circRNA circARNT2 Suppressed the Sensitivity of Hepatocellular Carcinoma Cells to Cisplatin by Targeting the miR-155-5p/PDK1 Axis

Yueyong Li,1 Yingjun Zhang,2 Shuai Zhang,3,4 Deyou Huang,5 Baosheng Li,5 Gengcheng Liang,1 Yingning Wu,4 Qiulan Jiang,1 Longhua Li,1 Cheng Lin,1 Zhonghen Wei,1 and Lingzhang Meng6

1Department of Interventional Medicine, the Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, China; 2Department of Medical Imageology, Human University of Medicine, Huaibou 418000, China; 3Department of Interventional Radiology, the Affiliated Hospital of Guizhou Medical University, Guiyang 550000, China; 4Department of Interventional Radiology, the Affiliated Cancer Hospital of Guizhou Medical University, Guiyang 550000, China; 5Department of Radiology, the Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, China; 6Center for Systemic Inflammation Research, School of Preclinical Medicine, Youjiang Medical University for Nationalities, Baise City 533000, Guangxi Province, China

Circular RNA (circRNA) is a novel subclass of non-coding RNA molecules that participate in development and progression of a variety of human diseases via sponging microRNAs (miRNAs). Until now, the contributions of circRNAs in chemoresistance of hepatocellular carcinoma (HCC) remain largely unknown. In the present study, we aimed to investigate the role of circRNA in cisplatin resistance of HCC. We investigated the expression of circRNAs in 5 paired cisplatin-sensitive and cisplatin-resistant HCC tissues by microarray analysis. The qRT-PCR analysis was to investigate the expression pattern of circARNT2 in HCC patient tissues and cell lines. Then, the effects of circARNT2 on cisplatin resistance, cell proliferation, and apoptosis were assessed in HCC in vitro and in vivo. circARNT2 was significantly upregulated in HCC tissues and cell lines. Overexpression of circARNT2 in HCC was significantly correlated with aggressive characteristics and served as an independent risk factor for overall survival in patients with HCC. In vitro experiments showed that knockdown of circARNT2 inhibited cell proliferation and enhances the cisplatin sensitivity of HCC cells. Furthermore, circARNT2 facilitates HCC progression in vivo. We demonstrated that circARNT2 acts as a sponge for miR-155-5p and verified that PDK1 is a novel target of miR-155-5p. In summary, our study demonstrated that circARNT2 modulates cisplatin resistance through miR-155-5p/PDK1 pathway. Our findings indicated that circARNT2 may serve as a promising therapeutic target for overcoming cisplatin resistance for HCC.

Circular RNA (circRNA) is a novel type of noncoding RNA with a covalently closed loop, which is generated by the back-splicing of pre-miRNA. More newly identified circRNAs have been found using high-throughput sequencing and via further functional validation. Increasing evidences indicate that circRNAs are implicated in several pathophysiological processes including human cancers. Aberrant expression of circRNAs has been frequently observed in various cancers. Moreover, circRNAs regulate malignant behaviors of cancer cells, such as proliferation, apoptosis resistance, migration, invasion, and drug resistance. Although several circRNAs have been reported to participate in the tumorigenesis and progression of HCC, the expressions and roles of circRNA in chemoresistance of HCC remain unclear.
In this study, we aimed to investigate the role of circRNA in cisplatin resistance of HCC. We identified 968 significantly dysregulated circRNAs in cisplatin-resistant HCC tissues. We focused on hsa_circ_0104670, which is located on chr15: 80767350–80772264 and derived from ARNT2 gene, and thus we termed as circARNT2. We further tested circARNT2 in HCC samples by qRT-PCR and the results showed that the expression of circARNT2 was markedly elevated both in HCC tissues and exosomes from HCC plasma. Gain-of function investigations showed that circARNT2 overexpression suppressed cancer cell growth in vivo and in vitro. Subsequent studies displayed that circARNT2 could sensitize HCC cells to cisplatin by targeting the miR-155-5p/PDK1 signaling axis. Our findings will provide new insights into the regulatory mechanisms of circARNT2 in tumor progression and cisplatin resistance of HCC.

RESULTS
Upregulation of circARNT2 Is Associated with Cisplatin Resistance in HCC
To investigate the role of circRNAs in cisplatin resistance of HCC, we performed circRNAs array to identify the differentially expressed circRNAs. A total of 9,857 circRNAs were detected in 5 pairs of cisplatin-resistant HCC tissues and cisplatin-sensitive HCC tissues by the circRNA microarray analysis. Among them, 968 circRNAs were significantly aberrantly expressed (p < 0.05 and fold-change [FC] > 2.0) between cisplatin-resistant HCC tissues and cisplatin-sensitive HCC tissues. Of these circRNAs, 538 were significantly upregulated and 430 were significantly downregulated in cisplatin-resistant
HCC tissues compared with cisplatin-sensitive HCC tissues. Among the 968 differentially expressed circRNAs, 246, including 132 upregulated ones and 114 downregulated ones, were verified as novel circRNAs; 722 circRNAs, including 406 upregulated and 316 downregulated ones, had been identified beforehand and listed in the circRNA database (circBase: http://www.circbase.org; Figure 1A). The 968 identified circRNAs were divided into five different categories on the basis of the way they were produced. Exonic circRNAs consisting of the protein-encoding exons accounted for 70.97% (687/968), intronic circRNAs from intron lariats comprised 8.99% (87/968), sense overlapping circRNAs that originated from exon and other sequence circRNAs comprised 18.08% (155/968), intergenic circRNAs composed of unannotated sequences of the gene and antisense circRNAs originating from antisense regions equally comprised 1.96% (19/968; Figure 1B). Hierarchical clustering was then performed to demonstrate the five most upregulated circRNAs (hsa_circ_0005394, hsa_circ_0001741, hsa_circ_0006916, hsa_circ_0102034, and hsa_circ_0104670) and five most downregulated circRNAs (hsa_circ_000567, hsa_circ_000458, hsa_circ_0001649, hsa_circ_0103809, and hsa_circ_0004018) expression patterns among the sets (Figure 1C).

The five most upregulated circRNAs were selected and validated by qRT-PCR using 82 HCC and paired non-tumorous tissue samples. As shown in Figure 1D–1H, qPCR results further confirmed that circ_0104670 was significantly increased in HCC tissues compared with adjacent tissues, and its expression was higher in cisplatin-resistant HCC tissues than in the cisplatin-sensitive tissues. By browsing the human reference genome (GRCh37/hg19), we identified that hsa_circ_0104670 (chr15: 80767350–80772264) is derived from ARNT2, with a spliced mature sequence length of 4,914 base pairs (bp), and thus we named it circARNT2. We verified its existence in many circRNA databases. According to the circBase database, circARNT2 is detected in normal human frontal cortex (http://www.circbase.org/cgi-bin/singleRecord.cgi?id=hsa_circ_0104670).

To further investigate the role of circARNT2 in HCC, we analyzed the relationship between circARNT2 expression in HCC tissues and clinicopathological characteristics of HCC patients. Using the median expression level of circARNT2 as cutoff value, patients who expressed circARNT2 equal to or greater than the average level were assigned to the “circARNT2 high” group. As shown in Table 1, high expression of circARNT2 in HCC tissues was significantly correlated with tumor size (p = 0.003), distant metastasis (p = 0.031), and TNM stage (p = 0.023) but not related to gender, age, and differentiation. In addition, further Kaplan-Meier survival analyses revealed that the HCC patients with high circARNT2 level had shorter overall survival than the patients had low circARNT2 level (p = 0.012, Figure 2A). We also found that circARNT2 was significantly increased in cisplatin resistant HCC cell lines (Hep3B-R and Huh7-R) compared with the parental HCC cell lines (p < 0.01; Figure 2B). These results suggest that circARNT2 is closely associated with cisplatin resistance in HCC.

Table 1. Association of circARNT2 Expression with Clinicopathological Features of HCC Patients

| Characteristics          | circARNT2 High (n = 41) | Expression Low (n = 41) | p     |
|--------------------------|------------------------|------------------------|-------|
| Age, y                   |                        |                        |       |
| ≥50                      | 21                     | 24                     | 0.657 |
| Gender                   |                        |                        |       |
| Female                   | 19                     | 22                     | 0.659 |
| Serum AFP                |                        |                        |       |
| ≥20                      | 20                     | 22                     | 0.268 |
| Tumor size               |                        |                        |       |
| ≥5.0 cm                  | 14                     | 18                     | 0.003 |
| TNM stage                |                        |                        |       |
| III/IV                   | 8                      | 18                     | 0.031 |
| Distant metastasis       |                        |                        |       |
| Yes                      | 6                      | 16                     | 0.023 |

To further validate the expression level of circARNT2 on cisplatin resistance, we constructed circARNT2 shRNA and circARNT2 overexpression vector and performed loss- and gain-of-function studies by knocking down or overexpressing circARNT2 in HCC cells. First, we knocked down the expression of both circARNT2 and ARNT2 mRNA. Hep3B-R and Huh7-R cells were transfected with three kinds of circARNT2 shRNA (respectively sh-circARNT2 #1, sh-circARNT2 #2, or sh-circARNT2 #3) or GFP lentivirus (sh-CTL), and the sequence only in the linear transcript (si-ARNT2). As expected, shRNA directed against the backsplice sequence knocked down only the circular transcript and did not affect the expression of linear species, and small interfering RNA (siRNA) targeting the sequence in the linear transcript knocked down only the linear transcript and did not affect the expression of the circular transcript in Hep3B-R and Huh7-R cells (Figures S1A–S1F; p < 0.01). Due to the highest efficiency of interference, sh-circARNT2 #3 was chosen for the subsequent experiments. Meanwhile, we infected Hep3B and Huh7 cells with the circARNT2 overexpression adenovirus (circARNT2 OE) or control GFP adenovirus (circARNT2 CTL). The qRT-PCR assay indicated the relative abundance of circARNT2 in Hep3B and Huh7-R cells with the circARNT2 overexpression adenovirus (circARNT2 OE) or control GFP adenovirus (circARNT2 CTL). The qRT-PCR assay indicated the relative abundance of circARNT2 in Hep3B and Huh7-R cells infected with adenovirus (Figures S1G and S1H; p < 0.01). We found that inhibition of circARNT2 significantly inhibited cells proliferation (Figures 2C and 2D) and induced apoptosis (Figures 2E and 2F) in Hep3B-R and Huh7-R cells compared with negative control. In addition, circARNT2 downregulation sensitized Hep3B-R and Huh7-R cells to cisplatin (Figures 2G and 2H).

circARNT2 Knockdown Inhibited the Growth of HCC

Furthermore, the tumor suppressive effects of circARNT2 downregulation were also confirmed in vivo. Hep3B-R cells stably infected with sh-circARNT2 or sh-CTL were subcutaneously injected into each
mouse. Our results showed that the tumor volumes in nude mice injected with sh-circARNT2-transfected Hep3B-R cells were smaller than in the control nude mice (Figure 3A). Tendencies in tumor weight were consistent with those in tumor volume (Figure 3B). The proliferative marker ki67 expression was decreased in tumor tissues of nude mice injected with sh-circARNT2-transfected Hep3B-R cells (Figure 3C).

Confirmation of the Circular Structure and Subcellular Localization of circARNT2

Next, we investigated the stability and localization of circARNT2 in HCC cells. Total RNAs from Hep3B-R and Huh7-R cells were isolated at the indicated time points after treatment with Actinomycin D, an inhibitor of transcription. Then qRT-PCR was performed to measure the level of circARNT2 and ARNT2 mRNA. The results showed that the half life of circARNT2 exceeded 24 h, whereas that of circARNT2 mRNA was about 4 h in both Hep3B-R and Huh7-R cells (Figures 4A and 4B). Furthermore, we found that circARNT2 was resistant to RNase R digestion (Figures 4C and 4D). These data confirmed that circARNT2 was a circular RNA. We then investigated the localization of circARNT2. The qRT-PCR of RNAs from nuclear and cytoplasmic fractions indicated that circARNT2 was predominantly localized in the cytoplasm of Hep3B-R and Huh7-R cells (Figures 4E and 4F). Collectively, the above data suggested that circARNT2 harbored a loop structure and was predominantly localized in the cytoplasm.

circARNT2 Functioned as a Molecular Sponge of miR-155-5p in HCC Cells

Given that many circRNAs can function as miRNA sponges in the cytoplasm, we determined whether circARNT2 may also bind to miRNAs as a sponge and regulate targets via the competitive endogenous RNA (ceRNA) mechanism. We therefore analyzed the sequence of circARNT2 using the miRanda algorithm and identified 199 miRNA-binding sites (Table S1); however, five miRNAs with relatively high scores (miR-155-5p, miR-1197, miR-155-5p, miR-1228, and miR-1236) were finally selected.

It is well known that miRNAs usually silence gene expression by combining with the Argonaute 2 (AGO2) protein and form the RNA-induced silencing complex (RISC). In the context of the ceRNA mechanism, it might be a prevalent phenomenon that AGO2 could bind with both circRNAs and miRNAs. We therefore conducted an RNA immunoprecipitation (RIP) assay to pull down RNA transcripts that bind to AGO2 in Hep3B-R cells. Indeed, endogenous circARNT2 was efficiently pulled down by anti-Ago2 (Figure 5A). To further detect whether circARNT2 could sponge miRNAs, we performed a miRNA pull-down assay using biotin-coupled miRNA mimics (miR-155-5p, miR-1197, miR-155-5p, miR-1228, and miR-1236). Interestingly, circARNT2 was only efficiently enriched by miR-155-5p, but not by the other three miRNAs (Figure 5B). In order to further validate the interaction, circARNT2 sequence containing the putative or mutated miR-155-5p binding site was cloned into the downstream of luciferase reporter gene, generating wild-type...
(WT)-circARNT2 or mutant (MUT)-circARNT2 luciferase reporter plasmids. Then the effect of miR-155-5p on WT-circARNT2 or MUT-circARNT2 luciferase reporter systems was determined. The results showed that miR-155-5p mimic considerably reduced the luciferase activity of the WT-circARNT2 luciferase reporter vector compared with negative control, while miR-155-5p mimic did not pose any impact on the luciferase activity of MUT-circARNT2-transfected Hep3B-R cells (p < 0.01, Figure 5C). In a further RIP experiment, circARNT2 and miR-155-5p simultaneously existed in the production precipitated by anti-AGO2 (p < 0.01, Figure 5D), suggesting that miR-155-5p is circARNT2-targeting miRNA. Furthermore, silencing of circARNT2 did not affect the expression of miR-155-5p, and transfection of miR-155-5p mimics did not affect the expression of circARNT2 (Figures 5E and 5F), which indicated circARNT2 functions as a miRNA sponge without affecting the expression of sponged miRNAs.

miR-155-5p Suppressed Cisplatin-Resistant HCC Tissues Resistance of HCC Cells
The qRT-PCR analysis indicated that there was a decreasing trend in miR-155-5p levels from normal liver tissues to cisplatin-sensitive HCC tissues and then to cisplatin-resistant HCC tissues, and the differences among the three groups were significant (p < 0.01; Figure 6A). We also confirmed that the expression of miR-155-5p was obviously decreased in cisplatin-resistant cells than that in cisplatin-sensitive cells, indicating the opposite result to circARNT2 expression (p < 0.01; Figure 6B). To gain insight into whether circARNT2 affected cisplatin resistance of HCC cells via modulation of miR-155-5p, we further performed rescue assays to confirm how miR-155-5p modulated cisplatin resistance. We transfected miR-155-5p mimics or inhibitors into HCC cells and the proliferation curves were performed. Our results showed that Hep3B cells transfected with miR-155-5p inhibitors grew at a dramatically higher rate as compared with controls (Figure 6C; p < 0.01), whereas miR-155-5p mimics markedly inhibited the cell growth in Hep3B-R cells when compared with cells transfected with miR-NC (Figure 6D; p < 0.01). Moreover, cell proliferation assay proved that downregulation of circARNT2 markedly inhibits the cell growth of Hep3B-R cells, whereas sh-circARNT2#3-induced decrease of cell growth was partially restored by miR-155-5p inhibition (Figure 6E; p < 0.01). Furthermore, flow cytometry analysis indicated that circARNT2 knockdown dramatically aggravated cisplatin-induced apoptosis of Hep3B-R cells, however, sh-circARNT2#3-triggered apoptosis was attenuated after cotransfection with miR-155-5p inhibitor (Figure 6F; p < 0.01). Together, these data hinted that inhibition of miR-155-5p could significantly reversed circARNT2-mediated cisplatin resistance in HCC cells.

miR-155-5p Inhibits PDK1 and Promotes Autophagy
We sought to explore potential target genes of miR-155-5p. Bioinformatics analysis by using the TargetScan and FindTar algorithm predicted one putative and highly conserved miR-155-5p binding site within the 3’ UTR of PDK1 (Figure 7A). Then, we focused on the transcriptional regulation of PDK1 expression by miR-155-5p. We constructed a luciferase reporter gene plasmid containing PDK1 WT 3’ UTR and its MUT 3’ UTR (Figure 7A). The dual luciferase reporter gene assay showed that the fluorescence enzyme activity was significantly decreased after co-transfection with the PDK1 WT 3’ UTR construct and miR-155-5p mimics. In contrast, the fluorescence enzyme activity was nearly unchanged after co-transfection with the PDK1 MUT 3’ UTR construct and miR-155-5p mimics (Figure 7B). To determine the expression levels of PDK1 in HCC, we investigated the PDK1 expression in the HCC tissues by qRT-PCR. The results showed that PDK1 expression was significantly upregulated in HCC specimens compare with that in the adjacent normal tissues (p < 0.01; Figure 7C). To further confirm the effects of circARNT2 on PDK1 expression, we transfected HCC cells with the circARNT2 siRNA and detected the PDK1 mRNA levels by qRT-PCR. The results showed that knockdown of circARNT2 expression significantly reduced the PDK1 mRNA levels in SR-HepG2 cells (Figure 7D). Moreover, inhibition of circARNT2 mediated decrease of PDK1 mRNA expression was significantly recuperated following miR-155-5p inhibitors (Figure 7D).

Previous studies have shown that PDK1 is a critical checkpoint for autophagy dysfunction. To determine the role of miR-155-5p in autophagy, we transfected miR-155-5p mimics into Hep3B-R cells and found that the expression levels P62, a marker of autophagy, was significantly reduced in miR-155-5p-overexpressing cells compared to the
corresponding control cells (Figure 7E). Taken together, the results indicate that autophagy activity could be enhanced with upregulation of miR-155-5p expression. Taken together, our results indicate that circARNT2 positively regulated PDK1 expression by interacting with miR-155-5p, and this is then followed by the inhibition of autophagy.

**DISCUSSION**

In this study, we explored the effect of circARNT2 on the chemosensitivity of HCC and demonstrate the regulatory mechanism of miR-155-5p/PDK1 signaling pathway. We first discovered that circARNT2 is frequently upregulated in HCC, and its expression significantly correlated with poor clinicopathologic characteristics. Second, our data showed that the high expression of circARNT2 correlated with poor patient prognosis, indicating its applicability as a promising prognostic biomarker in HCC. Third, we demonstrated that the inhibition of circARNT2 reversed the cisplatin resistance of HCC cells and thus inhibited the progression of HCC. Fourth, we revealed that circARNT2 acts as a ceRNA and regulates PDK1-induced autophagy by competing with miR-155-5p. These results suggested that circARNT2 may have the potential to regulate the cisplatin resistance of HCC, in turn promoting the progression of HCC.

In the last decade, improved drug therapy agents have significantly prolonged the survival of HCC patients with advanced diseases. Cisplatin, the first-generation of the platinum chemotherapeutic drugs, can inhibit DNA replication and transcription by forming crosslinks between DNA double strands and exhibits broad-spectrum antitumor activity. Cisplatin is one of the most commonly used chemotherapeutic agents to treat advanced HCC. However, the acquisition of multi-drug resistance (MDR) to cisplatin is still a major obstacle for HCC patients to obtain a satisfactory curative effect. The role of circRNAs and the underlying mechanisms in HCC has been reported before. However, more specific mechanisms of circRNAs in drug resistance of HCC need to be further teased out. Using a circRNA microarray assay, we analyzed aberrantly expressed circRNAs between cisplatin-resistant HCC tissues and cisplatin-sensitive HCC tissues. Results showed that 538 were significantly upregulated and 430 were significantly downregulated in cisplatin-resistant HCC tissues compared with cisplatin-sensitive HCC tissues. Then, we demonstrated that circARNT2 was upregulated in HCC tissues and associated with cisplatin resistance in HCC. To further validate whether circARNT2 functionally required for cisplatin resistance, we performed loss-of-function studies by knockdown circARNT2 in cisplatin-resistant HCC cell lines (Hep3B-R and Huh7-R). Meanwhile, we overexpressed circARNT2 in Hep3B and Huh7 cells. Loss-of-function experiments revealed that knockdown of circARNT2 inhibited the cisplatin-induced cell apoptosis and cell mobility of cisplatin-resistant cells. Gain-of-function experiments revealed that ectopic expression of circARNT2 promoted proliferation and promoted apoptosis of cisplatin sensitive cells, compared with negative control-transfected cells. In addition, xenograft experiments revealed that circARNT2 knockdown inhibited the growth of HCC in vivo.
circRNAs may act as transcription regulators or as sponges for small RNA regulators, which compete for microRNA (miRNA) activity in the process of regulating cell proliferation. Most circRNAs have miRNA-binding sites that can be used as miRNA sponges to inhibit the regulation of miRNAs on downstream target genes by a large number of miRNAs in cancers. Herein, circARNT2 has been shown to target miRNA-155-5p using bioinformatics tools. Intriguingly, the ectopic expression of miRNA-155-5p reduced the luciferase activity of the WT-circARNT2 luciferase reporter vector compared with negative control, while miR-155-5p mimic did not pose any impact on the luciferase activity of MUT-circARNT2-transfected cells. Furthermore, endogenous circARNT2 and miRNA-155-5p were pulled down by a special AGO2 antibody. Ultimately, we found that circARNT2 enhances the cisplatin resistance, mainly through interaction with miRNA-155-5p, and miRNA-155-5p mimics reversed circARNT2-mediated cisplatin resistance effects. Taken together, all of the data suggest that miRNA-155-5p recognizes and binds to circARNT2 without promoting the degradation of circARNT2.

In this study, we identified circARNT2 as a new interactive molecule of miRNA-155-5p, also confirmed that PDK1 was a new downstream target of miRNA-155-5p. PDK1 is now widely studied in malignant tumors because PDK1 can serve as an important junction point for multiple cell signaling pathways. The results of qRT-PCR showed that PDK1 expression in HCC specimens was significantly upregulated compared with that in the adjacent normal tissues. circARNT2 could control the PDK1 level by provoking miRNA-155-5p. Previous studies have shown that PDK1 is a critical checkpoint for autophagy dysfunction. Autophagy is an important metabolic process for maintaining cell homeostasis. Autophagy reduces protein synthesis and increases protein degradation, thereby inhibiting the proliferation of primary cancer cells and tumor growth. We found that the expression of miR-155-5p contrasted with the activity of autophagy marker protein (P62), indicating that upregulation of miR-155-5p induces autophagy in HCC cells.

In conclusion, our study revealed that circARNT2 is frequently activated in cisplatin-resistant HCC tissues and cell lines and associated with a poor survival outcome. These results indicate that circARNT2 functions as an oncogene by sponging miR-155-5p, leading to PDK1 upregulation, and finally sensitizes HCC cells to cisplatin. Therefore, our findings provide significant evidence to further elucidate the therapeutic use of circRNA in HCC.

MATERIALS AND METHODS

Clinical Specimens
A total of 82 pairs of HCC and tumor-adjacent tissues were collected from patients who underwent hepatectomy at the Affiliated Hospital...
None of HCC patients received any pre-operative treatments, such as RFA, TACE, immunotherapy, and targeted therapy. The tissue samples were confirmed by two histopathologists. All samples were immediately snap-frozen in liquid nitrogen and subsequently stored at –80°C until RNA extraction.

**Cell Culture**

HCC cell lines Hep3B, Huh-7, and the normal human liver cell line LO2 were purchased from the Chinese Academy of Sciences Cell Bank Type Culture Collection. The cells were cultured with DMEM and RPMI-1640 (GIBCO, Carlsbad, CA) together with 10% fetal bovine serum (GIBCO) at 37°C in an atmosphere containing 5% CO2. The cisplatin-resistant Hep3B (Hep3B-R) and Huh7 (Huh7-R) cells were prepared according to the method previously described.

**circRNA Microarray Analysis**

Total RNA was extracted from patients with cisplatin-resistant or cisplatin-sensitive HCC using the RNeasy Mini Kit (QIAGEN, GmBH, Hilden, Germany) according to the manufacturer’s instructions. The adjacent tissues were used as control. Patients with cisplatin-resistant HCC were defined as those with persistent disease more than 2 months, and those with recurrent disease more than 2 months after completion of chemotherapy containing cisplatin. Patients with cisplatin-sensitive HCC were defined as those without local residual lesions or recurrence at 2 months after completion of chemotherapy containing cisplatin. Purified total RNA was quantified using the NanoDrop 2000 spectrophotometer. The total RNA was sent to Aksomics (Shanghai, China) to analyze circRNA expression profiles. Differentially expressed circRNAs were identified as FC > 2 and adjusted p <0.05.

**TCGA Dataset Analysis**

The data and the corresponding clinical information of patients were collected from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/). We used the edgeR package of R packages to perform the difference analysis (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) and the pheatmap package of R packages to perform the cluster analysis (https://cran.r-project.org/web/packages/pheatmap/index.html). Sva R package was used to
remove the batch effect. Genes with adjusted p values < 0.05 and absolute FCs >1.5 were considered differentially expressed genes. Kaplan-Meier survival curves were drawn to analyze the relationships between genes and overall survival in the survival package. The corresponding statistical analysis and graphics were performed in R software (R version 3.3.2).

RNA Isolation and qRT-PCR

RNA was totally extracted from the cells and tissue using the with TRizol reagent (1 mL; Invitrogen) based on the manufacturer’s protocol. The testing for miRNA extraction was mirVana miRNA isolation kit (Ambion, Austin, TX, USA). After isolation, the RNA concentration in the RNA solution was determined using Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at 80°C for further use. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

siRNA and Plasmid Construction and Cell Transfection

For transfections, cells at the confluence of 50%–80% were infected with 1 × 10^6 recombinant lentivirus-transducing units and 6 μg/mL Polybrene (Sigma, Shanghai, China). Stably transfected cells were selected via treatment with 2 μg/mL puromycin for 2 weeks. Stably transfected cells were picked via flow cytometry for subsequent assays. Plasmid, lentivirus, miRNA inhibitor, and miRNA mimics used in this study were purchased from GenePharma (Shanghai, China), pHBV1.3 copy was purchased from Miaolingbio (Wuhan, China). Lipofectamine 3000 (Invitrogen, CA, USA) was utilized for transfection.

CCK-8 Assay

After transfection, the cells mixed with 10 mL of CCK-8 solutions per well and incubated for further 1 h at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was measured for absorbance at 450 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The optical density values of each well represented the survival/proliferation of HCC cells.

Flow Cytometric Analysis

Transfected cells were harvested after transfection by trypsinization. After the double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide was done by the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s recommendations, the cells were analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a Cell Quest software (BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells and then the relative ratio of early apoptotic cells were compared with control transfection from each experiment.
Tumor Xenograft in Nude Mice
Animal experiments were approved by the Ethical Committee for Animal Research of the Affiliated Hospital of Youjiang Medical University for Nationalities. Ten nude mice (5 mice per group, male, 2 months old) were purchased from Shanghai Experimental Animal Center (Shanghai, China). Mice were subcutaneously injected into the back with 1 × 10^6 SR-HepG2 cells transfected with si-circARNT2 or si-NC suspended in 100 μl Hank’s balanced salt solution. The tumor size was measured every 3 days with a caliper, and tumor volume was calculated according to the formula: volume = length × width^2/2. All mice were sacrificed on day 21 after inoculation. The resected tumor masses were harvested for subsequent weight and qRT-PCR analysis.

Actinomycin D and RNase R Treatment
To block transcription, we added 2 mg/mL Actinomycin D or dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) as a negative control into the cell culture medium. For RNase R treatment, total RNA (2 μg) was incubated for 30 min at 37°C with or without 3 U/μg of RNase R (Epicenter Technologies, Madison, WI, USA). After treatment with Actinomycin D and RNase R, qRT-PCR was performed to determine the expression levels of circARNT2 and ARNT2 mRNA.

Isolating RNAs from Nucleus and Cytoplasmic Fractions
The nuclear and cytoplasmic fractions were isolated using PARIS Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Briefly, cells were collected and lysed with cell fractionation buffer, followed by centrifugation to separate the nuclear and cytoplasmic fractions. The supernatant containing the cytoplasmic fraction was collected and transferred to a fresh RNase-free tube. The nuclear pellet was lysed with Cell Disruption Buffer. The cytoplasmic fraction and nuclear lysate were mixed with 2X Lysis/Binding buffer, followed by centrifugation to separate the nuclear and cytoplasmic fractions. The nuclear and cytoplasmic fractions were eluted with Elution Solution. U6 snRNA and 18S rRNA were employed as internal controls.

Luciferase Reporter Assays
The luciferase reporter assays were carried out with the help of the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The WT circARNT2 or MUT circARNT2 that had the predicted miR-155-5p binding site was established and integrated into pmiR-GLO-dual-luciferase-reporter vector. Cotransfection of circARNT2-WT or circARNT2-MUT was carried out with miR-155-5p mimics or negative control into HCC cells with the use of Lipofectamine 2000. Subsequent to transfection for a period of 48 h, the luciferase activities were measured in accordance with the guidelines of the manufacturer. In the same manner, pmiR-GLO-PDK1-WT or pmiR-GLO-PDK1-MUT were constructed, together with cotransfecting with miR-155-5p mimics or negative control into cells. 48 h following the transfection, the relative luciferase activities were detected.

RIP
Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) were used for RIP. Cells were lysed in complete RNA lysis buffer, then cell lysates were incubated with RIP buffer containing magnetic beads conjugated with human anti-AGO2 antibody (Millipore) or negative control mouse immunoglobulin G (IgG; Millipore).

Statistical Analysis
Results are presented expressed as mean ± SD (standard deviation). Student’s t test was performed to measure the difference between two group and differences between more than two groups were assessed using one-way ANOVA. p <0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.08.037.

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