Down-regulation of circular RNA C3 in hepatocellular carcinoma and its potential clinical implication

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Abstract

Background

Hepatocellular carcinoma (HCC) is a common primary malignant tumor with an increasing incidence worldwide. Circular RNAs (circRNAs) have been demonstrated in playing significant roles in the processes of physiology and pathology. However, the expression patterns and function of circRNAs in HCC remain unclear.

Methods

The expression of circular RNA C3 (circC3) in HCC tissues, para-cancer tissues and normal liver tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR), and their associations with clinicopathological features and prognosis were analyzed. Using in vitro models, the role of circC3 in HCC proliferation, apoptosis and glycolysis was evaluated.

Results

Compared with para-carcinoma tissues and normal tissues, CircC3 expression was significantly reduced in HCC tissues. The downregulation of CircC3 levels in HCC was significantly correlated with tumor number ($p < 0.001$), tumor size ($p < 0.001$), tumor invasion ($p = 0.026$) and TNM stage ($p = 0.024$). Cell experiments revealed that overexpression of circC3 resulted in inhibited proliferation, increased apoptosis and downregulated the expression of GLUT1 and LDHA. Lower expression of circC3 was significantly associated with poor prognosis. ROC curves showed that the circC3 and circSOX5 serve as a biomarker to distinguish HCC from para-carcinoma tissues.

Conclusions

Our study revealed that circC3 could inhibit HCC progress by promoting apoptosis, inhibiting proliferation and glycolysis. CircC3 may serve as a potential diagnosis and prediction biomarker for HCC.

1. Introduction

HCC is one of the most common malignant tumors, and its mortality ranks third around world[1, 2]. Because HCC is prone to metastasis and recurrence, the 5-year survival rate of patients is still less than 6% [3, 4], and the deaths of patients caused by HCC exceed 600 thousand per year worldwide[5]. Although the level of diagnosis and treatment of liver cancer continues to improve, HCC at mid- to late-stage results in a poor long-term survival rate[6]. Current therapies such as surgery, chemotherapy, and transplantation are not effective in treating advanced HCC[7]. Consequently, it is very necessary for us to investigate novel markers for HCC diagnosis and identify effective therapeutic targets of HCC.

CircRNA is a type of endogenous non-coding RNA (ncRNA) that exhibit developmental stage, tissue and cell-type specific[8, 9]. CircRNAs have no 5 cap and 3 poly (A) tail, and cannot be degraded by exonuclease, so are more stable than linear RNA[9]. Recent research has demonstrated that some circRNAs play a significant role in the progression and treatment of HCC. Research showed that down-regulation of cSMARCA5 is an independent risk factor for the overall survival and recurrent-free survival of HCC patients after resection surgery[5]. Han et al. found that circMTO1 acts as the sponge of microRNA-9 to suppress HCC progression, and the decrease of circMTO1 in HCC tissues may serve as a prognosis predictor for poor survival of patients [10].

In this study, we focused our attention on circC3 (http://www.circbase.org/cgi-bin/singlerecord.cgi?id=hsa_circ_0002130). CircC3 was derived from C3 gene and located at chr19:6702137–6702590. Researches show that complement component 3 (C3) was related to the progress of HCC. It has been reported that the 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC[11]. Xu. et al. reported that activated hepatic stellate cells (HSCs) exert immunosuppressive effects in HCC by producing C3[12]. However, the mechanism of CircC3 in HCC has not been elucidated. We expect our study would contribute to the diagnosis and treatment of HCC.

2. Materials And Methods

2.1. Sample collection

A total of 121 pairs of HCC tissues and para-carcinoma tissues, 33 control liver tissues (liver tissues from patients with liver hemangioma) and 31 cirrhosis tissues were collected from patients who underwent surgery from May 2015 to May 2019, in the Chengdu First People's Hospital and Sichuan Cancer Hospital. Tissue samples were immediately soaked in RNAlater@ RNA Stabilization Solution (Invitrogen, CA, USA) after surgery, then stored at −80 °C until use. All patients were confirmed by pathological diagnosis of liver biopsy. Clinical features of patients with HCC are shown in Table 1. All the healthy controls were with no hepatitis, hepatic diseases and abnormal biochemical results of liver function tests. Patients who had an additional history of solid organ tumors, underwent radiotherapy, chemotherapy or targeted therapy were excluded.

2.2 RNA extraction and qRT-PCR analysis

Samples of patient tissues and cell lines were lysed using TRIzol Reagent (Invitrogen, CA, USA). Total RNA was isolated and reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). QRT-PCR was performed using iTaq Universal SYBR Green supermix (Bio-Rad, Hercules, CA, USA) on a CFX96 system (Bio-Rad, CA, USA). The relative mRNA expression was analyzed by the comparative threshold cycle method followed by normalization to GAPDH expression. Relative mRNA expression was calculated by the 2-ΔΔ Ct method. The primer sequences used in this paper were shown in Supplementary table1.

2.3 Cell culture and transfection

Human HCC cell lines (Huh7, LM9 and 97L) were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were maintained at 37 °C in a humidified incubator with 5% (v/v) CO_2. HCC cell lines were authenticated by short tandem repeat (STR) profiling with ABI 3130 sequencing system (ABI, USA). The open reading frame (OFR) of circC3 was amplified from 97L cells and subcloned into pcDNA 3.1. Transfection was performed with FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacture's protocol.

2.4 Cell proliferation assay

Cell proliferation assay was performed using CCK8 (Dojindo, Japan) according to the manufacturer's instructions. At 24 h after transfection, the cells were seeded into 96-well plates with 1000 cells per well and cultured for 0, 24, 48, 72 or 96 h, respectively. About 10 μl CCK8 reagent was added to the wells and incubated for 2 h at 37 °C. The absorbance was measured at 450 nm. Each experiment was replicated three times.

2.5 Apoptosis detection

At 24 h after transfection, the level of apoptosis was detected with Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China) on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) and Tunel assay (Roche, Cat. No: 11 684 817 910) according to the manufacturer's protocol. Fluorescent images were captured using a Zeiss fluorescence microscope at magnification of ×200. Apoptosis rate was calculated according to the formulas: number of TUNEL positive cells/number total cells ×%.

2.6 Western blotting

HCC cells were lysed by RIPA lysis buffer (Wu han Goodbio technology CO., LTD Hubei, China). The concentration of total protein was determined by BCA protein concentration assay kit (Wu han Goodbio technology CO., LTD Hubei, China). Then sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred, soaked and incubated with anti-GLUT1 and anti-LDHA (1:1000; Wuhan proteintech group Hubei, China), anti-GAPHD (1:3000; Wuhan Goodbio technology CO., LTD Hubei, China) antibodies at 4 ° C overnight. The proteins were detected using a luminescence method (ECL Western Blotting detection with IgG antibodies respectively 1:5000 dilution). The GAPDH was used as the loading control. Quantification of protein bands was carried by using Quality One software.

2.7 Statistical analysis

Data were presented as the mean±standard deviation (SD). Differences within multiple groups were tested by analysis of One-way analysis of variance (ANOVA) followed by the Bonferroni correction for post hoc t test. Differences between two groups were tested by Student’s t test. The steps of ROC curve analysis were as follows: (1) Calculate the predicted probabilities. The Binary Logistic process of SPSS

was used for logistic regression, then obtained the logistic regression equation, and a new variable containing the prediction probability of each individual was generated in the working data table of SPSS. (2) ROC curve analysis. The ROC curve was performed using GraphPad, the prediction probability was used as the test variable. The AUC, 95% CI, P value, sensitivity and specificity could obtain from the ROC curve. Statistical significance was set at $P < 0.05$.

3. Results

3.1. CircC3 significantly down-regulated in HCC tissues and showed HCC-stage-specific expression

To investigate the expression pattern of circC3, we first analyzed the specificity of the amplified circC3 product. The melting curve analysis showed that the amplified product yielded a single peak and the sequence was completely consistent with that from circBase (Fig. 1). These results showed that circC3 could specifically be amplified using the quantitative real-time PCR (qPCR) method we established.

As shown in Fig. 2A, circC3 expression was significantly lower in HCC tissues compared with cirrhosis and control liver tissues, and its levels in cirrhosis tissues were significantly lower than those in control liver tissues. In HCC patients, circC3 expression in carcinoma tissues was significantly lower than in matched para-carcinoma tissues (Fig. 2B).

Correlation between circC3 and clinical characteristics are summarized in Table 1. Statistical analysis indicated that circC3 expression was significantly negatively correlated with tumor number, tumor size, tumor invasion and TNM stage. (Fig. 2C–E). Nevertheless, we found no significant association between circC3 expression and other clinicopathological parameters, such as gender, age, alcoholism and tumor differentiation.

3.2. The biological function of circC3 in HCC cell lines

The expression of circC3 was measured in HCC cell lines. As shown in Fig. 3A, endogenous circC3 expression was the lowest in Huh7 compared with other HCC cell lines. Thus, we choose Huh7 for overexpression assays. The efficiency of transfection is examined by qRT-PCR. After transfection, the expression of circC3 was significantly overexpressed (Fig. 3B). The CCK8 assay results showed that circC3 inhibited cell proliferation (Fig. 3C). Then, we investigated the role of circC3 in apoptosis in Huh7 cells. The Annexin V-FITC/PI apoptosis assay and TUNEL results indicated that circC3 overexpression promoted apoptosis in Huh7 cells (Fig. 3D-F).

Glucose transporter 1(GLUT1) and lactate dehydrogenase A (LDHA) were reported to play a pivotal role in promoting glycolysis. Aerobic glycolysis acts as a hallmark of liver cancer and is responsible for the regulation of proliferation, immune evasion, invasion, metastasis, angiogenesis, and drug resistance in HCC[13], while the regulation mechanism of circC3 to glycolysis in HCC has not been fully elucidated. We found that overexpression of circC3 resulted in significantly decreased expression of GLUT1 and LDHA by western blotting (Fig. 3G). We predicted that circC3, which is 195 bp in length, serves as a binding

platform for miRNAs. As predicted by CircBase and Circular RNA Interactome, we found 14 binding sites for miRNAs related to circC3. Researches show that there were 6 miRNAs which can bind with circC3 were related to glycolysis (Fig. 3H).

3.3. CircC3 may serve as a potential diagnosis and prediction biomarker for HCC.

Our observation showed that compared with high expression, low expression of circC3 was related to the poor progression-free-survival (PFS) (Fig. 4A, p = 0.008). Researches have shown that circSOX5 also regulate the biological properties of HCC[14]. In our study, we also found that circSOX5 expression was significantly lower in HCC tissues compared with para-carcinoma tissues (Fig. 4B), suggesting that these circRNAs might act as antioncogene in HCC. However, the diagnostic value of these circRNAs has not been fully elucidated. The area under the ROC curve (AUC) for each individual circRNA was 0.856, and 0.8521 (Table 2). Furthermore, the combination analysis of two circRNAs resulted in an increased AUC of 0.907 with 76.52% of sensitivity and 90.16% of specificity in discriminating HCC from para-carcinoma tissues (Table 2; Fig. 4C).

4. Discussion

HCC is a universal malignancy tumor, which usually progresses to an advanced stage because of late diagnosis. [15, 16]. The growing evidence is usable to verify the underlying mechanisms of circRNAs in the progression of HCC. Research show that CircRNA-SORE is transported by exosomes to spread sorafenib resistance in HCC via stabilizing YBX1[17]. CircRNA-5692 could inhibit the progression of HCC by sponging miR-328-5p to enhance the expression of DAB2IP[18]. However, knowledge about the function of circC3 in HCC is still limited. Our results showed that circC3 expression had a tendency to decrease gradually from normal liver tissues to cirrhosis liver tissues and further to HCC tissues, indicating that continuous reduction of circC3 might play central roles in cancerization and tumorigenesis. Furthermore, decrease of circC3 was associated with unfavorable clinicopathologic features (including larger tumor size, more intrahepatic metastases and advanced tumor stage) in HCC patients, suggesting that circC3 might serve as an antioncogene in HCC. We further confirmed this mechanism by in vitro experiments.

Our in vitro experiments showed that circC3 plays a critical tumor-suppressive role in HCC progression by inhibiting the proliferation and glycolysis, promoting apoptosis of HCC cells. GLUT1 and LDHA are regulated by noncoding RNAs, leading to changes of glucose uptake and lactate production levels, and play a vital role in the proliferation and apoptosis of HCC[7, 19, 20]. The downregulation of LDHA can induce apoptosis and growth arrest in mouse HCC model[21]. In our study, circC3 overexpression led to the downregulation of LDHA and GLUT1, that was agreeing with apoptosis increase and proliferation inhibition. It was reported that most circRNA promote glycolysis related pathway in tumor by sponging miRNAs [22–26]. For example, hsa_circ_0002130 promote osimertinib-resistant in non-small cell lung cancer (NSCLC) cells by sponging miR-498 to regulate GLUT1, HK2 and LDHA [27]. CircATP2B1 captured miR-326-3p/miR-330-5p and decreased the suppression of PKM2, thus aiding the aerobic glycolysis and

proliferation of gastric cancer cells[28]. However, Bo. et al. reported that circ_0006677 inhibits the progression and glycolysis in NSCLC by sponging miR-578 and regulating SOCS2 expression. There was putative binding site of miR-578 (187-194bp) related to circC3 (Fig. 3K). We suggested that by combining with miR-578, circC3 could exert an inhibitory effect on glycolysis related pathway in HCC. Thus, further works should be implemented to identify detailed molecular mechanisms of circC3 functioning in glycolysis of HCC.

Our results show that low expression of circC3 was associated with worse prognosis, indicating that the level of circC3 in HCC tissue could distinguish patients with high risk of recurrence and metastasis from patients with low risk even within known clinical risk factors. For these patients, we need to pay special attention to their prognosis and make individualized therapeutic schedule after hepatectomy. The single circRNA or combined circRNA could both distinguish HCC tissues well from para-carcinoma tissues by ROC analysis, and combined circRNA was related to a higher AUC. So, we considered that circC3 might be an ideal diagnostic and prognostic biomarker for HCC. However, the value of circC3 in HCC diagnosis should be further validated in a prospective with large-scale clinical study.

5. Conclusion

In conclusion, our study reveals that circC3 suppress HCC progression via promoting apoptosis, inhibiting proliferation and glycolysis. CircC3 may serve as a potential diagnosis and prediction biomarker for HCC.
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Declarations

Ethics approval and consent to participate

All patients who participate the study sign the written informed consent. The investigation was approved by Medical Institutional Review Board of Chengdu First People's Hospital and Sichuan Cancer Hospital.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

Funding

The authors have no financial support to declare.

Authors' contributions

Li Zuhua conceived and designed the experiments. Luo Min and Shi Yidou performed the experiments. Qing Keqin and Li Hongxia analyzed the data. He Weiyang wrote the paper.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

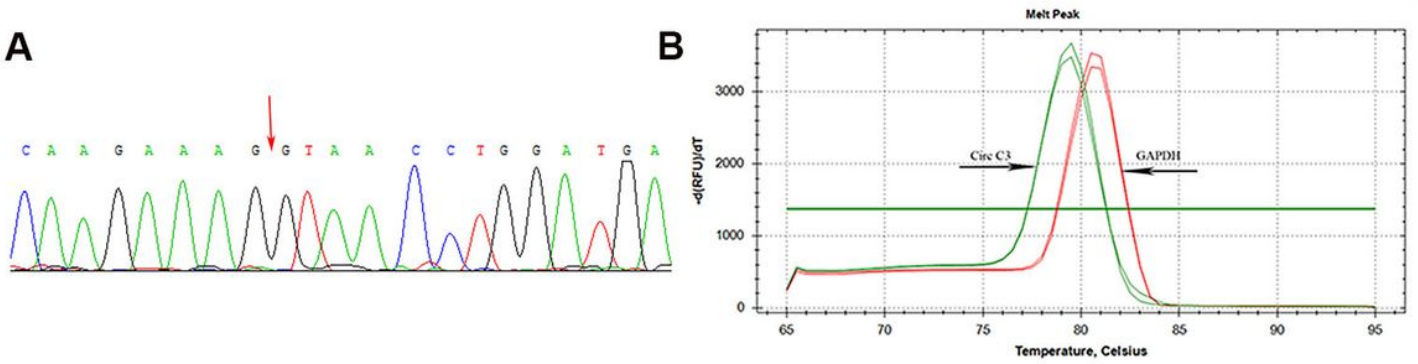

Figure 1

CircC3 was specifically amplified by real-time PCR. A. The sequence of circC3. The splicing junction site was indicated by red arrow. B. The melt curve of circC3 and GAPDH.

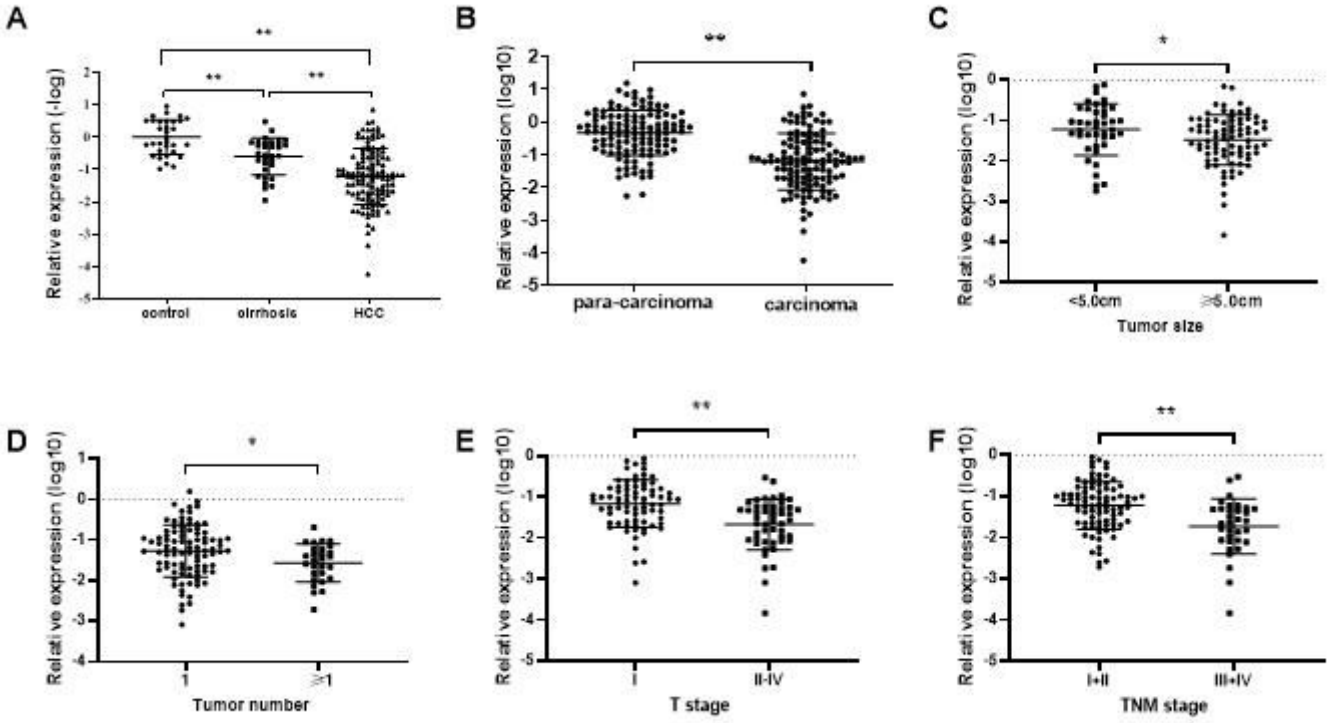

Figure 2

CircC3 was downregulated in HCC tissues and was associated with tumor size, tumor number, cancer invasion and TNM stage. A. The circC3 expression in healthy control, cirrhosis and HCC liver tissues. B. The circC3 expression was significantly downregulated in carcinoma compared with para-carcinoma of HCC liver tissues. C. Reduced circC3 was associated with tumor size (p = 0.024). D. Decreased circC3 was

corrected with tumor number (p = 0.024). E. Reduced circC3 was associated with cancer invasion (p <0.001). F. Decreased circC3 was related with TNM stage (p< 0.001). **, p < 0.01, *, p < 0.05.

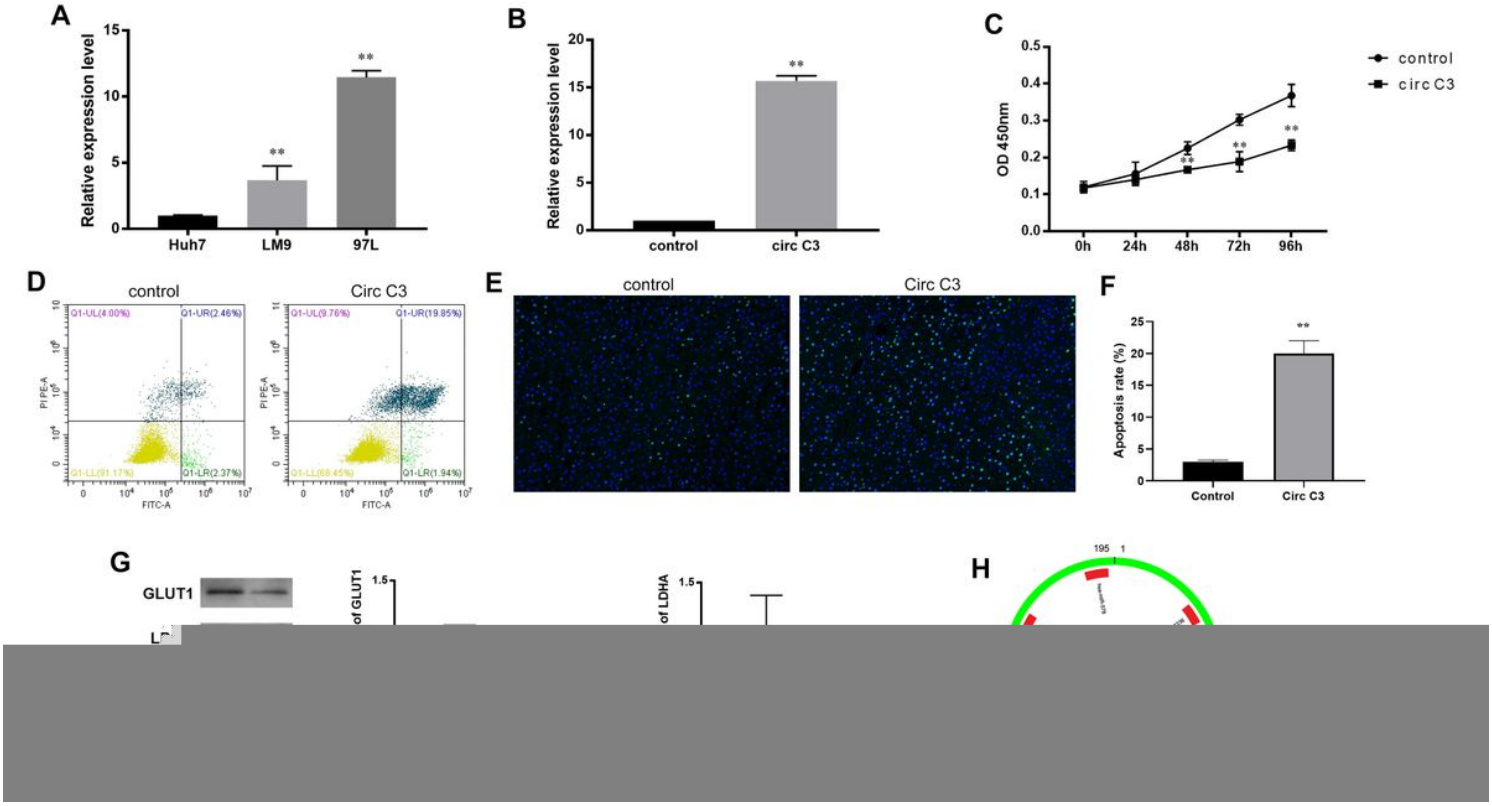

Figure 3

Overexpression of circC3 inhibited proliferation and promoted apoptosis in Huh7 cells. A. The expression patterns of circC3 in HCC cells. B. The expression of circC3 was significantly increased after transfection with circC3. C. Overexpression of circC3 inhibited proliferation detected by the CCK8 assay. D-F. Overexpression of circC3 increased the apoptotic detected by the Annexin V-FITC staining (D) and the Tunel assay (E). n =3, magnification, ×200 in each group. F. The statistical results of apoptosis rate. G Overexpression of circC3 decreased the expression of GLUT1 and LDHA detected by western blotting. H. Schematic drawing showing the putative binding sites of miRNAs related to circC3. **, p < 0.01, *, p < 0.05.

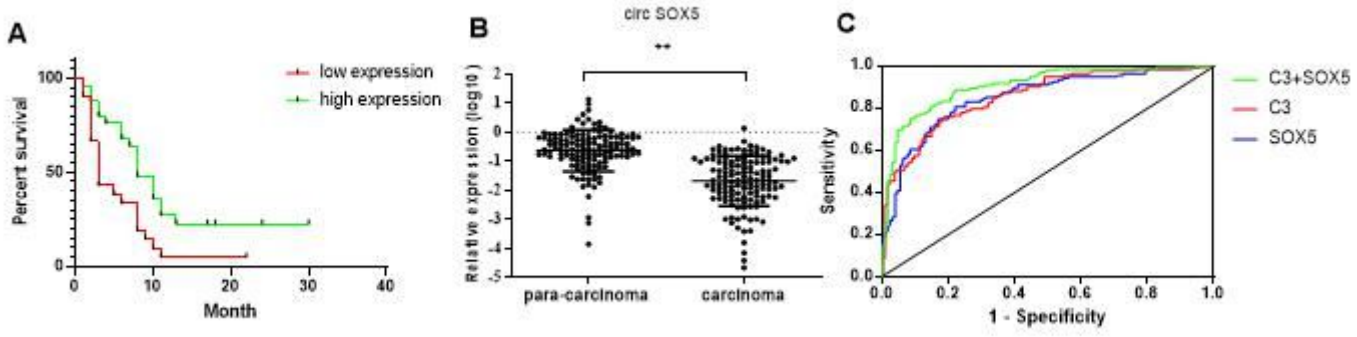

Figure 4

Log-rank analysis and ROC analysis for circRNAs in tissue samples. A Log-rank analysis showed low level of circC3 was related to poor PFS. B. CircSOX5 expressions was significantly downregulated in carcinoma compared with para-carcinoma. C. AUC indicates the performance of single or combined circRNAs in discriminating HCC from para-carcinoma. **, $p < 0.01$, *, $p < 0.05$.

Supplementary Files

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- Table1.xlsx
- Table2.xlsx
- SuplementaryTable1.xlsx