Cisplatin resistance-associated circRNA_101237 serves as a prognostic biomarker in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is a leading cause of cancer-associated mortality worldwide. Despite clinical advances, the survival rate of patients with HCC remains low, as most patients are diagnosed with HCC when they are already at the advanced stage. Certain circular RNAs (circRNAs) are closely associated with the development of liver cancer. In the present study, a circRNA array was performed to screen differentially expressed circRNAs in HCC tissues. The further analysis focused on the newly identified circRNA_101237, the host gene of which, cyclin-dependent kinase 8, is located at chr13:26974589-26975761. CircRNA_101237 was determined to be upregulated in tumor tissue and serum of patients with HCC as compared with that in paracancerous tissues and the serum of healthy controls, respectively. In addition, the expression of circRNA_101237 was associated with tumor size, lymph node metastasis, distant metastasis and TNM stage. Univariate and multivariate analysis indicated that serum circRNA_101237 levels were an independent predictor of survival prognosis in patients with HCC. The overall survival of patients with high expression of circRNA_101237 was reduced compared with that of patients with low expression of circRNA_101237. Of note, cisplatin induced the expression of circRNA_101237 in HCC cells in a dose- and time-dependent manner *in vitro*, and the levels of circRNA_101237 in the serum of patients with cisplatin-resistant HCC and in cisplatin-resistant Huh7 cells were increased. The present study provided novel insight into the use of circRNA_101237 as a diagnostic biomarker for HCC and a potential therapeutic target.

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Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-associated mortality worldwide. Although the clinical application of sorafenib, regorafenib and nivolumab has achieved significant progress in the treatment of HCC, the survival rate of patients with HCC remains low, as most patients with HCC are diagnosed at an advanced stage (1). Cisplatin and various platinum agents have become standard drugs for the treatment of HCC (2). However, as cisplatin resistance may occur, the survival benefit for patients with advanced HCC is unsatisfactory (3). To improve the survival of patients with HCC, novel biomarkers for early diagnosis and the development of novel therapeutic targets for cisplatin-resistant HCC are required (4).

Liver cancer is associated with hepatitis B virus (HBV), HCV and non-alcoholic fatty liver disease (5). However, the molecular mechanisms of the genesis of HCC remain poorly understood. Recently, new evidence has demonstrated that different types of non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long ncRNAs and partially circular RNAs (circRNAs), have a role in HCC (6). High-throughput next-generation sequencing analysis has identified a large number of circRNAs that are involved in liver cancer through interactions with miRNAs or proteins (7,8).

CircRNAs are generated from backsplicing of exons and introns, forming a circular exonic circRNA, a circular intronic RNA and an exon-intron circRNA. CircRNAs have a covalently closed continuous loop structure. They cannot be degraded by RNA exonuclease or RNase R. Therefore, circRNAs are suitable as diagnostic biomarkers for tumors, including HCC (9). CircRNAs are abundantly expressed in tissues, blood and microvesicles and are highly conserved among different species. CircRNAs are able to regulate gene expression at the transcriptional or post-transcriptional level (10). CircRNA is involved in various biological processes, including HCC cell proliferation, apoptosis and metastasis (11). For instance, overexpression of *Homo sapiens* circRNA_0001649 inhibits the proliferation and invasion of HCC cells (12). CircRNA circMTO1 (mitochondrial tRNA translation optimization 1) inhibits HCC progression by acting as a sponge for miRNA-9. Reduced levels of circMTO1 in HCC tissue may be used as a predictor of poor survival (13).

In the present study, a circRNA array was performed to screen for differentially expressed circRNAs in HCC tissues. The study then focused on the newly identified circRNA_101237, whose encoding gene is located at chromosome (chr)13:26974589-26975761 and which is produced by backsplicing of exons 10, 11 and 12 of cyclin-dependent kinase (CDK)8 (Fig. 1). CircRNA_101237 is upregulated in tumor tissues and serum of patients with HCC compared with that in paracancerous tissues and serum of healthy controls, respectively. In addition, the association between serum circRNA_101237 levels and the clinical outcome in patients with HCC was assessed.

Materials and methods

Patients and samples. A total of 100 HCC cancer tissues and matched adjacent tissues were collected from Huainan First People's Hospital and the First Affiliated Hospital of the Medical College of Anhui University of Science and Technology (Huainan, China) between September 2013 and September 2017. The serum samples from another independent cohort, including 120 healthy individuals who came for physical examination and 234 patients with HCC were also collected from Huainan First People's Hospital and the First Affiliated Hospital of the Medical College of Anhui University of Science and Technology between September 2013 and September 2017. The general demographic and clinicopathological characteristics of 234 patients with HCC are shown in Table SI. The medical records of HCC patients with clinical TNM staging and survival information were collected. Patients with cisplatin-resistant HCC were defined as those with persistent disease at >6 weeks and those with recurrent disease at >2 months after completion of cisplatin-based chemotherapy. Patients with cisplatin-sensitive HCC were defined as those without local residual lesions at 6 weeks or no recurrence at 2 months after completion of cisplatin-based chemotherapy. The cisplatin-based chemotherapy regimen consisted of doxorubicin 60 mg/m^2, followed by cisplatin 60 mg/m^2 infused over 30 min on day 1. Chemotherapy cycles were repeated every 21 days for 3 cycles (14).

Cell culture and treatment. The HCC cell lines HCCLM3, Hep3B and MHCC97-H were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All of the cells were grown routinely in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37˚C in a humidified atmosphere with 5% CO_2.

The HCC cell lines were exposed to cisplatin at 0, 0.5, 1 and 2 μg/ml for 48 h or at 1 μg/ml for 0, 12, 24 or 48 h, and the expression of circRNA_101237 was then assessed by reverse transcription-quantitative (RT-q) PCR.

Cisplatin-resistant cells. Parental Huh7 cells were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cisplatin-resistant cells (Huh7/DDP) were established as previously described (15). Briefly, Huh7 cells were treated with a low concentration (10 ng/ml) of cisplatin (Sigma-Aldrich; Merck KGaA) for 72 h. The medium containing 10 ng/ml of cisplatin was refreshed every 3 days for a total of 5 times. Further resistance was established by gradually raising the concentration of cisplatin in the culture solution until a target resistance of 5 μg/ml cisplatin was acquired.

CircRNA array. A total of 3 HCC tissues and the matched adjacent tissue samples were randomly selected for circRNA microarray. The circRNA microarray data were analyzed using Arraystar Human circRNA Array V2 analysis (Arraystar) by Kangchen BioTech Inc. In brief, total RNAs were digested with RNase R to remove linear RNAs and enrich circular RNAs. The enriched circular RNAs were amplified and transcribed into fluorescent circRNA utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar). The labeled circRNAs were hybridized onto the Arraystar Human circRNA Array V2 (8x15K; Arraystar). Following washing of the slides, the arrays were scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Differentially expressed circRNAs were then identified by analyzing the fold change, as well as the P-value. The threshold for significantly up- and downregulated genes was set as fold change >2.0 and P<0.05.

RT-qPCR analysis. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed to complementary DNA by using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (cat. no. 11766050; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR amplification was then performed with TaqMan Fast Advanced Master Mix (cat. no. 4444558; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. β-actin expression was assessed as an endogenous control. qPCR was performed using the following conditions: 50˚C for 2 min, 95˚C for 2 min and 40 circles of 95.0˚C for 1 sec and 60˚C for 20 sec. The primers used were as follows: circRNA_101237 forward, 5'-TGAGCT TGTGAGTGAGTGGT-3' and reverse, 5'-GCAAGGAGAATG GCGAGATG-3'; β-actin forward, 5'-TTGTTACAGGAAGTC CCTTGCC-3' and reverse, 5'-ATGCTATCACCTCCCCTG TGTG-3'. The $2^{-\Delta\Delta Cq}$ method was used to analyze the qPCR data (16).

Statistical analysis. Experiments were performed as three independent replicates. Values are expressed as the mean ± standard deviation and statistical analysis was performed using SPSS 17.0 statistical software (SPSS, Inc.). Differences among the groups were estimated by Student's t-test or one-way analysis of variance with Tukey's post-hoc test. The cases of HCC were divided into a high circRNA_101237 expression group (expression above the mean value) or otherwise into a low circRNA_100053 expression group. Good prognosis of patients with HCC was defined as a five-year overall survival probability of ≥60%, while a lower probability was defined as poor prognosis. The overall survival rate estimates over time were calculated using the Kaplan-Meier method with log-rank tests. The association

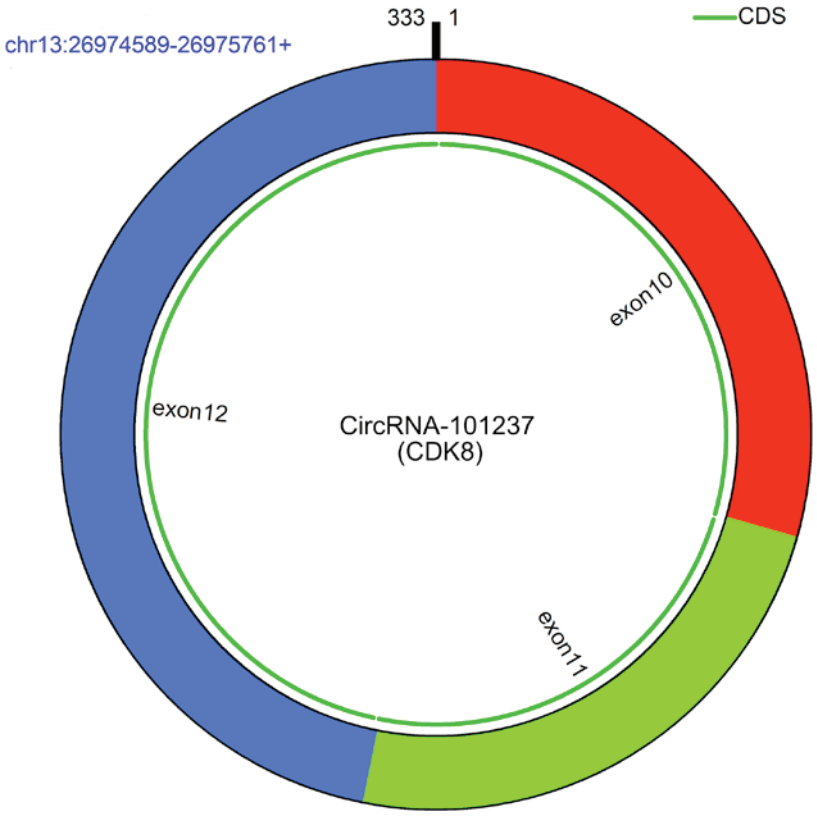

Figure 1. Schematic depicting the composition of circRNA_101237 (encoded by CDK8). circRNA, circular RNA; CDK8, cyclin-dependent kinase 8; chr, chromosome.

between circRNA_101237 expression and clinicopathological variables of HCC patients was evaluated using the Chi-squared test. Univariate and multivariate logistic regression analyses using the Cox proportional hazards model were performed to analyze prognostic factors. P<0.05 was considered to indicate statistical significance.

Results

CircRNA_101237 is upregulated in tumor tissues and peripheral blood serum from patients with HCC. To investigate the role of circRNAs in HCC, circRNA microarray was performed to screen differentially expressed circRNAs. Upregulation was seen in 65 circRNAs and downregulation was seen in 87 circRNAs in HCC tissues vs. adjacent controls. As indicated in the heatmap, circRNA_101237 was significantly upregulated in HCC tissues compared with that in the adjacent controls (Fig. 2A). The expression of circRNA_101237 was further confirmed in 100 HCC tissues and the adjacent tissues. The quantitative results suggested that circRNA_101237 was significantly increased in HCC tissues compared with that in the adjacent tissues (Fig. 2B). To further investigate whether circRNA_101237 in the peripheral blood may serve as a diagnostic biomarker for HCC, circRNA_101237 expression was measured in an independent cohort, including serum samples from 234 patients with HCC and 120 healthy controls. CircRNA_101237 was also significantly upregulated in serum samples from patients with HCC compared with those from healthy controls (Fig. 2C).

High circRNA_101237 expression is associated with poor outcome for patients with HCC. The association between circRNA_101237 expression and clinicopathological features of HCC patients was then analyzed. The HCC patients were stratified into a high circRNA_101237 group and low circRNA_101237 group based on the mean level of circRNA_101237. The results indicated that the expression of circRNA_101237 was associated with tumor size (P<0.001), lymph node metastasis (P=0.006), distant metastasis (P=0.0002), TNM stage (P=0.0002) and Barcelona Clinic Liver Cancer (BCLC) stage (P<0.001; Table I). In addition, univariate analysis suggested that the serum levels of circRNA_101237 (hazard ratio=3.29, P=0.01), as well as the tumor size (hazard ratio=3.24, P=0.01), lymph node metastasis (hazard ratio=2.76, P=0.03), distant metastasis (hazard ratio=5.72, P=0.01), BCLC stage (hazard ratio=2.87, P=0.02) and TNM stage (hazard ratio=4.15, P=0.03) were significantly associated with the prognosis of patients with HCC (Table II). Multivariate analysis revealed that the serum levels of circRNA_101237 (hazard ratio=3.42, P=0.02), tumor size (hazard ratio=3.14, P=0.03), lymph node metastasis (hazard ratio=3.76, P=0.02), distant metastasis (hazard ratio=4.35, P=0.01), BCLC stage (hazard ratio=3.25, P=0.03) and TNM stage (hazard ratio=3.93, P=0.03) were independent prognostic factors for the survival of patients with HCC (Table III). The association between serum levels of circRNA_101237 in HCC patients and overall survival was then further analyzed. The Kaplan-Meier survival curves indicated that the patients with high circRNA_101237 expression had a significantly poorer

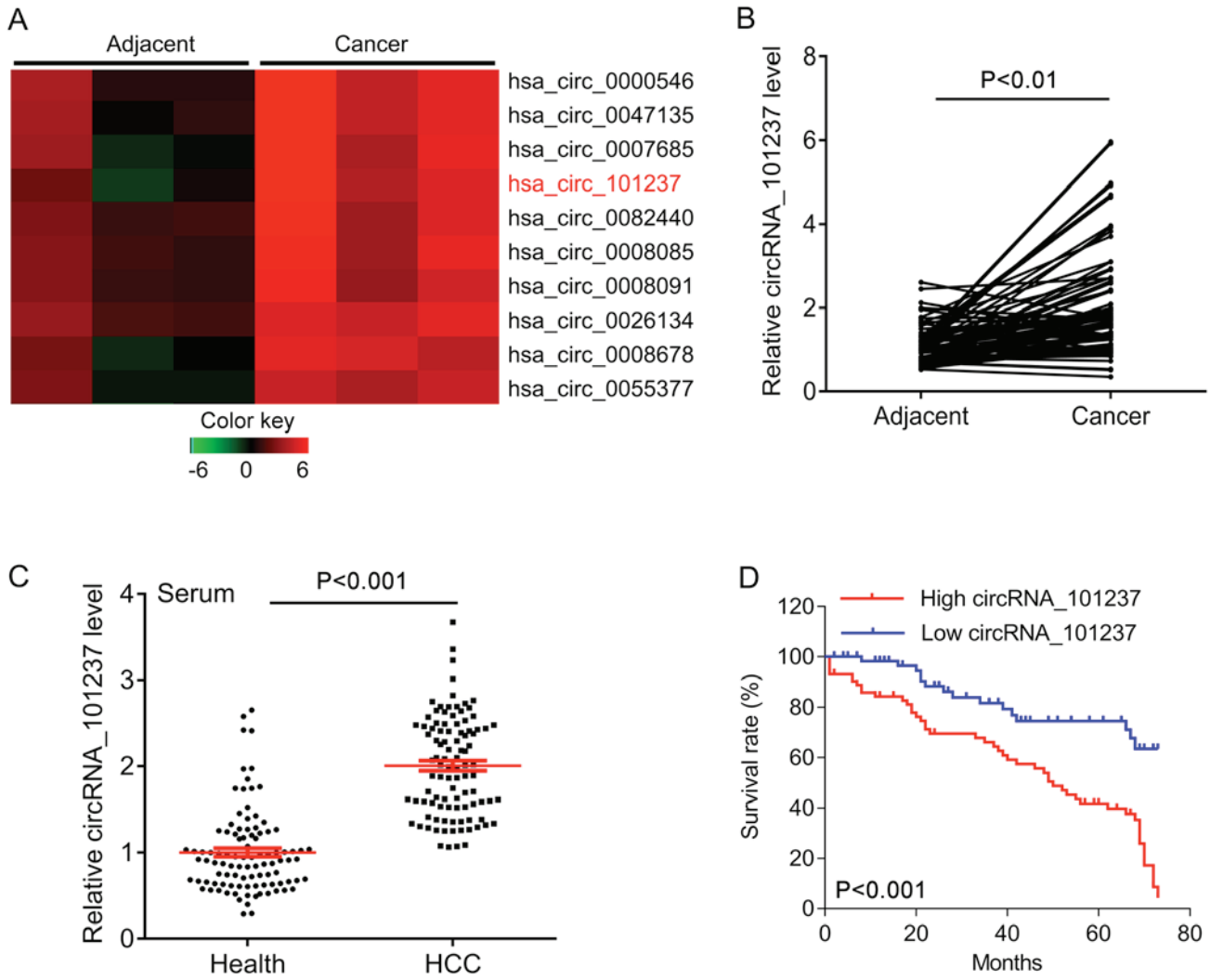

Figure 2. CircRNA_101237 expression in HCC tissues. (A) Heatmap for differentially expressed circRNAs in HCC tumor tissues and the matched adjacent tissues (n=3). (B) RT-qPCR was performed to determine the expression of circRNA_101237 in tumor tissues (n=100) and adjacent tissues (n=100). (C) RT-qPCR was performed to assess the expression of circRNA_101237 in serum samples from patients with HCC (n=234) and healthy controls (n=120). (D) Kaplan-Meier survival curves were drawn to compare the survival of patients with high circRNA_101237 expression (n=148) and those with low circRNA_101237 expression (n=86). RT-qPCR, reverse transcription-quantitative PCR; HCC, hepatocellular carcinoma; circRNA_101237, circular RNA 101237; hsa, *Homo sapiens*.

overall survival than those with low circRNA_101237 expression (P<0.001; Fig. 2D). Overall, the results suggest that circRNA_101237 expression has a negative impact on the prognosis of patients with HCC.

Circ_101237 expression is associated with cisplatin resistance in patients with HCC. Of note, circRNA_101237 was increased by ~3-fold in the serum of cisplatin-resistant HCC patients (n=50) compared with that in cisplatin-sensitive patients (n=62) (Fig. 3A). In addition, the expression of circRNA_101237 in liver cancer cell lines was determined, and the results indicated that circRNA_101237 was significantly upregulated in the MHCC97-H and Huh7 cell lines as compared with that in HCCLM3 cells (Fig. 3B). Of note, circRNA_101237 levels in cisplatin-resistant Huh7/DDP cells was higher than that in the parental cells (Fig. 3B). In addition, HCCLM3, Hep3B, MHCC97-H and Huh7 cells were confirmed to respond to cisplatin by upregulating circRNA_101237 in a time- and cisplatin dose-dependent manner (Fig. 4). These results suggest that circRNA_101237 may be used as a biomarker for HCC diagnosis and cisplatin resistance in patients with HCC.

Discussion

In the present study, a novel circRNA, circRNA_101237, was identified as a diagnostic biomarker for HCC and a potential therapeutic target. CircRNA_101237 was upregulated in HCC tumor tissues compared with that in adjacent tissues. It was further confirmed that circRNA_101237 is upregulated in serum samples from patients with HCC. The upregulation of circRNA_101237 was positively associated with tumor size, lymph node metastasis, distant metastasis and TNM stage of HCC patients. In addition, univariate and multivariate analysis suggested that circRNA_101237 is an independent predictor of prognosis in patients with HCC. These results indicate that circRNA_101237 has a key role in the development of HCC.

If cancer is diagnosed at an early stage, the 5-year survival rate of HCC patients is better (probably >70%) (17).

Table I. Clinical association between serum circRNA_101237 levels and clinicopathological characteristics of patients with hepatocellular carcinoma.

	Serum circRNA_101237		
Variable	Low expression (n=86)	High expression (n=148)	χ^2 test P-value
Age (years)			0.4070
<50	56	88	
≥50	30	60	
Sex			0.1460
Male	64	96	
Female	22	52	
Tumor size (cm)			<0.001
<3	60	48	
≥3	26	100	
Lymph node metastasis			0.0060
N0-1	52	61	
N2-4	34	87	
Distant metastasis			0.0002
No	57	60	
Yes	29	88	
TNM stage			0.0002
I-II	54	55	
III-IV	32	93	
BCLC stage			<0.001
0 or A	32	13	
B	26	34	
C	20	56	
D	8	45	
ALBI grade			0.089
1	34	42	
2	26	65	
3	26	41	
Diabetes mellitus			0.147
No	72	112	
Yes	14	36	
Body mass index (kg/m^2)			0.48
<30	54	86	
≥30	32	62	

BCLC, Barcelona Clinic Liver Cancer; ALBI, albumin-bilirubin; circRNA_101237, circular RNA 101237.

Early diagnosis of HCC is difficult due to inflammation and cirrhosis. Therefore, there is an urgent requirement to develop novel biomarkers for early diagnosis of HCC (11). Recently, serum alpha-fetoprotein, phosphatidylinositol-3, osteopontin, Golgi protein-73 and various miRNAs have been reported as promising early biomarkers of HCC, providing insight into the mechanisms that drive tumorigenesis, which may lead to the development of more effective treatment strategies (18). In the present study, circRNA_101237 was revealed as a novel biomarker for HCC. However, in the present cohort of HCC patients, no detailed information was available regarding HBV, HCV and non-alcoholic fatty liver disease. As the influence of hepatitis/NAFLD was unknown, it was not reasonable to analyze the association between circRNA_101237 and cirrhosis in patients with liver cancer. Furthermore, in the future, analysis of the correlation

Table II. Univariate analysis of prognostic factors for patients with hepatocellular carcinoma.

Variable	Hazard ratio (95% confidence interval)	P-value
Age (≥50/<50 years)	1.06 (0.870-1.142)	0.17
Sex (male/female)	1.02 (0.943-1.165)	0.34
Tumor size (≥3/<3 cm)	3.24 (1.820-7.322)	0.01
Lymph node metastasis (N2-4/N0-1)	2.76 (1.346-5.276)	0.03
Distant metastasis (yes/no)	5.72 (2. 703-9.352)	0.01
TNM stage (III-IV/I-II)	4.15 (1.492-7.626)	0.03
Serum circRNA_101237 levels (high/low)	3.29 (2.632-8.544)	0.01
BCLC stage (C-D/0-B)	2.87 (1.375-6.432)	0.02
ALBI grade (III/I-II)	1.2 (0.978-3.651)	0.06
Diabetes mellitus (yes/no)	1.08 (0.774-1.692)	0.17
Body mass index (≥30/<30 kg/m^2)	1.03 (0.641-1.863)	0.36

BCLC, Barcelona Clinic Liver Cancer; ALBI, albumin-bilirubin; circRNA_101237, circular RNA 101237.

Table III. Multivariate analysis of independent prognostic factors for patients with hepatocellular carcinoma.

Variable	Hazard ratio (95% confidence interval)	P-value
Tumor size (≥3/<3 cm)	3.14 (2.522-5.541)	0.03
Lymph node metastasis (N2-4/N0-1)	3.76 (1.765-8.547)	0.02
Distant metastasis (yes/no)	4.35 (3.431-8.651)	0.01
TNM stage (III-IV/I-II)	3.93 (2.086-6.322)	0.03
BCLC stage (C-D/0-B)	3.25 (2.268-8.634)	0.03
Serum circRNA_101237 levels (high/low)	3.42 (2.215-6.532)	0.02

BCLC, Barcelona Clinic Liver Cancer; ALBI, albumin-bilirubin.

between circRNA_101237 and recently used biomarkers may provide meaningful information for early diagnosis of HCC. These points will be further investigated in future studies by our group.

The present results indicated that circRNA_101237 levels were increased in cisplatin-resistant HCC tumor tissues and cisplatin-resistant Huh7 cells, and cisplatin exposure induced an increase in circRNA_101237 expression, suggesting that circRNA_101237 may be a biomarker of cisplatin resistance in patients with HCC. Cisplatin has been widely used to treat a variety of cancer types, including HCC (19). Patients with HCC initially respond to cisplatin therapy but resistance frequently occurs, which is associated with increased DNA repair, altered cell accumulation and increased drug efflux mediated by multidrug resistance proteins (20). It has been reported that cisplatin resistance in HCC may occur through the loss of Runt-associated transcription factor 3 and upregulation of cyclophilin B (21,22). Recently, certain key biomarkers of cisplatin resistance have revealed novel molecular mechanisms of resistance (23). The role of circRNAs in the development of chemotherapeutic drug resistance has also been highlighted (24-26). For instance, hsa_circ_0004674 is increased in chemoresistant osteosarcoma cells and tissues and is associated with poor prognosis (27). Upregulation of circRNA-MTO1 promotes monastrol-induced cytotoxicity and reverses monastrol resistance by inhibiting Eg5 (28). CircRNA-PVT1 (plasmocytoma variant translocation) is significantly upregulated in osteosarcoma, serum and chemoresistant cell lines, including those with doxorubicin and cisplatin resistance. CircRNA-PVT1 knockdown overcomes the resistance of osteosarcoma cells to doxorubicin and cisplatin (29). The present study indicated that patients with cisplatin-resistant HCC and cisplatin-resistant Huh7/DDP cells had increased levels of circRNA_101237, but it remains elusive whether circRNA_101237 knockdown is able to inhibit HCC cell proliferation and sensitize HCC cells to cisplatin. These biological roles of circRNA_101237 in HCC cells will be demonstrated in the future and the underlying mechanisms will be revealed.

In conclusion, the present study indicated that circRNA_101237 is upregulated in tissues and serum of patients with HCC and may serve as a diagnostic and prognostic biomarker. In addition, the levels of circRNA_101237 were increased in the serum of cisplatin-resistant HCC patients and in cisplatin-resistant Huh7 cells. The present

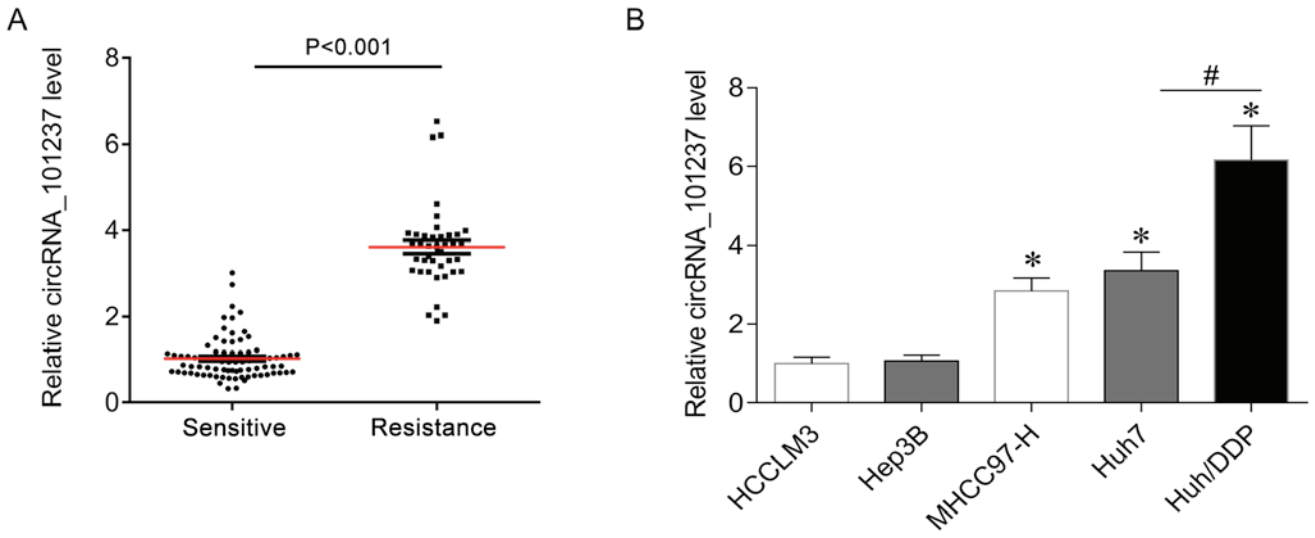

Figure 3. Expression of circRNA_101237 is associated with cisplatin resistance. (A) RT-qPCR was performed to assess the expression of circRNA_101237 in serum samples of patients with cisplatin-sensitive HCC (n=50) and those with cisplatin-resistant HCC (n=35). (B) RT-qPCR was performed to determine the expression of circRNA_101237 in liver cancer cells. *P<0.05 vs. HCCLM3 cells; #P<0.05, Huh7/DDP vs. Huh7. Huh/DDP, cisplatin-resistant Huh7 cells; RT-qPCR, reverse transcription-quantitative PCR; HCC, hepatocellular carcinoma; circRNA_101237, circular RNA 101237.

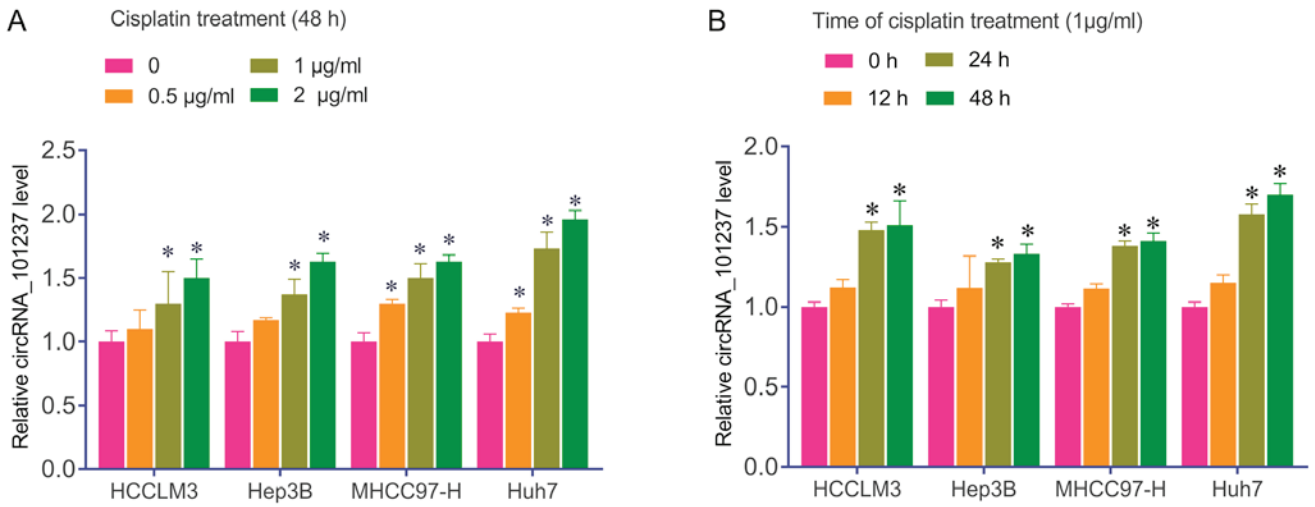

Figure 4. Changes in circRNA_101237 expression in hepatocellular carcinoma cell lines in response to cisplatin treatment. (A) Cells were treated for 48 h with cisplatin at 0, 0.5, 1 and 2 μg/ml. (B) Cells were treated with cisplatin at 1 μg/ml for 0, 12, 24 or 48 h. Reverse transcription-quantitative PCR was performed to assess the expression of circRNA_101237. *P<0.05 vs. controls. circRNA_101237, circular RNA 101237.

results provide evidence that circRNA_101237 may be used as a diagnostic biomarker for HCC and a potential therapeutic target for overcoming cisplatin resistance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL made substantial contributions to the design of the study. SZ, JW and YW analyzed and interpreted the patient data. SZ and JW performed the cell biological experiments. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Huainan First People's Hospital and the First Affiliated Hospital of the Medical College of Anhui University of Science and Technology (Huainan, China). All subjects provided written informed consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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