European Review for Medical and Pharmacological Sciences 2020; 24: 12124-12130

Circular RNA ZNF292 affects proliferation and apoptosis of hepatocellular carcinoma cells by regulating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: The purpose of this study was to explore the function of circular ribonucleic acid (circRNA) zinc finger protein 292 (ZNF292) in hepatocellular carcinoma (HCC).

MATERIALS AND METHODS: The expression of circRNA ZNF292 in Huh-7 cells was knocked down by small interfering RNAs (siRNAs), and the effect of circRNA ZNF292 knockdown on the proliferation of Huh-7 cells was analyzed by Cell Counting Kit-8 (CCK-8) assay and colony formation assay. Then, flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were adopted to analyze the impacts of circRNA ZNF292 knockdown on the cycle distribution and apoptosis of Huh-7 cells. Besides, the influences of circRNA ZNF292 knockdown on Wnt/β-catenin signaling pathway and its downstream molecules were detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting.

RESULTS: Compared with those in siRNA-normal control (NC) group, the proliferation of Huh-7 cells was significantly inhibited and their cloning ability was remarkably weakened ($p<0.05$), the proportion of cells in S phase was decreased while that in G1 phase was increased ($p<0.05$), the apoptosis rate of Huh-7 cells was higher and the number of apoptosis was larger in siRNA-2# knockdown group ($p<0.05$). Besides, in Huh-7 cells with circRNA ZNF292 knockdown, the expressions of Axin, β-catenin, phosphorylated signal transducer and activator of transcription 3 (p-STAT3), p-STAT5, Cyclin A and Cyclin-dependent kinase 2 (CDK2) were down-regulated, while the expressions of STAT3 and STAT5 did not change remarkably.

CONCLUSIONS: Knock downing circRNA ZNF292 leads to cell cycle arrest in G1 phase, thus suppressing cell proliferation and promoting cell apoptosis. The regulatory mechanism of circRNA ZNF292 may involve the regulation of cell cycle and related genes.

Key Words:
Hepatocellular carcinoma, Circle RNA ZNF292, Wnt/β-catenin, Proliferation, Apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary tumor in the liver, whose main pathogenic factors are hepatitis and liver cirrhosis caused by drinking, fatty liver, and hepatitis viruses[1]. HCC is characterized by a high incidence rate, late detection, and poor prognosis[2]. At present, HCC is primarily treated by surgical resection and liver transplantation, which are only for tumors in the early stage[3]. A study holds that the occurrence and development of HCC result from the synergistic effects of multiple factors and stages, and are correlated with the participation of various oncogenes, related tumor suppressor factors and non-coding ribonucleic acids (RNAs)[4]. Therefore, it is extremely necessary to find effective therapeutic targets for HCC.

Circular RNAs (circRNAs) are a new type of RNA molecules formed by covalent ring closure, which is widely found in eukaryotes. CircRNAs come from gene exon or intron regions and are abundant in mammalian cells. Existing research[5,6] has indicated that most circRNAs are conserved in different species. Besides, circRNAs are relatively stable due to their cyclic structure and resistance resulting from RNase R degradation. Due to their specific expressions, complexity of regulation, and crucial roles in diseases, circRNAs are attracting more and

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more public attention[7]. As circRNAs are closed loops without 5'-3' polarity and poly-(A) tails, they are more stable than linear RNAs and less likely to be degraded by RNA exonucleases or RNase R. Therefore, circRNAs can be used as potential targets for tumor therapy or diagnostic biomarkers, and they need to be further researched[8].

CircRNA zinc finger protein 292 (ZNF292) is a kind of circRNA discovered to be expressed under anoxic conditions. The silencing of circRNA ZNF292 inhibits the formation of extracorporeal tubes, suggesting that it may be related to the incidence rate, development, and prognosis of tumors[9]. In this study, circRNA ZNF292 was assumed to play a vital role in HCC. The effects of circRNA ZNF292 on the proliferation and apoptosis of HCC cells were explored *in vitro* through Huh-7 cell line, so as to reveal the possible action mechanism of ZNF292.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) high-glucose medium and fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), bicinchoninic acid (BCA) kit (Sigma-Aldrich, St. Louis, MO, USA), GeneJuice™ transfection reagent (Merck, Billerica, MA, USA), enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA), First Strand complementary deoxyribonucleic acid (cDNA) Synthesis kit and SYBR Green polymerase chain reaction (PCR) Master Mix (TaKaRa, Otsu, Shiga, Japan), 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), Cell Counting Kit-8 (CCK-8) detection kit (Sigma-Aldrich, St. Louis, MO, USA), in situ cell death detection kit (Roche, Basel, Switzerland) and a flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell Culture and Transfection

The human HCC Huh-7 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured under standard conditions (DMEM basal medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 37°C and 5% CO_2). Huh-7 cells were plated in a 6-well plate for 24 h and transfected with GeneJuice™ transfection reagent based on the transfection procedure for 6 h according to the manufacturer's protocol. Then fresh culture medium was added, and cells were harvested after 48 h in all experiments. CircRNA ZNF292 small interfering RNAs (siRNAs) were designed and synthesized by GenePharma (Shanghai, China).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from transfected cells using TRIzol and reversely transcribed into cDNAs using the First Strand cDNA Synthesis kit. The SYBR Green PCR Master Mix was employed to analyze the expression levels of circRNA ZNF292 and mRNAs on 7500 real-time PCR system. PCR conditions: denaturation at 95°C for 10 min, at 95°C for 15 s and at 60°C for 60 s for a total of 40 cycles. Finally, multiple changes in the expressions of circRNA ZNF292 and mRNAs were normalized using $2^{-\Delta\Delta Ct}$ relative quantification method. The primers for qRT-PCR are as follows (Table I).

Cell Proliferation Analysis

Cell proliferation was measured by CCK-8, and the specific operation steps were strictly carried out in accordance with the instructions. In short, the transfected cells (2×10^3) were inoculated into a 96-well plate and cultured for 24 h, 48 h, and 72 h. At different times, they were incubated with 10 μL of CCK-8 solution per well for 2.5 h in the dark. The absorbance at 450 nm was measured to reflect the proliferation activity of cells. In the

Table I. Primer sequences.

Gene	F/R	Sequence (5′-3′)
CircRNA ZNF292	F	GCTCAAGAGACTGGGGTGTG
	R	AGTGTGTGTTCTGGGGCAAG
U6	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT
GAPDH	F	ATCCACGGGAGAGCGACAT
	R	CAGCTGCTTGTAAAGTGGAC

meantime, colony formation assay was adopted to determine the ability of cell clone formation. Briefly, the transfected cells (1×10^3) were seeded into the 6-well plate and cultured for 14 d. After cells were fixed with methanol, they were stained with 0.1% crystal violet solution, and colonies were counted.

Cell Cycle Analysis

The transfected Huh-7 cells were collected and added with pre-cooled 75% ethanol, followed by standing overnight at 4°C. Then, the cells were washed with phosphate-buffered saline (PBS), added with RNase, and digested at 37°C. After that, propidium iodide (PI) was added, staining was carried out at 4°C in the dark for 30 min, and the cells were detected on the machine within 24 h.

Cell Apoptosis Analysis

AnnexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was utilized to determine the apoptosis rate by flow cytometry according to the manufacturer's protocol. Cells were digested with trypsin, harvested, washed twice with PBS, and resuspended in binding buffer. After that, the cells were incubated with AnnexinV-FITC and PI away from light at room temperature for 15 min, and the stained cells were then detected *via* a BD FACSCalibur flow cytometer. Subsequently, the number of apoptotic Huh-7 cells was analyzed and measured *via* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Next, Huh-7 cells collected after transfection were fixed on a cover glass, and the apoptosis number was evaluated using *in situ* cell death detection kit according to the instructions. Thereafter, Huh-7 cells collected after transfection were incubated with 50 μL of TUNEL mixture (containing 50 μL of enzyme solution and 450 μL of labeling solution) at 37°C for 60 min. Finally, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at 37°C for 15 min, washed three times with PBS, and observed using a fluorescence microscope.

Western Blotting Analysis

Cells (3×10^5) were inoculated into each well of the 6-well plate, cultured under standard conditions for 48 h, collected, lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and precipitated by centrifugation at 12,000 g and 4°C for 10 min. The total protein concentration was measured using the bicinchoninic acid (BCA) kit, and 60 μg protein samples were separated in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Thereafter, the membrane was blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated with primary antibody overnight at 4°C. Next, the membrane was washed with Tris Buffered Saline and Tween-20 (TBST) and incubated with secondary antibody for 1 h at room temperature, after which, the ECL kit was used to display immunoreactive bands.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was adopted to analyze data. Each experiment was conducted in triplicate, and the numerical value was expressed as mean ± standard error of measurement. Two-tailed Student's *t*-test was employed to analyze the statistical differences between groups. $p<0.05$ represented that the difference was statistically significant.

Results

Knocking Down CircRNA ZNF292 Inhibited the Proliferation of Huh-7 Cells

To explore the role of circRNA ZNF292 in regulating the proliferation of HCC cells, the expression of circRNA ZNF292 in Huh-7 cell line was knocked down by circRNA ZNF292 siRNAs (Figure 1A). Compared with siRNA-NC, the three target sequences, siRNA-1#, -2#, and -3#, all displayed the knockdown capability ($p<0.05$), which was the most exceptional in siRNA-2#, so siRNA-2# was selected for the following *in-vitro* function experiment of circRNA ZNF292. CCK-8 results revealed that compared with siRNA-normal control (NC), siRNA-2# knockdown significantly inhibited the proliferation of Huh-7 cells (Figure 1B, $p<0.05$). Furthermore, the effect of circRNA ZNF292 knockdown on the cloning ability of Huh-7 cells was further confirmed by colony formation assay. It was found that compared with that in siRNA-NC group, the cloning ability in siRNA-2# knockdown group was notably reduced (Figure 1C, $p<0.05$).

Knocking Down CircRNA ZNF292 Resulted in Cell Cycle Arrest in G1 Phase

Since the expression of circRNA ZNF292 exerted an inhibitory effect on the proliferation of

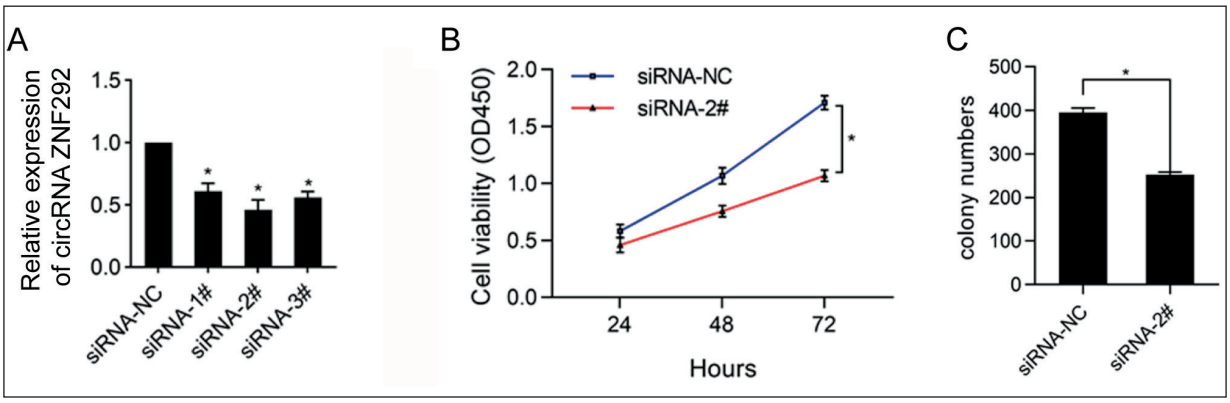

Figure 1. Knocking down circRNA ZNF292 inhibits the proliferation of Huh-7 cells. **A,** Effect of circRNA ZNF292 knockdown detected via qRT-PCR. **B,** Proliferation ability of Huh-7 cells in circRNA ZNF292 knockdown group and control group detected via CCK-8. **C,** Cloning ability of Huh-7 cells in circRNA ZNF292 knockdown group and control group analyzed by colony formation assay (*p<0.05).

Huh-7 cells, whether the knockdown of circRNA ZNF292 affects the distribution of Huh-7 cell cycle was researched. The results showed that compared with those in siRNA-NC group, the proportion of cells in S phase was decreased, while that in G1 phase was increased in siRNA-2# knockdown group (Figure 2, p<0.05). The above data indicate that the knockdown of circRNA ZNF292 results in cell cycle arrest in G1 phase and further suppresses cell proliferation.

Knocking Down CircRNA ZNF292 Promoted the Apoptosis of Huh-7 Cells

At 48 h after transfection, the apoptosis of Huh-7 cells with circRNA ZNF292 knockdown was detected by flow cytometry. According to the results, the apoptosis rate of Huh-7 cells in siRNA-2# knockdown group was higher than that in siRNA-NC group (Figure 3A, p<0.05). TUNEL assay further confirmed the effect of circRNA ZNF292 knockdown on the apoptosis of Huh-7 cells. It was discovered that siRNA-2# knockdown group had more apoptotic cells than siRNA-NC group (Figure 3B, p<0.05).

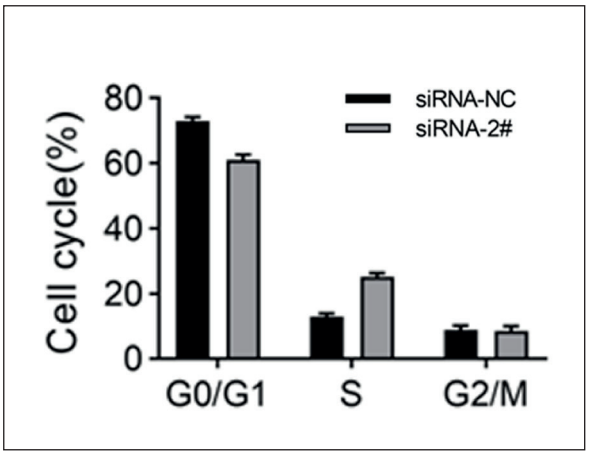

Figure 2. Effects of circRNA ZNF292 knockdown on the cycle distribution of Huh-7 cells detected *via* flow cytometry (*p<0.05).

Influences of Knocking Down CircRNA ZNF292 on the Wnt/β-catenin Signaling Pathway and its Downstream Molecules

Cell cycle process can be regulated by the Wnt/β-catenin signaling pathway (Axin, β-catenin, STAT3, and STAT5) and its downstream molecules such as Cyclin A and cyclin-dependent kinase 2 (CDK2). In Huh-7 cells knocked down by circRNA ZNF292, the expressions of Axin, β-catenin, phosphorylated (p)-signal transducer and activator of transcription 3 (STAT3), p-STAT5, Cyclin A, CDK2 were found to be down-regulated, while those of STAT3 and STAT5 did not change predominantly (Figure 4), indicating that ZNF292 silencing modulates cell cycle through the Wnt/β-catenin signaling pathway to inhibit tumor formation.

Discussion

At present, HCC has become the most common cancer worldwide. This malignant tumor leads to a large proportion of cancer-related deaths every year, especially in China[10]. Up

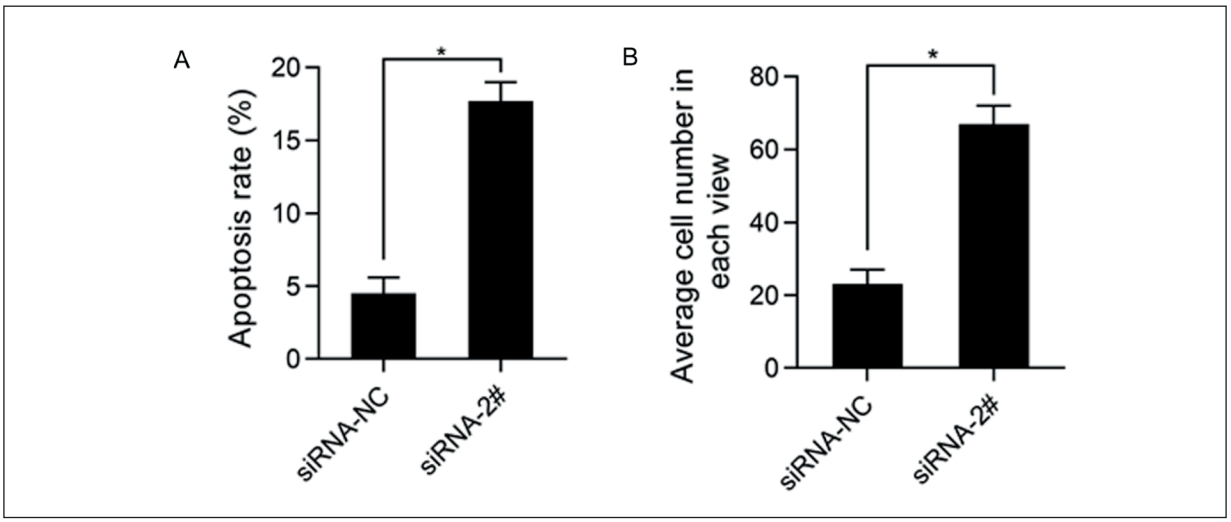

Figure 3. Knocking down circRNA ZNF292 promotes the apoptosis of Huh-7 cells. **A,** Effect of circRNA ZNF292 knockdown on the apoptosis rate of Huh-7 cells examined via flow cytometry. **B,** Effect of circRNA ZNF292 knockdown on the number of apoptotic Huh-7 cells detected *via* TUNEL assay (*p<0.05).

to now, the main treatment methods for HCC include radical resection and chemotherapy. In the past decades, efforts and progress have been made in HCC treatment. However, the prognosis of HCC patients is still very poor, and the 5-year survival rate is lower than 25% due to the high frequency of tumor recurrence and metastasis[11]. Therefore, it is very urgent to find new biomarkers for HCC diagnosis and identification of effective therapeutic targets due to the above severe challenges.

CircRNAs are newly discovered non-coding RNAs with little protein coding potential, accounting for more than 90% of human transcriptomes[12]. CircRNA comes from reverse splicing of precursor mRNAs and are covalently closed transcripts, so they are more stable and tolerant than linear RNAs[13]. High-throughput sequencing shows that the expression of circRNAs is cell-, tissue- or development-specific, indicating that circRNA may have specific biological function[14]. Increasing evidence reveals that circRNAs have

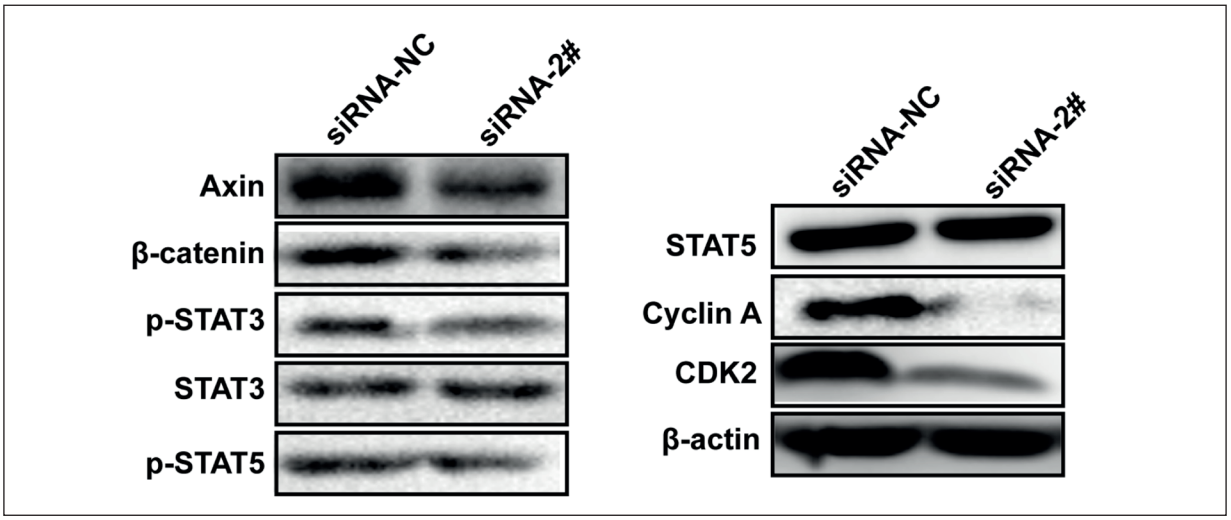

Figure 4. Influences of circRNA ZNF292 knockdown on the Wnt/β-catenin signaling pathway and its downstream molecules detected *via* qRT-PCR and Western blotting (*p<0.05).

become potential media of tumor biology through various mechanisms[15]. Han et al[13] analyzed the expression profile of human circRNA in HCC tissues and identified that circMTO1 is evidently down-regulated in HCC tissues. The survival period of HCC patients with a low circMTO1 expression is shortened, and circMTO1 silencing in HCC can down-regulate the p21 expression of oncogenic miR-9, thus facilitating the proliferation and invasion of HCC cells.

Boeckel et al[9] demonstrated that circRNA ZNF292 can be up-regulated under hypoxic conditions, such as the up-regulation observed during the formation of human umbilical vein endothelial cell tubes, and it also verified the role of circRNA ZNF292 in tumorigenesis. In this research, circRNA ZNF292 was assumed to play a vital role in HCC, and the effects of circRNA ZNF292 on the proliferation and apoptosis of HCC cells were explored *in vitro* through Huh-7 cell line. It was discovered that knocking down circRNA ZNF292 leads to cell cycle arrest in G1 phase, thus suppressing cell proliferation and promoting cell apoptosis.

Cyclin A is a key molecular regulator in the transition of cell cycle from S phase to G2 phase, which functions by activating CDK2. This regulatory pathway is involved in cell proliferation and cell tube formation, and is closely associated with tumor development and pathogenesis[16,17]. Besides, it Chen et al[18] showed that proline-rich protein 11 also participates in cell cycle regulation and is related to tumor malignancy. The results of the present study demonstrated that the proportion of cells in S phase was decreased, while that of cells in G1 phase was increased after circRNA ZNF292 silencing, resulting in the arrest in G1 phase. Axin, β-catenin, and STAT3/5 are components of the Wnt/β-catenin signaling pathway, which play vital roles in cell growth, differentiation, and cancer processes. Axin is believed to be a tumor suppressor gene related to other colorectal tumor suppressor genes such as APC that have central functions of degrading β-catenin, a downstream signal molecule of the Wnt/β-catenin signaling pathway, regulating the biological function of many tumor cells, and preventing the activation of Wnt/β-catenin signaling pathway[19,20]. It was found that the silencing of circRNA ZNF292 was capable of significantly down-regulating the expressions of β-catenin, p-STAT3, p-STAT5, Cyclin A, and CDK2. The above results indicate that signal transduction in the STAT3/5/β-catenin pathway participates in the potential mechanism of circRNA ZNF292 and exerts a regulatory effect in HCC cells.

Conclusions

We reveal that circRNA ZNF292 plays a pivotal role in the proliferation and apoptosis of HCC cells. The regulatory mechanism of circRNA ZNF292 may involve the regulation on cell cycle and related genes, but more in-depth research is needed to further understand the regulatory mechanism of circRNA ZNF292.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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