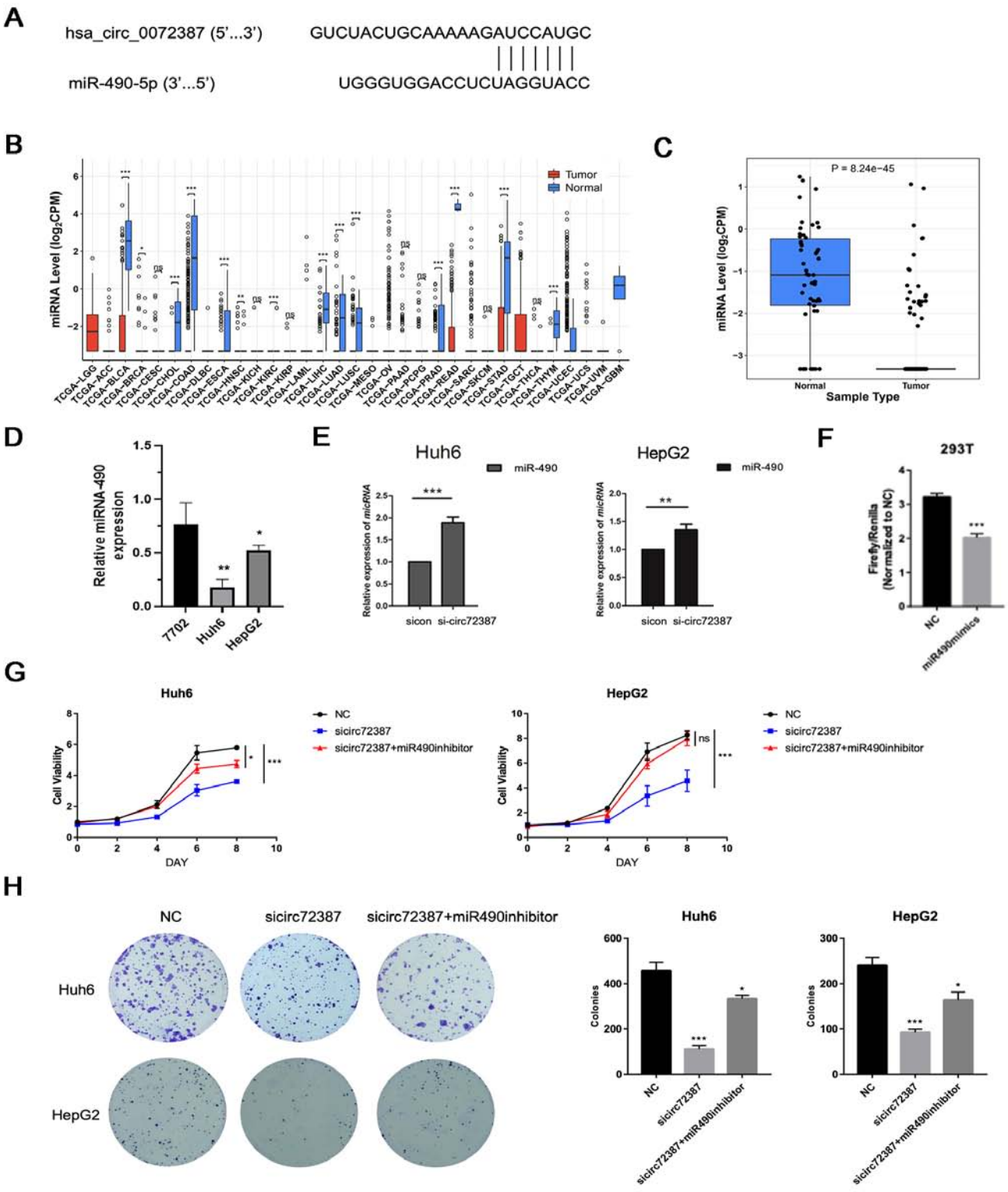

Figure 3: Circ72387 Serves as a Sponge of MiR490 in HB. a) The potential binding sites of miR490 on circ72387. b) The expression of miR490 in various cancers by CancerMIRNome. c) The expression of miR490 in TCGA-LIHC by CancerMIRNome. d) Relative expression of miR490 in HB cell lines (HepG2 and Huh6) compared to normal human hepatocyte (7702) by qRT-PCR. e) Relative expression of miR490 in HB cell lines (HepG2 and Huh6) transfected with si-con and si-circ72387. f) Dual-Luciferase assays verified the interaction of circ72387 with miR490. g) The viability in treated HB cell detected by Cell Counting Kit-8 (CCK8) assays. h) Colony formation assays.

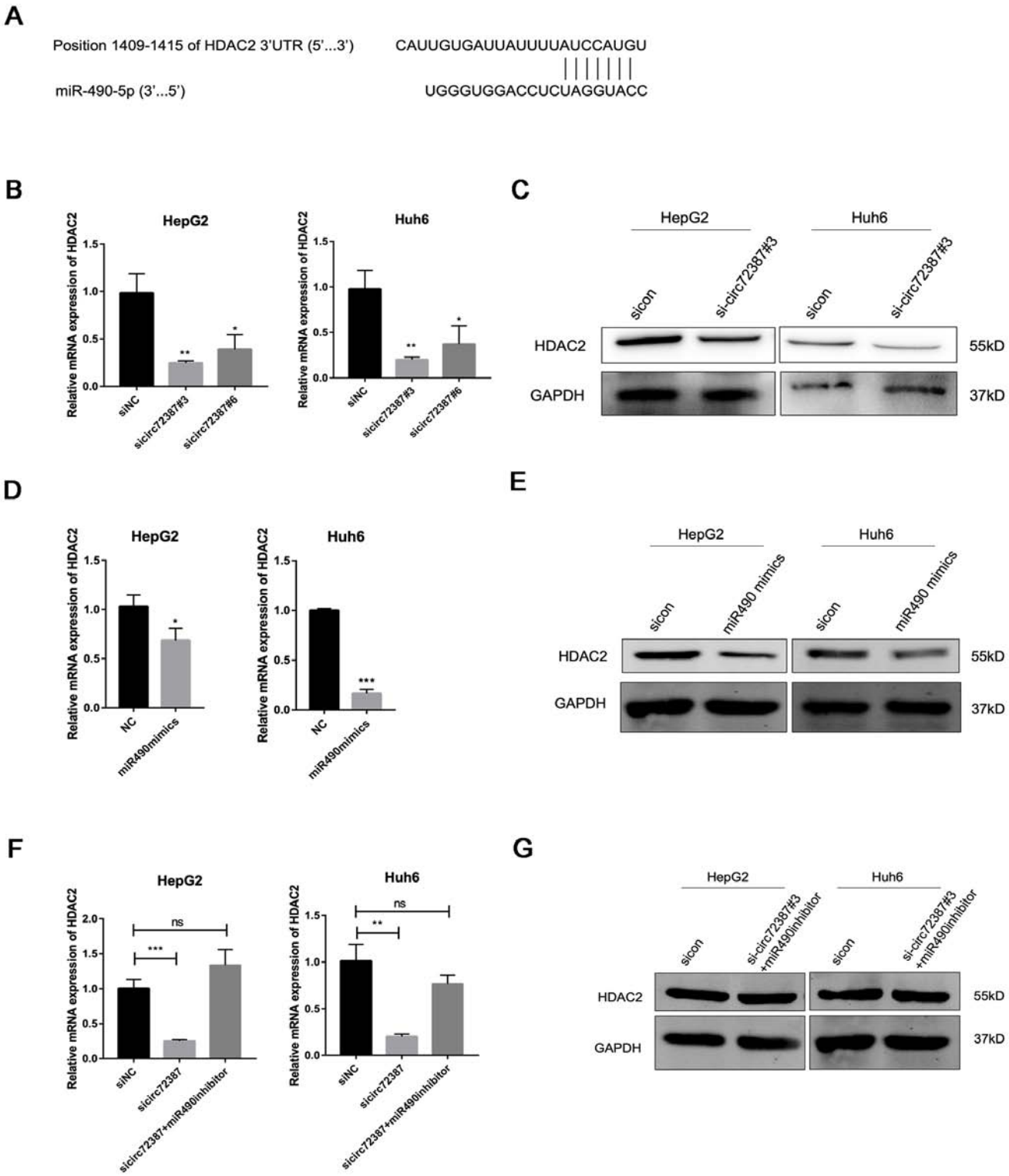

Figure 4: HDAC2 is a Downstream Target of MiR490 in HB Cells. a) The potential binding sites of HDAC2 on miR490. b) Relative mRNA expression of HDAC2 in HB cell lines (HepG2 and Huh6) transfected with si-NC, si-circ72387#3 and si-circ72387#6. c) The expression of HDAC2 in HB cell lines compared with circ72387 silenced analyzed by western blot. d) Relative mRNA expression of HDAC2 in HB cell lines compared with miR490 overexpressed by qRT-PCR. e) The expression of HDAC2 in HB cell lines compared with miR490 overexpressed analyzed by western blot. f) Relative mRNA expression of HDAC2 in HB cell lines transfected with si-NC, si-cir72387 and si-cir72387+miR490inhibitor by qRT-PCR. g) The expression of HDAC2 in HB cell lines compared with si-cir72387+miR490inhibitor transfected analyzed by western blot.

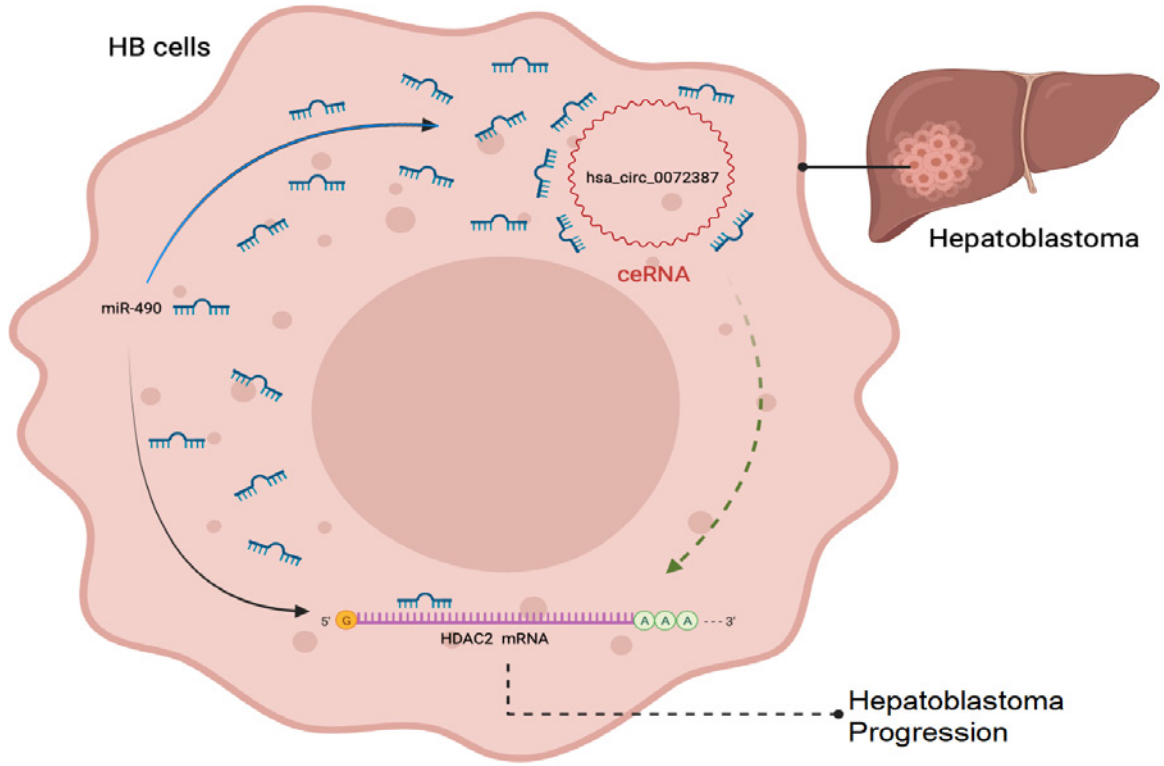

Figure 5: A schematic diagram of circ72387 miR-490/HDAC2 regulatory axis.

of HB proliferation by circ72387 except miR490. Altogether, these results indicated that circ72387 regulated proliferation and viability of HB cells by sponging miR490.

HDAC2 is a downstream target of MiR490 in HB cells

Since MiRNAs can lead to translational suppression and gene silencing by binding to target mRNAs, Targetscan (http://www.targetscan.org/mamm_31/) was used to predict the down-stream target mRNA by which Histone Deacetylase 2 (HDAC2) was selected (Figure 4a). We observed that the relative mRNA and the protein expression of HDAC2 were inhibited after silencing circ72387 (Figure 4b and 4c) or overexpressing miR490 (Figure 4d and 4e). Moreover, miR490inhibitor could rescue the relative mRNA (Figure 4f) and the protein expression (Figure 4g) of HDAC2 in HB cells induced by the silence of circ72387. Taken together, these data proved that circ72387 served as a molecular sponge of miR490 to regulate the expression of HDAC2.

Discussion

Hepatoblastoma is a common type of primary malignant hepatic tumor in in infants and toddlers, whose mechanism still remains unknown. Because of its nonspecific symptoms and incipient development, nearly 20% of newly diagnosed HB patients observed metastasis. A lack of early-diagnosis and treatment biomarker lead to a poor prognosis in HB patients [4,5]. In recent years, dysregulation of circRNAs has been suggested to have a close association with the occurrence and progress of many kinds of cancers [11,12]. In this work, we confirmed that circ72387 promotes the proliferations of HB via regulating miR490/HDAC2 axis. Previous studies have revealed that circRNAs are rich in eukaryotic cells and are determined as potential prognostic biomarkers for tumors due to their abundance and stability [7,31]. Though the exact function of most circRNAs in HB remain unclear, Liu et al. confirmed that circRNA plays a role in the pathogenesis of hepatoblastoma by bioinformatics analysis [13]. Meanwhile, hsa_circ_0000594 has also been reported to play a role as ceRNA in hsa_circ_0000594/mir-217/SIRT1 axis in the development of HB, which can be a new diagnosis biomarker for HB (Song et al. 2019). Interestingly, a research in 2020 showed that circ_0043800, a circRNA originated from STAT3, can both be induced by Gli2 and up-regulate STAT3 and Gli2 through miR-29a/b/c-3p which broadened the molecular mechanism of circRNA (Khan et al. 2020). Our previous research also suggested that circHMGCS1/miR-503-5p/IGF-PI3K-Akt regulatory axis could affect the proliferation and the apoptosis of HB cells and is a potential diagnostic and prognostic biomarker for HB patients [32]. However, the exact roles of circ72387 in HB remain undefined. In this study, we observed an upregulation of circ72387 in HB tissues and cells. Additionally, interference of circ72387 could effectively inhibit the proliferation rather than the apoptosis of HB cells. Collectively, these findings showed that circ72387 was a potential tumor promoter in HB.

Emerging evidences showed that circRNAs can modulate their downstream target genes expression in many cancers by acting as miRNA sponges to suppress the activity of miRNAs [7,33]. Therefore, CircInteractome was applied to predict the down-stream target miRNA of circ72387. Results indicated that circ72387 could bind to miR490, which were confirmed by a series of experiments in HB. Previous studies have reported that miR490 acts as an antioncogene

in various cancers. Fan et al. demonstrated that miR490 repress the progress of Triple-Negative Breast Cancer (TNBC) [34]. Vinchure et al. clarified that miR490 inhibits the migration and EMT in glioblastomas by regulating the expression of TGIF2 [35]. Especially, Dai et al. confirmed that lncRNA-SNHG15 served as a molecular sponge for miR-490-3p while miR-490-3p directly targets HDAC2 [18]. HDACs are frequently up-regulated in various types cancer, where they usually promote cell proliferation and viability [36,37]. In 2019 Tang et al. confirmed that [38] p300/YY1/miR-500a-5p/HDAC2 regulatory axis regulates cell proliferation in human colorectal cancer. Previous study found that HDAC2 overexpression was associated with tumor proliferation and higher Ki67 expression in breast cancer tumors [39]. A recent study confirmed the anti-tumor activity of miR-646 by targeting HDAC2 to suppress the proliferation ability of human breast cancer [40]. This proposal is in accordance with the result in our study that the relative expression of miR490 in HB is suppressed while over-expression of miR490 can significantly inhibit the expression of HDAC2. However, the exact mechanisms of HDAC2 in hepatoblastoma require more investigation. It is worth mentioning that we also discovered that there might be other downstream genes playing roles in the regulation of HB cells by circ72387 except miR490 (Figure 3g and 3h). More researches need to be done to explain for the complete mechanism of circ72387 in future.

Taken together, these findings showed that circ72387 could promote the proliferation of HB cells through miR-490/HDAC2 regulatory axis. Our study might explain a possible underlying mechanism of HB progression and provide a promising circRNA-targeted therapy for HB (Figure 5).

Conclusion

In this study, we focused on the regulatory network based on circular RNAs' role as Competitive Endogenous RNAs (ceRNAs) in hepatoblastoma. We observed an increase in the expression of circ72387 in HB tissues and cells, which could lead to an enhanced proliferation of HB cells. In addition, circ72387 interacts with miR490 as ceRNAs and then competitively binding with HDAC2, which up-regulates HDAC2, a tumor-proliferation associated gene. The early detection of circ72387 may pave a way for the early-diagnosis of HB. Furthermore, our results also indicate that circ72387 takes part in the proliferation of HB, which suggests that the blockade of circ72387 might be a promising circRNA-targeted therapy for HB.

Contributions

HG and RY conducted most of the experiments and drafted the article. CX helped conduct the experiments and edited the manuscript. JL, LL and XS coordinated the project, conceived and designed the experiments and edited the manuscript. All authors read and approved the work reported.

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