Hsa-circRNA-0015004 promotes hepatocellular carcinoma development via miRNA-330-3p/RCC2 axis

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Article

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Abstract

Background: Although circular RNAs (circRNA) have been demonstrated to modulate tumor initiation and progression, their roles in the proliferation of hepatocellular carcinoma (HCC) are still poorly understood.

Methods: Based on the analysis of GEO data (GSE12174), hsa-circRNA-0015004 (circ-0015004) was screened and validated in 80 sets of HCC specimens. Subcellular fractionation analysis was designed to determine the cellular location of circ-0015004. Colony formation and cell counting kit-8 were performed to investigate the role of circ-0015004 in HCC. Dual-luciferase reporter gene assays, RNA immunoprecipitation and chromatin immunoprecipitation were employed to verify the interaction among circ-0015004, miR-330-3p and regulator of chromatin condensation 2 (RCC2).

Results: The expression level of circ-0015004 was significantly upregulated in HCC cell lines and HCC tissues. HCC patients with higher circ-0015004 levels displayed shorter overall survival, and higher tumor size and TNM stage. Moreover, knockdown of circ-0015004 significantly reduced HCC cell proliferation in vitro and inhibited the growth of HCC in nude mice. Mechanistic studies revealed that circ-0015004 could upregulate the expression of RCC2 by sponging miR-330-3p, thereby promoting HCC cell proliferation. Furthermore, we identified that Ying Yang 1 (YY1) could function as an important regulator of circ-0015004 transcription.

Conclusions: This study systematically demonstrated the novel regulatory signaling of circ-0015004/miR-330-3p/RCC2 axis in promoting HCC progression, providing insight into HCC diagnosis and treatment from bench to clinic.

Highlights

- The expression level of circ-0015004 was significantly up-regulated in HCC cell lines and HCC tissues.
- HCC patients with higher circ-0015004 level had shorter overall survival, higher tumor size and TNM stage.
- circ-0015004 can up-regulate the expression of RCC2 by sponging miR-330-3p, thereby promoting the proliferation of HCC cells.

1 Introduction

Hepatocellular carcinoma (HCC), ranking as the third leading cause of cancer-related deaths in the world, accounts for about 90% of all cases of primary liver cancers (1–3). Its primary etiological factors encompass cirrhosis and HBV infection (4). Despite notable process in alternative therapies, surgical resection is still the most important way to treat HCC (5), albeit with a dishearteningly low 5-year survival (6). Hence, it is urgent to investigate the molecular mechanism of HCC and explore the novel potential biomarkers and therapeutic targets for HCC.
Circular RNAs (circRNAs), a subset of non-coding RNA molecules that primarily originate from pre-mRNA's reverse splicing process (7), have been implicated in many stages of tumor progression, including tumor apoptosis, differentiation, metastasis, and proliferation (8, 9). It's noteworthy that circ-ZEB1 was shown to play an important role in reinforcing the advancement of HCC, and the circ_0000098/miR-383/MCUR1 axis has been instrumental in orchestrating tumorigenesis in HCC (10, 11). Nevertheless, the precise roles and underlying mechanisms of circRNAs in HCC remain largely unknown. Among them, circ-0015004, stemming from the succinate dehydrogenase C (SDHC) gene, situated on chr1:161293403–161326630, has been found to drive proliferation, invasion, and migration in renal cell carcinoma (12). However, its role in HCC remains enigmatic.

Small RNA molecules known as microRNAs (miRNAs), characterized by their short length comprising 22 nucleotides, have the potential to exert either oncogenic or suppressive effects on tumors (13). There is an emerging acknowledgment regarding circRNAs acting as competitive endogenous RNAs (ceRNAs), possessing the capability to influence the expression of subsequent miRNAs (14). Within the context of HCC, the circRPN2 functions to restrain cell proliferation and glycolysis through modulation of the miR-183-5p/FOXO1 axis (15). Similarly, the suppression of hsa_circRNA_104348 has been shown to hinder the advancement of HCC through targeting the miR-187-3p/RTKN2 axis (16). Furthermore, miRNAs have exhibited their capacity to modulate mRNA levels by engaging with the 3′-untranslated regions (UTRs) (17).

In this study, based on the analysis of GEO data (GSE12174), circRNA-0015004 was screened and validated in HCC specimens. We demonstrate that Ying Yang 1 (YY1)-regulated circRNA-0015004 promotes hepatocellular carcinoma development via miRNA-330-3p/regulator of chromatin condensation 2 (RCC2) axis.

2 Materials and methods

2.1 Human HCC tissue

This study was approved by the Medical Ethics Committee of Wujin Traditional Chinese Medicine Hospital. Informed consent was available for each patient. Tissue specimens were collected from 80 patients who underwent surgical procedures between March 2018 and June 2020. None of these individuals had undergone any preoperative chemotherapy or radiotherapy treatments. Tissue samples were meticulously collected and promptly preserved in liquid nitrogen for subsequent analysis. In parallel, an all-encompassing compilation of clinicopathological data and survival outcomes for all individuals involved was meticulously collated.

2.2 Cell culture

The HCC cell lines, including HCC-LM3, MHCC-97H, Hep3B, HepG2, and Huh-7, and the normal liver cell line L02, were from the Shanghai Academy of Sciences. The cultivation of all cellular varieties was meticulously administered at a temperature of 37°C, employing RPMI 1640 medium (Gibco, NY, USA).
supplemented with 10% concentration of fetal bovine serum (Gibco), in addition to 100 mg/mL of streptomycin and 100 U/mL of penicillin. Subsequently, the cultured cells were maintained within a regulated environment infused with 5% CO2, fostering an optimum milieu for their expansion and proliferation.

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

For total RNA extraction, a procedure utilizing Trizol reagent was executed for both tissue samples and cellular specimens. The extracted RNA underwent a subsequent reverse transcription process, yielding single-stranded cDNA. Following this, a thorough analysis via qRT-PCR was conducted using the SYBR Green Master Mix (Takara, Tokyo, Japan). GAPDH functioned as the intrinsic reference for both mRNA and circRNA analysis, whereas U6 was exclusively utilized as the internal reference for mRNA quantification. The $2^{-\Delta\Delta CT}$ method was used for precise computation of the expression level relative to the reference levels. Detailed information regarding the primers utilized can be found in Table S1.

2.4 Western blot analysis

Protein extraction from cells or tissues was performed using RIPA lysis buffer. The extracted proteins were subjected to separation via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene fluoride membrane (Bio-Rad, CA, USA). To detect protein expression levels, a rabbit anti-RCC2 (CST; 3667S) polyclonal antibody was employed, with anti-GAPDH (CST; 92310SF) utilized as an internal control. The utilization of HRP-conjugated secondary antibodies (CST; 7074S) in conjunction with ECL Plus from EMD Millipore in Billerica, MA, USA, facilitated the precise detection of protein expression levels.

2.5 Experiments involving RNase R treatment

Following the amalgamation of RNase R and RNA acquired from Geneseed Biotech in Guangzhou, China, the composite underwent an incubation period of 20 minutes at 37°C. Subsequently, an additional series of qRT-PCR analyses was undertaken to quantitatively assess the mRNA levels of both GAPDH expression and circ-0015004. This comprehensive procedure enabled a precise assessment of the targeted RNA molecules, offering valuable insights into their expression profiles.

2.6 Subcellular localization

To elucidate the subcellular localization of circ-0015004, we employed a PARIS Kit sourced from Invitrogen in Carlsbad, CA, USA. This kit facilitated a precise determination of the cellular compartment where circ-0015004 predominantly resides, offering crucial insights into its functional role.

2.7 Cell transfection

siRNAs for targeting circ-0015004 (siCirc1, siCirc2) and YY1 (si-YY1), and a negative control siRNA (NC) (RiboBio, Guangzhou, China) were transfected into MHCC and Hep3B cells. After 48-hour transfection, the transfection efficacy was determined by qRT-PCR analysis. In addition, si-miR-330-3p, miR-330-3p mimics
and their corresponding NC counterparts (RiboBio, Guangzhou, China) were transfected into the cells by a transfection reagent (Lipofectamine 3000, Invitrogen). The specific sequences employed for these transfections are specified in Table S2.

### 2.8 CCK-8 assays

Following a 48-hour transfection duration, the assessment of cellular proliferation was performed using a Cell Counting Kit-8 (CCK-8) (Vazyme, Nanjing, China). The cells were evenly distributed into a 96-well plate (Invitrogen), with a seeding density of $1 \times 10^3$ cells per well. Subsequent to seeding, measurements were taken at 450 nm optical density (OD) at intervals of 24, 48, 72, 96, and 120 hours. To initiate the detection process, each well was treated with 10 µl of the CCK-8 reagent, followed by an additional one-hour incubation period at 37°C.

### 2.9 Colony formation assays

Following resuspension, the transfected cells were evenly distributed onto six-well plate (Invitrogen), with each well containing 500 cells. Then, the cells were allowed to culture under continuous temperature conditions for a period spanning 10 days. Post-culture, the cells underwent three washes with PBS, followed by fixation using methanol for 10 minutes. Subsequent staining with 0.1% crystal violet was carried out, allowing for photographic documentation and subsequent cell counting.

### 2.10 Bioinformatics analysis

The GSE12174 dataset constitutes a gene expression library containing information on circRNA expression levels in normal liver tissue and liver cancer tissue. This dataset was obtained from the Gene Expression Omnibus (GEO) database, a publicly accessible repository dedicated to storing and sharing genetic data.

To analyze the dataset, the GEO2R online tool was used. GEO2R is a web-based tool that provides a simple and user-friendly interface for analyzing gene expression data.

In this instance, the screening criteria employed adjusted P value < 0.05 and included logFC < 1. These specific criteria were utilized to pinpoint dysregulated circRNAs, which denote circRNAs exhibiting either upregulation or downregulation in HCC tissues compared to normal liver tissues.

Subsequently, the Starbase database was utilized to forecast the target genes of two dysregulated circRNAs identified in the GSE12174 dataset: circ-0015004 and miR-330-3p. Starbase is a web-based platform that provides a variety of tools for predicting the functions and targets of circRNAs. In this case, it was used to identify the genes that are likely to be regulated by these circRNAs.

Finally, the JASPAR database was used to predict possible upstream regulators of the dysregulated circRNAs. JASPAR is a database of transcription factor binding profiles, which are used to predict the regulatory elements in the genome. In this case, it was used to identify transcription factors that may be involved in the regulation of the circRNAs and their target genes.
2.11 Dual-luciferase reporter gene assays.

For predicting binding sites, we utilized online bioinformatics platforms (Circinteractome, miRwalk). Following this, elaborately engineered recombinant luciferase vectors encompassing both mutant (MUT) and wild-type (WT) sequences of circ-0015004 and RCC2 were constructed. These sequences were subsequently integrated into the pMIR-REPORT vector obtained from Ambion in Waltham, MA, USA. Co-transfection experiments were subsequently conducted in MHCC and Hep3B cells using the negative control (NC), miR-330-3p mimics, or si-YY1, assisted by Lipofectamine 3000 from Invitrogen. Following a 48-hour incubation, the luciferase activity was assessed utilizing the dual-luciferase reporter gene detection system obtained from Promega Corporation.

2.12 Examination employing RNA immunoprecipitation (RIP) analysis.

For the conduction of RIP analysis, a RIP kit was procured from Merck in Darmstadt, Germany. Briefly, MHCC and other cells were lysed using buffer for RIP lysis. The lysates were subsequently exposed to magnetic beads that had been previously attached to either the anti-AGO2 or anti-IgG antibody. This incubation was performed at 4°C for 6 hours. Then, the magnetic beads underwent a thorough washing process followed by digestion with proteinase K. Subsequent scrutiny of the purified RNA was carried out through qRT-PCR analysis, providing valuable insights into the interactions between the target molecules and AGO2.

2.13 Chromatin immunoprecipitation (ChIP).

The ChIP assay was conducted using a ChIP Chromatin Immunoprecipitation Kit acquired from Millipore in Massachusetts, USA. Formaldehyde was used to initiate cross-linking within the chromatin, leading to its fragmentation via sonication into smaller sections. Following this fragmentation process, magnetic beads, pre-coated with either anti-YY1 (CST; 46395S) or anti-IgG (CST; 3900S) antibodies, were employed for immunoprecipitation. Following the collection of enriched chromatin fragments and the removal of cross-linking, a subsequent qRT-PCR analysis was conducted.

2.14 Statistical analysis

Data in this study were statistically assessed and graphed by using GraphPad Prism 8 (La Jolla, CA, USA). The Data were presented as the mean ± standard deviations (SD). Differences between two or more groups were evaluated by the Student’s t test and ANOVA. P < 0.05 was defined as statistically significant.

2.15 Methods statement

The experiments have approved by the institutional and licensing committee, including any relevant details. The article have confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

3 Results
3.1 The circ-0015004 level is upregulated in HCC tissues and HCC cell lines

Dysregulated circRNAs in HCC tissues were identified through an assessment of the GSE12174 dataset. Based on the criteria of absolute log-fold change (\(|\log FC|\)) and adjusted P value, we observed a substantial elevation in circ-0015004 expression in HCC tissues (Fig. 1A). In accordance with data acquired from the UCSC Genome Browser, circ-0015004 originated from the back-splicing process involving exons 2, 3, 4, and 5 within the SDHC gene (Fig. 1B). Then, an assessment employing qRT-PCR was conducted on a cohort consisting of 80 individuals diagnosed with HCC. We observed a remarkable upregulation in the expression levels of circ-0015004 in HCC tissues compared to controls (Fig. 1C). To ascertain the clinical significance of the elevated expression of circ-0015004, a comprehensive analysis of the clinicopathological features among HCC patients was carried out. By employing the median expression level as the dividing threshold, patients were categorized into two distinct segments: a high-expression cohort consisting of 40 samples and a low-expression cohort consisting of 40 samples. According to the data presented in Table 1, the levels of circ-0015004 expression exhibited variations in accordance with the tumor size and TNM stage. Nevertheless, there was no notable correlation observed with respect to age, gender, HBV infection, or liver cirrhosis. Moreover, individuals manifesting escalated circ-0015004 expression levels displayed a less promising prognosis when compared to those exhibiting lower expression levels (Fig. 1D). Additionally, circ-0015004 was highly expressed in all five HCC cell lines (HepG2, MHCC-97H, Huh7, Hep3B, and HCC-LM3) (Fig. 1E). After undergoing RNase R treatment, the circ-0015004 expression levels in MHCC-97H and Hep3B cells remained relatively unaltered, suggesting the stable presence of circ-0015004 in these cells (Fig. 1F-G). These findings strongly imply the potential role of circ-0015004 in HCC.
Table 1
The correlation between hsa-circ-0015004 expression and clinicopathological features in 80 HCC patients.

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3.2 Downregulation of circ-0015004 inhibits HCC cell proliferation

Given the elevated expression of circ-0015004 in MHCC-97H and Hep3B cells, we focused on these lines for subsequent in vitro assessments. qRT-PCR analysis revealed cytoplasmic predominance of circ-0015004 (Fig. 2A). After transfection with si-NC, si-Circ1, and si-Circ2, both si-Circ2 and si-Circ1 significantly suppressed the expression of circ-0015004 (Fig. 2B). Remarkably, the reduction of circ-0015004 impeded cellular proliferation (Fig. 2C). Furthermore, circ-0015004 knockdown markedly
restrained the colony-forming capability of MHCC-97H and Hep3B cells (Fig. 2D). These results demonstrate that downregulation of circ-0015004 inhibits HCC cell proliferation.

3.3 Circ-0015004 acted as a sponge for miR-330-3p

The target miRNAs of circ-0015004 were predicted by the https://circinteractome.nia.nih.gov/. As shown in Fig. 3A, there was a potential binding site between miR-330-3p and circ-0015004. Furthermore, miR-330-3p upregulation inhibited the luciferase activity of the circ-0015004 reporter plasmids, while there were no significant changes in luciferase activities under miR-330-3p binding site mutation (Fig. 3B). Moreover, RIP assays exhibited a more significant amplification of circ-0015004 and miR-330-3p during Ago2 immunoprecipitation compared to IgG immunoprecipitation (Fig. 3C). Additionally, a reciprocal relationship was detected between the expression levels of miR-330-3p and circ-0015004 in both HCC cell lines and tissue. There was a significant negative correlation between miR-330-3p and circ-0015004 expression levels (Fig. 3D–F). Notably, circ-0015004 knockdown significantly enhanced miR-330-3p expression in MHCC-97H and Hep3B cells (Fig. 3G). Taken together, these results demonstrate that circ-0015004 functioned as a sponge for miR-330-3p.

3.4 MiR-330-3p impedes HCC cell proliferation

To uncover the governing process by which miR-330-3p influences the proliferation of HCC cells, we transfected miR-330-3p mimics into MHCC-97H and Hep3B cells. The efficacy of transfection was confirmed by qRT-PCR analysis (Fig. 4A). CCK-8 assays and colony formation analyses unequivocally showed that the enforced expression of miR-330-3p significantly restrained the growth capacity of HCC cells (Fig. 4B, C).

3.5 RCC2 acts a direct downstream target of miR-330-3p

To explore the regulatory mechanism of miR-330-3p in HCC cells, we employed miRwalk, miRTarbase, and miRDB to identify potential candidates for the target genes of miR-330-3p (Fig. 5A). Among the six candidate targets, RCC2 emerged prominently. RCC2 mRNA levels exhibited significant unregulation in both HCC cell lines and tissues (Fig. 5B, C). Analysis from the Kaplan-Meier Plotter database unveiled an association between elevated RCC2 expression and a poorer prognosis (Fig. 5D). Moreover, MHCC-97H and Hep3B cells were transfected with the pMIR-REPORT luciferase vector containing both WT) and mutant RCC2-3' UTR sequences, alongside either miR-330-3p mimics or a negative control. As shown in Fig. 5E-G, there was a notable reduction in fluorescence intensity in the WT group after the transfection with miR-330-3p mimics, whereas there was no significant alteration in the mutant group. Circ-0015004 knockdown decreased the RCC2 expression (Fig. 5H, L). Additionally, elevated expression of miR-330-3p led to a noteworthy decrease in both RCC2 mRNA and protein levels (Fig. 5I, M). Conversely, the reduction of RCC2 triggered by the inhibition of circ-0015004 was counteracted upon miR-330-3p knockdown (Fig. 5J-K, N). Collectively, these results suggest that circ-0015004 upregulates RCC2 expression by decrease the expression of miR-330-3p.
3.6 YY1 functions as a transcriptional regulator of circ-0015004

To investigate the transcriptional regulation mechanism of circ-0015004 in HCC, we used JASPAR database to explore the potential transcription factors of circ-0015004 (Fig. 6A). Among these candidates, our focus zeroed in on YY1 for further investigation. The ChIP assay was applied to examine the enrichment of YY1 onto the circ-0015004 promoter, indicating the binding of YY1 on circ-0015004 promoter (Fig. 6B). Additionally, the dual-luciferase reporter gene assay showed that si-YY1 markedly diminished the fluorescence intensity in the WT group (Fig. 6C). Furthermore, YY1 knockdown significantly decreased the expression of circ-0015004 (Fig. 6D-E). Collectively, these results demonstrate the role of YY1 serving as a crucial regulator for circ-0015004 transcription.

3.7 Suppressing the expression of circ-0015004 inhibits HCC growth in vivo

Given downregulation of circ-0015004 inhibits HCC cell proliferation, we further examined the effect of circ-0015004 on tumour growth in vivo. The results showed that circ-0015004 knockdown markedly decreased tumour volume and weight compared to the controls (Fig. 7). These results suggested that circ-0015004 was involved in HCC growth in vivo.

Discussion

Emerging evidence underscores the pivotal role of aberrantly expressed circRNAs in HCC progression (18, 19). In recent years, numerous circRNAs have emerged as biomarkers due to their functional relevance in the context of HCC (7, 20). In this study, circ-0015004 was screened and validated from the GSE12174 dataset. We observed a remarkable upregulation in both HCC tissues and cell lines. Furthermore, we demonstrated the novel regulatory signaling of circ-0015004/miR-330-3p/RCC2 axis in promoting HCC growth.

It is well documented that circRNAs play as miRNAs sponges to regulate gene expression and cellular activities. Our study unraveled the role of circ-0015004 as an antagonistic RNA (ceRNA) countering miR-330-3p, subsequently enhancing the mRNA level of RCC2 in HCC. MiR-330-3p has been acknowledged for its suppressive effects on various human tumors, including colorectal cancer, melanoma, and ovarian cancer (21–23). Notably, miR-330-3p downregulation amplifies HCC cell proliferation (24, 25). In line with the previous studies, miR-330-3p expression was decreased in both HCC cell lines and tissues. Moreover, the increased miR-330-3p significantly restricted the growth of HCC cells, underscoring the potential function of circ-0015004 as a modulator of miR-330-3p in HCC.

RCC2 was shown to play a pivotal role in regulating RAC1 activity. RCC2 is of great interest because it is involved in multiple cellular mechanisms, including cell proliferation, invasion, migration, apoptosis, cell cycle progression, and DNA damage response(26–28). Noteworthy, RCC2 expression levels in HCC tissue
correlate with tumor stage, size, differentiation and metastasis, influencing patients’ median survival times. Moreover, ectopic RCC2 expression engenders an escalation in colony formation and HCC cell proliferation while influencing cellular apoptosis (29, 30). Our study highlighted an elevation in RCC2 expression within both HCC tissues and cell lines. Importantly, we demonstrated that circ-0015004 could upregulate the expression of RCC2 by sponging miR-330-3p.

YY1 is a transcription factor that is elevated in cancer cells and regulates genes involved in differentiation, cell cycle control and carcinogenesis (31–33). Its dysregulation is associated with a variety of cancers, and its expression is increased in HCC (34). YY1 promotes quaking expression via super-enhancer binding during EMT of HCC (32). Our study further demonstrated that YY1 upregulates circ-0015004 expression, consequently impacting miR-330-3p and RCC2 mRNA levels.

In summary, our findings suggest that circ-0015004, regulated by YY1, emerges as an oncogenic factor, promoting HCC cell proliferation via the miR-330-3p/RCC2 axis. These discoveries reveal promising avenues for potential targets in HCC therapy.

**Conclusions**

In the present study, we demonstrate that circ-0015004 upregulates RCC2 expression by sponging miR-330-3p, thereby promoting HCC cell proliferation. Furthermore, we identified that YY1 may function as an important regulator of circ-0015004 transcription. Our findings suggest that circ-0015004 may serve as potential biomarker or therapeutic target for HCC.

**Declarations**

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Contributions of Authors**

Jie Z and Tong Z conceptualized and formulated the experimental design. PW, KJW, JJQ and Jie Z conducted the experiments. LQS, Jun Z and Qiang Z scrutinized and analyzed the data. LQS, Jun Z and Qiang Z composed the manuscript.

**Ethics Approval and Consent to Participate**

This study received approval from the Ethics Committee of Wujin traditional Chinese medicine hospital.

**Funding**

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Data availability statement

The data of this study are available from the corresponding author upon reasonable request.

References


Figures
Figure 1

The elevation of circ-0015004 in HCC cell lines and tissues. (A) GSE12174 dataset was analyzed and circ-0015004 was selected as the research object. (B) Circ-0015004 was formed through the back-splicing of the 2,3,4 and 5 exons of the SDHC gene. (C) Expression of circ-0015004 in HCC tissues. (D) Survival analysis of HCC patients. (E) Expression analysis of circ-0015004 in L02 cell lines (F-G) and HCC verified the levels of circ-0015004 expression in MHCC-97h and Hep3B cell lines post RNase R treatment. **P < 0.01, ***P < 0.001.
Figure 2

**Downregulation of circ-0015004 inhibits HCC cell proliferation.** (A) An investigation into the localization of circ-0015004 within Experiments targeting the nuclear and cytoplasmic localization in MHCC-97h and Hep3B cells. (B) The levels of circ-0015004 expression in MHCC-97h and Hep3B cells following transfection. (C-D) CCK-8 and colony formation assays were conducted to evaluate the influence of circ-0015004 regarding cell proliferation in MHCC-97h and Hep3B cells. **P < 0.01, ***P < 0.001.
Circ-0015004 acted as a sponge for miR-330-3p. (A) Computational tools were employed to identify the sites where circ-0015004 and miR-330-3p complementarily bind. (B) A luciferase reporter examination uncovered miR-330-3p as a target of circ-0015004 in Hep3B and MHCC-97h cells. (C) The assessment of circ-0015004 and miR-330-3p expression levels in MHCC-97h and Hep3B cells was conducted via RIP analysis. (D) Relative miR-330-3p expression in HCC tissues compared to adjacent normal tissues. (E) Relative miR-330-3p expression in L02 and HCC cell lines. **P < 0.01, ***P < 0.001.
Figure 4

MiR-330-3p impedes HCC cell proliferation. (A) Expression levels of miR-330-3p were comparatively analyzed in transfected MHCC-97H and Hep3B cells. (B-C) To assess the impact of miR-330-3p on cell proliferation in both MHCC-97H and Hep3B cells, CCK-8 and colony formation assays were utilized. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5

RCC2 acts a direct downstream target of miR-330-3p. (A) Computational tools such as MiRDB, miRtarbase, and miRwalk were harnessed to pinpoint potential gene targets susceptible to the impact of miR-330-3p. (B-C) A comparative analysis conducted for the purpose of evaluating RCC2’s distinct expression in both HCC cell lines and tissues. (D) Analysis from the Kaplan-Meier Plotter database illuminates the association of elevated RCC2 expression with a more adverse prognosis. (E) Employing computational tools, potential sites for binding between miR-330-3p and RCC2 were identified. (F-G) Employed a luciferase reporter gene test highlighted the interaction between RCC2 in MHCC-97h and Hep3B cells and miR-330-3p. (H-K) The relative mRNA expression levels of RCC2 were assessed in Hep3B
and MHCC-97h cells post transfection. (L-N) The assessment of RCC2 protein expression levels was carried out in transfected MHCC-97h and Hep3B cells. **$P < 0.01$, ***$P < 0.001$.

**Figure 6**

**YY1 acts as a transcriptional regulator of circ-0015004.** (A) Examination of the Jaspar database unveiled the prospective binding locus of YY1 within the promoter region of circ-0015004. (B) Results from the ChIP assay revealed the occupancy of YY1 in the promoter segment of circ-0015004. (C) Assessment of comparative luciferase activity in both Hep3B and MHCC-97h cells. (D-E) Examination of the comparative levels of expression regarding rcc2 and circ-0015004 in Hep3B and MHCC-97h cells. **$P < 0.01$, ***$P < 0.001$.**
Figure 7

** Suppressing the expression of circ-0015004 inhibits HCC growth in vivo. ** (A-B) Visual representation of xenograft tumors. (C-F) Measurement of tumor weight and volume conducted in both the NC group and siCirc group (n = 5/group). **P<0.01, ***P<0.001.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
• TableS1.docx
• TableS2.docx