CircABC10 activates the miR-670-3p/HMG20A pathway to promote hepatocellular carcinoma progression

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Abstract

Background/aims: The dysregulation of circABC10 may play an early role in tumor progression. However, its function in liver cancer (HCC) is still unclear. Therefore, this experimental design is based on circABC10 to explore the pathogenesis of HCC.

Methods: The expression of circABC10 and miR-670-3p in HCC tissues was detected by RT-qPCR. CCK-8, Brdu incorporation, colony formation and transwell assays were used to determine the effect of circABC10 on HCC cell proliferation and migration. Target gene prediction and screening, luciferase reporter assays were used to validate downstream target genes of circABC10 and miR-670-3p. HMG20A expression was detected by RT-qPCR and Western blotting. The tumor changes in mice were detected by in nude mice.

Results: CircABC10 was significantly increased in HCC tissues and cell lines, and high CircABC10 expression was directly associated with low survival in HCC patients. Silencing of circABC10 inhibited proliferation and invasion of hepatocellular carcinoma. In addition, circABC10 acted as a sponge of miR-670-3p to upregulate HMG20A expression. In addition, overexpression of miR-670-3p or knockdown of HMG20A reversed the carcinogenic effects of circABC10 in HCC. There was a negative correlation between the expression of circABC10 and miR-670-3p, and a positive correlation between the expression of circABC10 and HMG20A in HCC tissues.

Conclusion: circABC10 promotes HCC progression by modulating the miR-670-3p/HMG20A axis, and circABC10 may be a potential therapeutic target for HCC.

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Keywords: circABC10, miR-670-3p, HMG20A, Liver cancer, Proliferation

Background

Primary liver cancer is the third leading cause of cancer-related death [1, 2]. The most common type of primary liver cancer is hepatocellular carcinoma (HCC) [3]. Currently, the first-line root therapy includes surgical resection and liver transplantation [4, 5]. However, due to the aggressive biological characteristics of liver cancer, the current first-line and second-line treatments are relatively ineffective, and the number of deaths is basically the same every year [6]. In addition, because liver cancer is characterized by rapid growth of tumor cells, liver metastasis can occur early, tumor malignancy rate is high and many are multidrug resistant, and its 5-year survival rate is generally within 5% [7]. How to more effectively intervene in the occurrence of liver cancer and treat patients with liver cancer has become a major and urgent problem. Therefore, it is extremely important to further explore the pathogenesis of liver cancer and find effective diagnosis and treatment methods.
As part of the non-coding RNA family, circular RNA (circ RNA) is a closed circular molecule. Circ RNA has been reported to be involved in multiple processes of tumor progression, which provides a new direction for cancer therapy [8, 9]. There is increasing evidence that circ RNA is involved in autophagy-related, apoptosis, cell cycle and proliferation, regulating tumor cell growth, apoptosis and cell cycle progression, suggesting that circ RNA may be a novel therapeutic target in cancer [10, 11]. Many studies had indicated that circ RNA is associated with HCC [12]. Circ ABCB10 is highly expressed in embryonic tissues and is not expressed in most adult tissues. It is highly expressed in tumor tissues such as breast cancer [13]. However, there is a lack of literature on the regulation of liver cancer by circABC10.

In the regulatory network of non-coding RNAs, the binding and competition between circ RNA and mi RNA provides new insights into our understanding of cancer development and progression [14]. Cell proliferation, apoptosis, differentiation, metabolism, and ontogenesis are all regulated by mi RNA. A large number of evidences indicate that the abnormal expression of mi RNA is closely related to the development of various human cancers [15]. Multiple studies of mi RNA expression profiles in HCC revealed multiple aberrantly expressed mi RNAs [16]. The expression levels of mi R-21, mi R-221, mi R-222 were significantly increased, and the expression levels of mi R-122a, mi R-125a, mi R-139 were significantly decreased [17, 18]. Several studies have shown that mi R-670 has elevated levels of expression in HCC. Up-regulation of mi R-670 expression promotes proliferation of liver cancer cells [19]. At present, the biological functions of circ RNA regulating mi RNA in HCC is not clear. It is necessary to carry out in-depth systematic research. Based on the above studies, it was hypothesized that circ ABCB10 may regulate the progression of HCC through mi R-670 expression. The main purpose of this study was to explore the mechanisms of action of circ ABCB10 in the regulation of HCC, and provide new ideas for the diagnosis and targeted therapy of liver cancer.

Materials and method

Patients and tissue specimens

40 HCC samples and paired paracancerous tissue specimens were collected from the clinical sample bank of the First Hospital of Jilin University. The study was approved by the the First Hospital of Jilin University Research Ethics Committee and all patients’ samples were signed with written consent. None of the patients received radiotherapy or chemotherapy.

Cell culture and transfection

Human HCC cell lines HepG2, PLC, SK-Hep1, HCCLM3, Huh7 and Hep3B) and human LO2 normal liver cell lines were purchased from the Cell Center of Shanghai Institute of Biological Sciences. The human HCC cell line HepG2 was cultured in DMEM medium, and the remaining cells were cultured in RPMI 1640 medium.

si-circABC10, si-HMG20A, mir-670-3p, miR-670-3p agomi (GenePharma, Shanghai, China) was transfected into cells using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). The sequence of si-circABC10 was as follow: 5'-TGGTGAAATAAAATATGCAGGA-3'. The human cDNA sequence of cirABC10 was cloned into the pcD-ciR vector to construct a circABC10 overexpression plasmid and transfected with Lipofectamine 2000 (Invitrogen).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). qRT-PCR was performed using a ViiATM 7 real-time PCR system (Life Technologies, Grand Island, NY). GAPDH and U6 were used as internal references. qRT-PCR specific experimental procedures were performed with reference to the literature [20]. The primer sequences were as follows: CircABC10 (divergent primer): Forward: 5’-CTTATCCACTTGGCCGGG-3’; CircABC10 (divergent primer) Reverse 5’-CGCGTAGATCTCAGGGG-3’. ABCB10 (converging primer): Forward: 5’-TCAATGCGTGTCTGGTTT-3’ ABCB10 (converging primer) Reverse: 5’-GGAGGAGACGATGTACCTACCA-3’. miR-670-3p: Forward: 5’-CTGATCGTGGAGAGAGTGTT-3’; miR-670-3p: Reverse: 5’-GGTCTCTGAGCATCGGGGCGG-3’. HMG20A: Forward: 5’-TTCGATGCAAGAAGGACGCGC-3’. HMG20A: Reverse: 5’-AGGCCGAGATTCCTGGTCC-3’. GAPDH: Forward: 5’-CGAGAAGATCCCGGGACGAC-3’. GAPDH: Reverse: 5’-TTGTGCAATACAGCGTGACGAC-3’. U6: Forward: 5’-GACAGATTCGGTCTGGGCGAC-3’. U6: Reverse: 5’-GATTACCGTCCGAGATCTGC-3’.

Animal research

Eighteen 6-week-old female BALB/c nude mice were randomly divided into two groups. Each mouse was injected subcutaneously with 10⁶ HepG2 cells to construct a mouse xenograft model. On the 9th day, mice were...
intratumorally injected with cholesterol-conjugated si-circABCB10, miR-670-3p agomir and a negative control (GenePharma). Tumor size was measured twice a week. All nude mice were sacrificed after 2 weeks and tumors were dissected for qPCR or western blot analysis experiments. The study was conducted in strict accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Cell proliferation assay
The transfected cells were seeded into 96-well plates and cell growth was measured every 24 h. The absorbance values were finally determined at 450 nm using a microplate reader (ELx 808; Bio-Tek Instruments Inc., Winoooski, VT, USA).

BrdU incorporation assay
The transfected cells were seeded at a density of 2000 cells per well in a 96-well plate format. 48 h after transfection, cell proliferation was analyzed using the BrdU Cell Proliferation Assay Kit (#5213S, Cell Signaling).

Cell clone formation assay
The transfected cells were seeded into a 6-well plate at a density of 4000 cells per well. After 2 weeks of culture, the cells were fixed with methanol, stained with 0.1% crystal violet, and the colonies were imaged and counted.

Transwell migration and invasion assay
The upper basement membrane of the Transwell chamber was pre-coated with 20 μg Matrigel and cultured overnight in a 24-well plate. The cell suspension was added to the upper chamber, the medium was added to the lower chamber. After 12 h of culture, it was washed with PBS for 3 times, fixed with 90% of the formaldehyde and then stained with the crystal violet solution for 15 min. The photograph was taken under an inverted microscope. Transwell migration assay was free of matrigel coating and the rest of the procedure was consistent with the invasion assay.

Dual luciferase reporter gene assay
The wild type or mutant sequence of the circABCB10 and HMG20A 3′-untranslated region (3′-UTR) was cloned into the pmirGLO vector. Cells were co-transfected with these reporter plasmids and miR-670-3p inhibitors or mimetics, respectively. Cells were co-transfected with these reporter plasmids and miR-584-5p inhibitors or mimics. Luciferase activity was measured using a dual luciferase assay system (Promega).

Western blot
The transfected cells were collected, total protein was extracted, and the protein concentration was quantified using the BCA Protein As-say Kit. The anti-HMG20A antibody (1:1000, Proteintech, Chicago, IL, USA) and anti-GAPDH antibody (1:1000, Abcam, Cambridge, UK) were added, and it was incubated overnight at 4°C. After that, it was incubated with 1:5 (v/v) labeled anti-rabbit secondary antibody for 1 h. Western blot analysis were performed with reference to the literature [21].

Computational analyses and bioinformatics
The circABCB10 and miRNAs expression of HCC specimens of TCGA data was downloaded from UALCAN (http://ualcan.path.uab.edu). TCGA data from OncoLnc (http://www.oncolnc.org) was used to analyse the correlation between circABCB10 expression and prognosis of HCC patients. Potential target miRNAs of circABCB10 were predicted by starBase v3.0 (http://starbase.sysu.edu.cn/). Potential targets of miRNA were predicted by TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/).

Statistical method
The monitoring data were analyzed by SPSS19.0 statistical software. The results of data analysis were showed as mean ± standard deviation (mean ± SD). Student’s t test (2 groups) and ANOVA (multiple groups) were conducted to analyze the difference. The gene expression levels in tumors of our cohort were compared with normal adjacent tissues by Wilcoxon test. Spearman’s correlation analysis were employed to explore the association between two variables. Overall survival curves were plotted using the Kaplan–Meier method and estimated by the log-rank test. P < 0.05 indicated the difference was significant.

Results
CircABCB10 was significantly overexpressed and predicted poor prognosis in HCC
As shown in Fig. 1a, the expression level of CircABC10 in HCC tissues was significantly increased compared with that in the paired adjacent normal tissues (n = 40) (Fig. 1a). Similarly, we determined the expression difference of circABCB10 between HCC and normal liver tissues in TCGA database. Statistical analysis of TCGA data from UALCAN indicated that the expression of circABCB10 was obviously higher than that in normal liver tissues (P < 0.001, Fig. 1b). The expression level of circABCB10 was significantly increased in the HCC cell lines (HepG2, PLC, SK-Hep1, HCCLM3, Huh7 and Hep3B) compared with that in the L02 normal liver cell line (Fig. 1c). The patients with HCC were divided into
low expression group (less than the median value) and high expression group (more than the median value). As shown in Fig. 1d, the overall survival of the circABCB10 high expression group (n = 20) was significantly lower than that of the circABCB10 low expression group (n = 20) (P < 0.01). Notably, survival analysis of TCGA data from OncoLnc (http://www.oncolnc.org/) consistently revealed that high circABCB10 level in HCC tissues predicted an obvious poor clinical outcomes of patients (P = 0.007, Fig. 1e). These data indicated that circABCB10 had a potential carcinogenic effect in HCC.

**Silencing of circABCB10 inhibits HCC growth in vitro**

As shown in Fig. 2a, siRNA effectively reduced circABCB10 expression, and had no significant effect on its linear isoform ABCB10 mRNA. As shown in Fig. 2b–d, compared with the control group, in Huh7 and HepG2 cells, the cell proliferation rate of the circABCB10-suppressed group was significantly decreased, and the BrdU incorporation and the number of cell colonies were significantly decreased (P < 0.01). As shown by Fig. 2e, the number of cell migration and invasion was significantly reduced in the circABCB10-suppressed group compared with that in the control group (P < 0.01). The above results indicated that circABCB10 exerted a carcinogenic effect and promoted the growth and metastasis of HCC cells.

**CircABCB10 served as a sponge of miR-670-3p**

Furthermore, it was predicted by bioinformatics and miR-670-3p was identified as a potential target for CircABCB10 (Fig. 3a). Luciferase reporter assays was performed using the WT-CircABCB10 or mutant (Mut)-CircABCB10 luciferase reporter plasmid to validate the predicted results. As shown in Fig. 3b, ectopic expression of miR-670-3p significantly inhibited the luciferase activity of WT-CircABCB10, but had no significant effect on the luciferase activity of Mut-CircABCB10. Furthermore, miR-670-3p expression was significantly decreased in the circABCB10 overexpression group compared with that in the normal group, while miR-670-3p expression was significantly increased in the circABCB10 silenced group (Fig. 3c). As shown in Fig. 3d, e, miR-670-3p was significantly down-regulated in HCC tissues compared with that in normal tissues in both our cohort of 40 HCCs and TCGA database (P < 0.01). In addition, a direct negative correlation between CircABCB10 expression and
Fig. 2. CircABCB10 promoted the malignant behavior of HCC cells. a CircABCB10 expression levels in Huh7 and HepG2 cells. b The effect of circABCB10 on cell viability was determined by CCK-8 method. c BrdU incorporation assay for the effect of circABCB10 on cell proliferation. d Colony formation assay for the effect of circABCB10 on cell proliferation. e Effect of the transwell assay circABCB10 on cell migration and invasion.*P < 0.05, **P < 0.01, ***P < 0.001 by t-test.
miR-670-3p levels in HCC tissues was found (Spearman \( r = -0.4723; \) Fig. 3f). Taken together, these results indicated that CircABCB10 may exert its biological function through miR-670-3p.

**CircABCB10 sponged and sequestered miR-670-3p to upregulate HMG20A expression**

Furthermore, it was predicted by bioinformatics and HMG20A was identified as a potential target for miR-670-3p (Fig. 4a). In order to validate the predicted results, luciferase reporter assays was performed using the WT-miR-670-3p or mutant (Mut)-miR-670-3p luciferase reporter plasmid. As shown in Fig. 4b, ectopic expression of HMG20A significantly inhibited the luciferase activity of WT-miR-670-3p, but had no significant effect on the luciferase activity of Mut-miR-670-3p. Compared with the control group, overexpression of miR-584-5p in Huh7 and HepG2 cells reduced the mRNA level of HMG20A, while overexpression of CircABCB10 increased the mRNA expression level of HMG20A. CircABCB10 and miR-670-3p co-transfection was able to partially abolish the effect of CircABCB10 and miR-670-3p on the mRNA expression level of HMG20A (Fig. 4c). The results were further confirmed by Western blot (Fig. 4d). Furthermore, it was found that circABCB10 expression was positively correlated with HMG20A expression in HCC tissues.
tissues (Fig. 4e). The results indicated that circABCB10 enhanced the expression level of HMG20A by targeting miR-584-5p.

miR-670-3p overexpression or HMG20A silencing effectively reversed circABCB10-induced promotion of HCC progression

As shown in Fig. 5a–c, overexpression of circABCB10 increased cell viability, BrdU-incorporated cell number and cell colony number. Co-transfection of circABCB10 with miR-670-3p and co-transfection of circABCB10 with si-HMG20A decreased the cell viability, number of BrdU-incorporated cells and number of cell colonies. Moreover, overexpression of circABCB10 increased cell invasion and migration, while co-transfection of circABCB10 with miR-670-3p and co-transfection of circABCB10 with si-HMG20A reduced cell invasion and migration (Fig. 5d, e). The data indicated that miR-670-3p overexpression or HMG20A silencing partially abolished the progression of circABCB10 induced HCC cells.

circABCB10 promoted HCC progression in vivo by regulating the miR-670-3p/HMG20A axis

Next, the effect of circABCB10 on HCC progression in vivo was analyzed. The results showed that circABCB10 knockdown significantly inhibited tumor weight and tumor volume in mice compared with that in the control group. CircABCB10 knockdown and miR-584-5p overexpression were able to further inhibit tumor weight and tumor volume in mice (Fig. 6a–c). Furthermore, as shown in Fig. 6d, compared with the control group, circABCB10 knockdown inhibited the expression of HMG20A, while circABCB10 knockdown and miR-584-5p overexpression further inhibited the expression level of HMG20A. These data indicated that circABCB10 can promote HCC progression by modulating the miR-670-3p/HMG20A axis.

Discussion

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world, and is the third in the world [22, 23]. The main cause of high mortality of liver cancer is liver cancer metastasis [24]. Although clinical interventions such as embolization chemotherapy (TACE) or preoperative percutaneous transhepatic artery occlusion (TAE), postoperative TACE and biotherapy are available, the overall effect of these measures is still very limited [25, 26]. The molecular mechanism of human liver cancer metastasis is not completely clear, and little is known about its molecular regulatory network.
Fig. 5 miR-670-3p overexpression or HMG20A silencing reversed circABCB10 induced HCC progression. **P < 0.01 vs control vehicle group, #P < 0.05 vs circABCB10

(a) CCK-8 for cell viability.
(b) BrdU incorporation assay for cell proliferation.
(c) Colony formation assay for colony proliferation.
(d) Transwell for cell migration and invasion.
Therefore, it is necessary to find and study molecules closely related to liver cancer metastasis, understand the mechanism of liver cancer metastasis, and prevent the occurrence of liver cancer metastasis. It is of great significance to diagnose the metastasis of liver cancer at the molecular level and to select appropriate targets for intervention and treatment.

It is worth noting that the study of previous tumor metastasis in the world mainly focused on the study of proteins and their coding genes, while ignoring the role of non-coding genes (Non-coding RNA, nc RNA) in tumor metastasis [27]. It was closely related to the occurrence and development of various diseases [28]. As part of the non-coding RNA family, circular RNA (circ RNA) is a closed circular molecule. Circ RNA is usually specific for tissue and developmental stages expression in a sexual manner, and high expression abundance [29]. Many evidence have indicated that circ RNA is associated with HCC [30]. Circ MTO1 is significantly down-regulated in HCC tissues and inhibits HCC progression by expanding mi R-9 [12]. In addition, the expression of circ MTO1 is decreased, and the survival rate of patients with HCC is decreased. Studies have found that the expression of CIRs-7 in HCC tissues is significantly higher than that in adjacent tissues [31]. circABCB10 is another up-and-coming circular RNA that has received increasing attention [32]. The results of this study showed that the expression of circABCB10 was significantly increased in cancer tissues and cells, and overexpression of circABCB10 reduced the survival rate of patients. Silencing of circABCB10 reduced cell viability and inhibited cell migration and invasion. In vivo experiments showed that circABCB10 silencing inhibited tumor growth. CircABCB10 can be used as an oncogene to control the development of liver cancer by inhibiting its expression.

As the target of circ RNA, mi RNA is the most widely studied non-coding RNA, which can regulate cell proliferation, differentiation and participate in the process of individual development [33]. Recent studies have shown that mi RNA has a wide regulatory effect on liver function, and has a certain relationship with liver diseases such as hepatitis, liver fibrosis and liver cancer [2]. For example, mi R-122 is significantly down-regulated in liver cancer, especially in liver metastasis. The overexpression of mi R-122 can not only significantly reduce the migration and invasion ability of liver cancer cells, but also inhibited liver cancer metastasis through the inhibition of angiogenesis [34]. It was screened miR-670-3p as a target gene for circABCB10 by database. CircABCB10 regulated its expression by targeting the 3′UTR of the miR-670-3p gene. And circABCB10 knockdown significantly increased the expression level of miR-670-3p. miR-670-3p was down-regulated in HCC tissues, and a negative correlation between circABCB10 expression and miR-670-3p levels was observed in HCC tissues. It was suggested that circABCB10 may play an oncogene role in HCC through sponge miR-670-3p.
The transcription factor HMG20A regulates cell cycle progression, DNA synthesis, and cell proliferation and apoptosis [35]. The latest research shows that HMG20A can regulate malignant tumors [36, 37]. This study found that HMG20A was a potential target for miR-670-3p, and that miR-670-3p regulated its expression by targeting the 3′UTR of the HMG20A gene. In addition, overexpression of miR-670-3p can down-regulate the expression of HMG20A, while CircABCB10 and miR-670-3p co-transfection partially abolished the effect of CircABCB10 and miR-670-3p on HMG20A expression. There was a positive correlation between circABCB10 and HMG20A expression. Moreover, overexpression of circABCB10 significantly increased the proliferation rate of HCC cells and increased the invasion and migration ability of cells. MiR-670-3p overexpression and si-HMG20A partially abolished the proliferation of circABCB10 and inhibited the invasion and migration of cells. The above results indicated that circABCB10 acted as miR-670-3p sponge to upregulate the expression level of HMG20A.

Conclusion
CircABCB10 promoted the proliferation and metastasis of HCC by up-regulating HMG20A as a sponge of miR-670-3p, suggesting that circABCB10 may be a potential oncogene of hepatocellular carcinoma.

Abbreviations
HCC: hepatocellular carcinoma; circ RNA: circular RNA

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Authors’ contribution
Guarantor of integrity of the entire study: YF, study concepts: DW, study design: YF, literature review: LF, clinical studies: YF, experimental studies: YF, LC, data acquisition: YF, statistical analysis: YF, XL, manuscript editing: YF, manuscript review: DW. All authors read and approved the final manuscript.

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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the Maternity and Child Care Center of Liuzhou. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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