Role of CircRNAs_100395 in Proliferation and Metastases of Liver Cancer

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Background:
Circular RNAs (circRNAs) are a kind of noncoding RNA with high cancer-specific expression, and great potential in regulating tumorigenesis. Among these, circRNA_100395 (circ_100395) has been reported to be downregulated in lung cancer, and participates in the process of tumor cell proliferation and metastasis. However, its expression and function in liver cancer remain unknown.

Material/Methods:
Quantitative real-time polymerase chain reaction (RT-qPCR) was used to evaluate the expression level of circ_100395 and microRNAs-1228 (miR-1228) in liver cancer samples and the adjacent non-tumor tissues. Cell proliferation, apoptosis, invasion, migration, and epithelial-mesenchymal transition (EMT) pathway of circ_100395 upregulated cells were analyzed using a Cell Counting Kit-8 (CCK-8), flow cytometry, Transwell assay, and Western blot analysis.

Results:
We found that circ_100395 was downregulated in cancerous liver tissues relative to the adjacent normal tissues. The overexpression of circ_100395 was negatively associated with tumor differentiation, microvascular invasion, and portal vein tumor thrombosis. However, patients with higher circ_10039 expression tended to have better postoperative disease-free survival time. Moreover, upregulation of circ_100395 in liver cancer cells inhibited cell proliferation, induced apoptosis, then silenced the EMT pathway and reduced migration and invasion abilities, while this anti-tumor effect was significantly reversed by the downstream target, miR-1228.

Conclusions:
circ_100395 appears to be a promising therapeutic target for liver cancer.

MeSH Keywords:
Carcinoma, Hepatocellular • Cell Proliferation • Epithelial-Mesenchymal Transition • Neoplasm Metastasis

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Background

Liver cancer is one of the most common malignant tumors in the world, with approximately 840,000 new cases annually [1,2]. Patients infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) have heightened susceptibility to liver cancer [3]. Although the biotherapy of liver cancer has made great progress in recent years, including molecular-targeted therapy and immunotherapy, the 5-year survival rate remains relatively poor [4,5]. Therefore, identifying novel biomarkers involved in the development and occurrence of liver cancer would be a promising way to improve the prognosis for patients.

Circular RNAs (circRNAs) are highly expressed in the cytoplasm of eukaryotic cells and have important roles in tumorigenesis and cancer progression [6–8]. circRNAs and the long noncoding RNAs (lncRNAs) are all noncoding RNA molecules, but circRNAs contains a circular structure with covalent bonds, without the 5’ end cap or 3’ end poly(A) tail found in lncRNAs [9]. Owing to this circular structure, circRNAs have greater stability in the cytoplasm, instead of being easily eliminated by RNA exonuclease [10]. In addition, the multifunctionality of circRNAs has been gradually elaborated, including the splicing and transcriptional regulation of specific RNAs and the interaction with RNA-binding proteins [11,12]. Moreover, recent studies revealed that circRNAs can act as miRNA sponges, then interfere with miRNA function and promote tumor development [13–15]. For example, Hongning Cai et al. found that circ-0000263 can downregulate the expression of miR-150-5p, then promotes cervical tumor growth [16]. Consequently, circRNAs could serve as a novel biomarker for liver cancer.

circRNA_100395 is a circRNA located at chr1: 173726114-17374498, and 4 exons in KLHL20 mRNA contribute to form this circRNA. Previous research found that circ_100395 is downregulated in lung cancer [17] and is closely related to positive lymph node metastasis rate and overall survival rate of patients with lung cancer. However, the relationship between circ_100395 and liver cancer is still unclear.

In this study, using publicly available bioinformatic algorithms, we explored the relationship between the circ_100395 and the development of liver cancer, showing that miR-1228 might be a downstream gene of circRNA_100395. Therefore, we assessed the biological function of circ_100395 and miR-1228 in liver cancer and explored the potential molecular mechanism.

Material and Methods

Tissue samples

Tumor tissues and adjacent normal tissues were obtained from 60 patients who underwent liver cancer surgical resection from 2008 to 2013. All of these tissues were confirmed to be hepatocellular carcinoma by 3 experienced pathologists. After excision, the tissues were quickly frozen, then stored at –80°C. This research complied with the Declaration of Helsinki was approved by the Ethics Committee of Southern Medical University. We obtained informed consent signed by the patients or their family members before collecting their tissues samples.

Cell culture

The liver cancer cell lines Huh7, BEL-7402, HepG2, HCCLM3, and MHCC97H, as well as the normal hepatocyte cell line LO2, were obtained from the BeNa Culture Collection (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) was used to incubate the cells. Then, these cells were cultured in a humidified atmosphere with 37°C and 5% CO2.

RNA interference

The circ_100395 cDNA plasmid (pcDNA3.1) was first developed by GenePharma (Suzhou, China), then the lentiviruses expressing circ_100395 and negative control were subsequently constructed by GenePharma (Suzhou, China). The Huh7 and HepG2 cells were collected and transplanted into 6-well plates. The seeded cells were incubated with circ_100395 lentivirus 24 h later. The multiplicity of infection (MOI) is 50 for Huh7 cells and 30 for HepG2 cells. After 48-h transfection, the Huh7 and HepG2 cells were again cultured with free medium containing 4 μg/mL polybrene. After 5 days, the stably transfected cells were collected for further study.

Real-time quantitative polymerase chain reaction (RT-qPCR) assays

Total RNA of frozen tissues and cell lines was extracted with Trizol reagent (TaKaRa), then the PrimeScript RT Master Mix (TaKaRa, Dalian, China) was used to transcribe 500 ng total RNA into cDNA. Subsequently, the SYBR Premix Ex Taq II Kit (TaKaRa) was used to detect the expression of circ_100395 by RT-qPCR assays, normalized to the expression of the endogenous control GAPDH. Fold changes were calculated by the 2^\(-\Delta\Delta Ct\) methods.

Cell proliferation assays

The circ_100395 upregulated stable cells were collected after transfection. Subsequently, the Huh7 and HepG2 liver cancer cells were transplanted into 96-well plates, then cultured in the DMEM medium supplemented with 2% FBS. The OD value of cells was detected at 24, 48, 72, and 96 h using a Cell Counting Kit-8 (CCK-8) kit (Dojido, Tokyo, Japan) following the manufacturer’s instruction. We added 100 μl free medium
containing 10% CCK-8 solution into each well, followed by culturing at a humid atmosphere with 37°C and 5% CO₂ for 1–4 h. Finally, the solution was then measured spectrophotometrically at 450 nm.

Flow cytometry analyses

The Huh7 and HepG2 liver cancer cells were collected after digestion by trypsin enzyme. Subsequently, the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (KeyGen, China) was used to stain the cells. Then, the collected cells were analyzed with a FACS Canto II flow cytometer (BD Biosciences).

Transwell assays

The Transwell chambers (0.8 μm; Corning, NY, USA) with or without Matrigel coating were used to analyze the cell migration or invasion abilities through Transwell assays. Into the lower chambers we added medium containing 20% PBS, while into the upper chambers we added 200 μl serum-free suspension containing 4×10⁴ cells. The bottom side of the chambers was fixed and stained with 0.5% crystal violet 24 h later. Finally, the migrated or invaded cells were analyzed through digital images captured via a microscope.

Western blotting assay

The Huh7 and HepG2 liver cancer cells were harvested and lysed using radioimmunoprecipitation (RIPA; Beyotime, Shanghai, China) buffer containing 1% protease inhibitor phenylmethylsulfonylfluoride (PMSF; Beyotime). Following that, proteins were extracted, then quantified using the bicinchoninic acid (BCA; Beyotime) method. The same amount of protein was separated by 10% sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE; Fdbio Science, Hangzhou, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Then, the membranes were incubated with TBST solution containing 5% FBS for 1 h to block the non-specific antibody, followed by culturing with primary antibodies at 4°C overnight. The primary anti-human antibodies used (all from Abcam; Cambridge, US) were E-cadherin (1:1000), N-cadherin (1:1000), Vimentin (1:1000), Snail (1:100), and GAPDH (1:3000). Subsequently, we co-cultured the membranes with the secondary antibody (1:10 000; Proteintech) for 1 h at room temperature. Finally, enhanced chemiluminescence (ECL; Millipore) was used to visualize the protein bands.

Dual-luciferase reporter assays

The 293T cells were incubated in 24-well plates with 5×10⁴ cells/well. Subsequently, these cells were transfected with pmirGLO-circ_100395-WT or pmirGLO- circ_100395-MUT plasmid, with miR-1228 mimics or miR-1228 NC. 48 hours later, passive lysis buffer (Promega) was applied to lyse cells, and the dual-luciferase reporter assays (Promega, Madison, WI, USA) was applied to calculate the relative luciferase activity through normalizing firefly luminescence to Renilla luminescence.

Statistical analyses

Results are shown as mean ±SD. We used GraphPad Prism 7.0 (GraphPad Software, Inc, CA, USA) or IBM SPSS Statistics 20.0 (IBM, IL, USA) for statistical analyses. Also, the statistical data were evaluated by 2-tailed t test, analysis of variance (ANOVA), or Pearson chi-squared test, whereas the Kaplan-Meier method and log-rank test were used to analyze survival data. Statistical significance was considered when P<0.05.

Results

Clinicopathologic features associated with circ_100395 expression

To explore the potential function of circ_100395 in liver cancer, we first detected circ_100395 expression in 60 paired liver cancer tissues. RT-qPCR assay showed lower circ_100395 expression in tumorous tissues than in adjacent normal tissues (Figure 1A). Liver cancer patients with lower expression of circ_100395 were more likely to have microvascular invasion (Figure 1B), portal vein tumor thrombosis (Figure 1C), and poor differentiation (Figure 1D). To further assess the relationship between the expression of circ_100395 and clinicopathological parameters, we divided the cohort into 2 groups according to median values. Consistent with data provided in Figure 1B-D, chi-square testing (Table 1) showed that circ_100395 expression level was negatively associated with tumor differentiation (P=0.017), microvascular invasion (P=0.020), and portal vein tumor thrombosis (P=0.002), and no significant difference was observed between circ_100395 expression and other parameters (sex, age, tumor size, AFP, capsule, cirrhosis, and TMN stage). Overexpression of circ_100395 in liver cancer patients was associated with longer disease-free survival time (Figure 1E). Our results suggest that circ_100395 suppresses liver cancer.

circ_100395 is highly associated with cell proliferation and apoptosis of liver cancer

To identify the biological role of circ_100395 in liver cancer, we first selected 2 liver cancer cell lines from among 5 liver cancer cell lines (Huh7, BEL-7402, HepG2, HCCLM3, and MHCC97H) based on the expression of circ_100395. RT-qPCR analysis (Figure 2A) showed that circ_100395 was downregulated in
liver cancer lines compared to LO2 cells. Furthermore, Huh7 and HepG2 cell lines showed relatively lower expression of circ_100395 among these 5 cell lines, and thus were chosen for further cellular experiments.

After the selection of cancer cells, we subsequently constructed circ_100395-overexpressed liver cancer cells using lentivirus-based transfection (Figure 2B, 2C). Compared to the non-treated cells (Blank group), no significant change in circ_100395 was observed in negative control cells (Control), while circ_100395 expression was dramatically increased in the overexpressed group (OE-circ_100395), indicating successful construction of circ_100395-overexpressed liver cancer cells. Then, the cell proliferation abilities of liver cancer cells were assessed by CCK-8 assay. The OD value was significantly lower in Huh7 OE-circ_100395 cells at 48, 72, and 96 h relative to the control group (Figure 2D). Consistently, the OD value change showed a similar tendency in HepG2 OE-circ_100395 cells (Figure 2E).

Cell apoptosis rates in control and OE-circ_10039 cells were tested by flow cytometry analysis. The FACS showed that the numbers of apoptotic cells significantly increased after overexpression of circ_10039 in Huh7 and HepG2 cell lines (Figure 2F, 2G). The above experiments indicate that circ_10039 exerts an anti-growth effect in liver cancer cell lines.

circ_100395 is closely associated with migration, invasion, and wound-healing abilities of liver cancer cells

Cellular metastasis ability is a major aspect of cancer tumor evaluation. Consequently, Transwell migration and invasion capabilities assays were carried out. After the upregulation of circ_100395, we observed a significant decrease in the migration and invasion abilities of liver cancer cells (Figure 3A, 3B). These findings suggest that circ_100395 plays a crucial role in the progression of liver cancer.
of circ_100395 in liver cancer cells, the numbers of migrated and invaded cells were significantly reduced in Huh7 (Figure 3A, 3C) and HepG2 (Figure 3B, 3D) cells. Furthermore, the wound scratch assays showed that the healing abilities were also significantly decreased in OE-circ_100395 liver cancer cell lines (Huh7 in Supplementary Figure 1, and HepG2 cells in Supplementary Figure 2). The above experiments indicated that circ_100395 exerts an anti-metastasis effect in liver cancer cell lines.

**circ_100395 is highly associated with the epithelial-mesenchymal transition (EMT) in liver cancer cells**

To study the possible mechanism of circ_100395 in anti-growth and metastasis abilities, we first detected the expression level of EMT-related markers through Western blot analysis. The epithelial marker (E-cadherin protein) was upregulated, while the mesenchymal markers (including N-cadherin, Vimentin, and snail proteins) were downregulated after overexpression of circ_100395 in both cell lines (Figure 3E, 3F).

**miR-1228 was negatively related to anti-tumor effect and EMT pathway induced by circ_100395 in liver cancer cells**

We used the Starbase bioinformatic tool to explore genes downstream of circ_100395 during the development of liver cancer; miR-1228 was found to be a potential downstream gene of circ_100395. Firstly, we assessed the expression of miR-1228 in OE-circ_100395 Huh7 and HepG2 cells by RT-qPCR. Compared to the control group, OE-circ_100395 Huh7 showed 70% decreased expression, while OE-circ_100395 HepG2 cells showed 55% decreased expression (Figure 4A). We predicted

### Table 1. Correlation analyses between relative Circ_100395 expression and clinicopathological parameters of patients with hepatocellular carcinoma.

| Clinicopathological parameters | Circ_100395 | χ² | P |
|-------------------------------|------------|----|---|
|                               | Low        |     |    |
| Gender                        |            |    |    |
| Male                          | 26 (52.0%) | 0.480 | 0.488 |
| Female                        | 4 (40.0%)  |    |    |
| Age (years)                   |            |    |    |
| <60                           | 26 (51.0%) | 0.131 | 0.718 |
| ≥60                           | 4 (44.4%)  |    |    |
| Tumor size (cm)               |            |    |    |
| <5                            | 16 (53.3%) | 0.267 | 0.606 |
| ≥5                            | 14 (46.7%) |    |    |
| Differentiation               |            |    |    |
| Well                          | 3 (21.4%)  | 8.127 | 0.017 |
| Moderate                      | 14 (50.0%) |    |    |
| Poor                          | 13 (72.2%) |    |    |
| AFP (μg/L)                    |            |    |    |
| <400                          | 16 (42.1%) | 2.584 | 0.108 |
| ≥400                          | 14 (63.6%) |    |    |
| Capsule                       |            |    |    |
| Positive                      | 25 (54.3%) | 1.491 | 0.222 |
| Negative                      | 5 (35.7%)  |    |    |
| Cirrhosis                     |            |    |    |
| Positive                      | 22 (52.4%) | 0.317 | 0.573 |
| Negative                      | 8 (44.4%)  |    |    |
| Microvascular invasion        |            |    |    |
| Positive                      | 19 (65.5%) | 5.406 | 0.020 |
| Negative                      | 11 (35.5%) |    |    |
| Portal vein tumor thrombosis  |            |    |    |
| Positive                      | 12 (85.7%) | 9.317 | 0.002 |
| Negative                      | 2 (14.3%)  |    |    |
| TNM                           |            |    |    |
| I+II                          | 20 (46.5%) | 0.739 | 0.390 |
| III+IV                        | 10 (58.8%) |    |    |

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Figure 2. Overexpression of circ_100395 inhibits proliferation and promotes apoptosis of liver cancer cells. (A) Expression of circ_100395 in 5 liver cancer cell lines (Huh7, BEL-7402, HepG2, HCCLM3, and MHCC97H) and the immortalized epithelial cell line (LO2), as detected by RT-qPCR assay. (B, C) Successful construction of circ_100395-overexpression liver cancer cell lines (OE-Circ_100395 Huh7 and HepG2 cells). (D, E) CCK8 assays of Huh7 and HepG2 cells after upregulation of circ_100395. (F, G) Flow cytometry images (left) and the static cell apoptosis rates (right) of Huh7 and HepG2 cells after upregulation of circ_100395. * P<0.05, ** P<0.01, *** P<0.001.
the potential binding site between circ_100395 and miR-1228 (Figure 4B). To further explore the relationship between miR-1228 and circ_100395, we used luciferase reporter assay, which identified that the luciferase activity of the wild-type cells with miR-1228 mimics was significantly decreased (Figure 4C), showing that circ_100395 can directly bind to miR-1228 and might act by inhibiting the expression of miR-1228.

To further explore their relationship, we assessed the expression of miR-1228 in OE-circ_100395 Huh7 cells (Figure 4D), showing that the expression of miR-1228 was lower than in OE-circ_100395 HepG2 cells. After transfection of miR-1228 mimics, the cell growth ability was increased (Figure 4E) and cell apoptosis rates decreased (Figure 4F). In addition, the Transwell assay results showed that cell migration and invasion abilities were inhibited by the upregulation of miR-1228 (Figure 4G). Moreover, Western blot results showed that the epithelial
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Emerging evidence has shown that circRNA is closely associated with the occurrence of malignant tumors, and its dysfunction can inhibit tumor growth and metastasis. Also, the down-regulation of the circRNA_100395 had been confirmed in lung cancer [17]. Consistently, our results revealed that the expression of circ_100395 in liver cancer was downregulated relative to the adjacent normal tissues, which also is closely associated with microvascular invasion, portal vein tumor thrombosis, and poor differentiation. Moreover, patients with higher expression of tend to have a longer disease-free survival. Therefore, circ_100395 may function as a novel therapeutic biomarker in liver cancer.

**Discussion**

Liver cancer is the fourth leading cause of cancer deaths in the world [1]. Due to the lack of typical symptoms and effective biomarkers in the early stage, many patients are diagnosed with advanced liver cancer and lose the opportunity for surgical intervention [5]. Therefore, early diagnosis would be an important way to prolong the survival of patients with liver cancer, and many studies have sought to and identify new molecular biological markers and therapeutic methods for liver cancer [18–20]. In the present study, we mainly explored the tumor suppressor role of circ_100395 in liver cancer and clarified its underlying mechanism.

Emerging evidence has shown that circRNA is closely associated with the occurrence of malignant tumors, and its dysfunction can inhibit tumor growth and metastasis. Also, the down-regulation of the circRNA_100395 had been confirmed in lung cancer [17]. Consistently, our results revealed that the expression of circ_100395 in liver cancer was downregulated relative to the adjacent normal tissues, which also is closely associated with microvascular invasion, portal vein tumor thrombosis, and poor differentiation. Moreover, patients with higher expression of tend to have a longer disease-free survival. Therefore, circ_100395 may function as a novel therapeutic biomarker in liver cancer.

The proliferation, migration, and invasion abilities are the major factors for the evaluation of malignant tumor progression, and many studies found that circRNAs participate in these pathologic processes [21,22]. Mengli Yang et al. revealed that circ_0034642 promotes glioma growth via the miR-1205/BATF3 axis [13]. In our study, the results of CCK-8 assay and flow cytometric analyses consistently revealed that the over-regulation of circ_100395 inhibits proliferation and induces more cell apoptosis in liver cancer. Moreover, we discovered that, compared with the negative control of liver cancer cells, the OE-circ_100395 cells inhibited metastasis abilities. These cellular findings, coupled with the clinical significance of circ_100395, indicate that upregulating circRNA_100395 expression might inhibit liver cancer growth and distant metastasis, thus improving outcomes. However, the potential mechanism of circ_100395 in anti-growth and metastasis effects remains unclear.

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelioid cells lose cell-to-cell adhesion and cell polarity and then transform into mesenchymal cells with increased migratory and invasive abilities [23,24]. A growing number of studies indicate that circRNA inhibits cell metastasis via suppression of the EMT pathway [25,26]. For example, circPRMT5 had been confirmed to regulate miR-30c expression, then trigger the EMT pathway and promote the metastasis of urothelial carcinoma of the bladder [27]. Similarly, Western blot analysis revealed that OE-circ_100395 can lead to upregulation of the epithelial marker (E-cadherin) and the downregulation of the epithelial marker and the upregulation of the mesenchymal markers N-cadherin, Vimentin, and snail proteins (N-ca) proteins in Huh7 and HepG2 cells after upregulation of circ_100395. (E, F) Expression of the epithelial marker, E-cadherin (E-ca), and the mesenchymal markers N-cadherin, Vimentin, and snail proteins (N-ca) proteins in Huh7 and HepG2 cells after upregulation of circ_100395. *** P<0.001.

**Figure 3.** Overexpression of circ_100395 suppresses migration, invasion, and epithelial-mesenchymal transition in liver cancer cells. (A, B) Transwell migration images (left) and number of migrated cells (right) of Huh7 and HepG2 cells after upregulation of circ_100395. (C, D) Transwell invasion images (left) and number of invaded cells (right) of Huh7 and HepG2 cells after upregulation of circ_100395. (E, F) Expression of the epithelial marker, E-cadherin (E-ca), and the mesenchymal markers N-cadherin, Vimentin, and snail proteins (N-ca) proteins in Huh7 and HepG2 cells after upregulation of circ_100395. *** P<0.001.

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of mesenchymal markers (N-cadherin, Vimentin, and snail) in liver cancer cell lines. Our results suggest that EMT pathway dysfunction is the mechanism underlying the anti-metastasis effect in OE-circ_100395 cell lines.

In addition, accumulating evidence indicates that circ RNAs interfere with the expression of microRNAs, then regulate cell proliferation through the cavernous mechanism [28,29]. Juan Zhang et al. proposed that circ_0008450 promotes hepatocellular carcinoma development through sponging miR-548p [30]. Using the bioinformatics software, we predicted that miR-1228 is the downstream gene of circ_100395. Interestingly, the dual-luciferase reporter assays in our research confirmed the direct binding between circ_100395 and miR-1228, while miR-1228 was found to be downregulated in OE-circ_100395 cells. We found that the addition of miR-1228 reversed the anti-growth effect induce by circ_100395 in liver cancer cells by promote cell proliferation and inhibiting cell apoptosis. Furthermore, miR-1228 also promotes cell migration and invasion and reverses the EMT pathway in OE-circ_100395 liver cancer cells. Taken together, these data consistently show that circ_100395 plays an important role in control of liver cancer cell proliferation, EMT, and metastasis through regulation of miR-1228.

Conclusions

In summary, overexpression of circ_100395 silences miR-1228 expression, then inhibits EMT pathway activity and inhibits liver cancer cells proliferation and metastasis and induces cell apoptosis. Therefore, circ_100395 appears to be a promising prognostic and therapeutic biomarker in liver cancer. However, the present study has some limitations: the mechanism by which miR-1228 regulates cell proliferation and metastasis remains unclear, and further research is needed to identify more potential targets for circ_100395 in regulating cell proliferation and metastasis.

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Conflict of interests

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Supplementary Figures

Supplementary Figure 1. Overexpression of circ_100395 suppresses wound-healing ability of Huh7 cells.

Supplementary Figure 2. Overexpression of circ_100395 suppresses wound-healing ability of HepG2 cells.

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