

circular RNA CircITCH (has-circ-0001141) suppresses hepatocellular carcinoma (HCC) progression by sponging miR-184

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Abstract

Background

Aberrant expression of circular RNA(circRNA)is involved in the occurrence of various diseases and tumor development, in which plays a vital role, including hepatocellular carcinoma (HCC). Nevertheless, the regulation mechanism and biological function of circITCH in hepatocellular carcinoma (HCC) remain unclear.

Methods

The expression level of circular RNA itchy E3 ubiquitin protein ligase (circ-ITCH) was identified and validated by real-time polymerase chain reaction (RT-qPCR) in HCC cell lines. The stability of circITCH was confirmed by Ribonuclease R (RNase R) assay. Subsequently, through silencing and overexpression of circITCH to investigate the functional roles of circITCH in HCC proliferation, invasion and apoptosis. We also carried out bioinformatics analysis, luciferase reporter assays to define the relationship between microRNA (miR)-184 and circITCH.

Results

CircITCH (hsa_circ_0001141) was a stable circRNA and downregulated in HCC cells. Overexpression of circITCH inhibited cell proliferation, migration, invasion and promoted apoptosis in vitro, whereas knockdown of circITCH had the opposite effects. Mechanistically, miR-184 could be sponged by circITCH, and its overexpression could mitigate the suppressive effects of circITCH overexpression on HCC progression. Through biological website to predict the target genes of miR-184 may be combined. Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to investigate mRNAs with significant functional enrichment and pathways, also which its relationship with HCC related pathway and immune cells.

Conclusions

Our findings reveal that circITCH served as a repressor to restrain HCC malignancy via miR-184. Therefore, circITCH may serve as a potential prognostic marker and therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC), as one most common type of primary liver cancer, ranks the third cause of leading cancer-related deaths worldwide [1]. With multiple causes, including HBV infection and liver cirrhosis are the major risk factors for HCC [2]. The development of HCC is a complex multistep process involving persistent inflammatory damage, including hepatocyte necrosis and regeneration associated

with fibrotic deposition [3]. Despite the development of numerous new alternative therapies, surgical resection still remains the mainstay of HCC treatment, but the metastasis and recurrence rates of HCC after surgery are still high [4]. Therefore, the most urgent needs are to elucidate the etiology and molecular mechanisms underlying the development and progression of HCC, and to find sensitive markers for the diagnosis and prognosis of HCC [5].

Currently, with the development of high-throughput sequencing techniques, transcriptomics has gradually become a focus of attention. Circular RNAs (circRNAs) are a class of endogenous non-coding RNA which has been found to be commonly expressed in eukaryotic genes [6], a family of covalently closed loop structure RNAs without 5'-cap and 3'-poly A tail, have been identified to play vital roles in human cancers [7]. The unique circular structure of circRNAs confers inherent resistance to degradation by exonuclease and makes them more stable than their linear parental genes [8–10]. Accumulating evidences about circRNAs have demonstrated that they participate in diverse biological processes including proliferation, invasion, metastasis, apoptosis and autophagy of malignant tumors [11, 12], which implying a great promise of circRNAs as predictive biomarkers and therapeutic targets in cancer [13, 14]. Recently, the function and mechanism of circRNAs in HCC have also been preliminary studied. CircFBLIM1 has been shown to act as a ceRNA that promotes the development of hepatocellular cancer by acting as a sponge of miR-346 [15]. Circ-0051443 is transmitted from normal cells to HCC cells via exosomes and suppresses the malignant biological behaviors by promoting cell apoptosis and arresting the cell cycle [16]. Circ-DB promotes HCC growth and reduces DNA damage via the suppression of miR-34a and the activation of deubiquitinating-related USP7 [17].

Circular RNA itchy E3 ubiquitin protein ligase (circ-ITCH) is located on human chromosome 20, 20q11.22, which derived from exons 7–14 of the itchy E3 ubiquitin protein ligase (ITCH) gene. It was demonstrated to function as a tumor suppressor in multiple human cancers, including ovarian cancer [18], bladder cancer [19], breast cancer [20], papillary thyroid cancer [21] and colorectal cancer [22] and so on. Study has found that expression of circ-ITCH in HCC tissues was significantly reduced, and a high level of circITCH was associated with favorable survival of HCC [23]. However, the function and regulatory mechanism of circITCH (circ-0001141) in HCC malignant behaviors remain largely unclear. The discovery of more circRNAs that regulates the development of HCC will provide a more effective choice for targeted therapy.

MicroRNAs (miRNAs) are small, mostly non-coding RNAs containing approximately 22 nucleotides, which are highly conserved across species and function in post-transcriptional regulation of gene expression [24]. The molecular functions of circRNA have been elucidated, such as the regulation of transcription, translation, protein binding. Remarkably, it has been widely reported that circRNA serves as “miRNA sponges” to sequester miRNAs from binding to their target mRNAs [25–26]. Herein, we focus on the underlying mechanisms of circITCH in HCC and identify whether circITCH could affect cell malignant phenotypes through competing endogenous RNAs (ceRNA) regulating cascades “circRNA-miRNA-mRNA”, which enhances the significance of the findings of the present study.

In this study, we confirmed the down-regulation of circITCH in HCC tissues and cells. Circ-0001141, as a ceRNA, inhibited cell proliferation, migration, invasion and promoted apoptosis by sponging miR-184. The functional complementation analysis was conducted to verify the functions of them and the relationships among the circRNA/miRNA/mRNA networks. This study might provide a novel insights and biomarker for HCC treatment.

Methods

Cell culture

Human normal liver cell lines (LO2) and HCC cell lines (Huh7, HCCLM3, SMMC-7721, MHCC97H, HepG2) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) and antibiotics (1% penicillin/streptomycin, Gibco, USA) in a humidified incubator with 5% CO₂ at 37°C.

RNase R treatment

To verify the stability of circITCH molecular structure, RNase R digestion experiment was carried out. 2µg of total RNA extracted from HCC cells were incubated with 3U/µg RNase-R (Geneseed Biotech, Guangzhou, China) at 37 °C for 15 minutes and 70 °C for 10 minutes. Then, the expression levels of linear β-actin and circITCH were determined by qRT-PCR.

RNA extraction and RT-qPCR

Total RNA was extracted from cell lines using TRIzol Reagent. A NanoDrop 2000 microspectrophotometer was used to determine the quality and concentration of total RNA. After spectrophotometric quantification, 1 µg of total RNA in a final volume of 20 µl was used for reverse transcription (RT) with a PrimeScript RT Reagent Kit (Tsingke, China). Then, Fluorescence quantitative PCR was conducted using TB Green Fast qPCR Mix (Tsingke, China). All primers were designed and synthesized by Tsingke (Guangzhou, China). The expression of small nuclear RNA U6 or β-actin genes was used as a control to calibrate the original concentration of miRNA, circRNA respectively. Target gene expression was calculated using the 2- $\Delta\Delta$ CT method. Related primer sequences are shown in Table 1.

Cell transfection

Small interfering RNAs (siRNAs) targeting the junction sequence of circITCH (si-circ-1, si-circ-2 and si-circ-3), miRNA-184 mimics, inhibitors and their related negative control oligonucleotides were designed and

synthesized by HanBio (Shanghai, China). All transfection operations were performed using Entranster™-R4000 (Engreen Biosystem, Beijing, China) according to the manufacturer's instructions. All the sequences used in the present study are listed in Table 2.

Construction of stable cell lines

To establish stable overexpression of circITCH in human HCCLM3 cell lines, we used control lentivirus HBLV-ZsGreen-PURO and purpose lentivirus HBLV-hsa_circ_0001141-Null-ZsGreen-PURO to infect HCCLM3 cells (HanBio, Shanghai, China). In the infected cells after 48 h, by adding and maintaining 1.0 μ g/ml puromycin kill not be infected cells effectively.

Thus under puromycin drugs to maintain stable strains of eventually get circITCH stable expression.

Dual luciferase reporter assay

According to the predictions of Circular RNA Interactome (circinteractome) and Starbase online software, circITCH possessed binding sites for miR-184 in the 3'-UTR region. To experimentally verify the possible miRNA target genes and binding sequences, the wild-type and mutant circITCH 3'-untranslated region (3'-UTR) sequences were synthesized and cloned into dual luciferase reporter plasmids (HanBio, Shanghai, China) containing the psiCheck2 promoter. HCCLM3 cells were inoculated into a 96-well plate and grown to 50%-70% confluence 24 h at 37°C, 5% CO₂. They were transfected in combination with the wild-type or mutant circITCH reporter gene plasmids and miR-184 mimic or miR-NC mimic. After 48 h, the activities of both firefly luciferase (LUC) and Renilla luciferase (RLUC) were measured with a Dual-Luciferase Reporter System Kit (Promega, USA).

RNA fluorescence in situ hybridization (FISH)

FISH kit (RiboBio) was used to detect the location of circITCH in HCC cells following the manufacturer's protocol. Briefly, cells were subjected to pre-hybridization solution for 30 min. Then, cy3-labelled circITCH probe were hybridized at 37°C overnight after pre-hybridization. Next, slides were washed and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). A confocal microscopy was applied to analyze results.

Cell counting kit-8 (CCK8) assay

For the CCK8 assay, transfected HCC cells in logarithmic growth phase were seeded into a 96-well plate at a density of 1×10^4 cells/well for 6 replicates. The proliferation of cells were monitored every 24 hours for 0, 24, 48, 72 h. 10 μ l of CCK 8 solution (KeyGene, Biotech, China) was added to each well and they were

maintained at 37°C for 2h away from light. The absorbance of each well at 450 nm was measured with a microplate reader.

5-Ethynyl-2'-deoxyuridine (EdU) assay

Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit (RiboBio, Guangzhou, China) was used to detect cell proliferation ability according to the manufacturer's instructions. The transfected HCC cells (1×10^4 cells for each group) were seeded in 96-well plates and cultured for 24 h, after, a 50 μ M EdU solution was added onto plates and incubated for 2h at 37 °C. Then, the cells were immobilized with 4% paraformaldehyde and stained with Apollo Dye Solution and Hoechst 33342. Finally, the images were acquired using a fluorescence microscope and the ratio of EdU-positive cells was calculated.

Transwell assay

To perform the invasion assay, after 24h of transfection treatment, HCC cells (1×10^5 /well) were prepared into the upper chamber (8- μ m pore size; Millipore) with 200 μ L serum-free medium, and 800 μ L of medium containing 20% fetal bovine serum was added into the bottom transwell chamber. After incubating at 37 °C with 5% CO₂ for 24 h, we gently scraped the cells on the upper chamber with cotton swabs, whereas the invasive cells on the bottom chamber were fixed with 100% methanol for 20 min and stained with 0.1% crystal violet for 15 min at normal temperature. Five fields were randomly selected under a microscope (200 \times magnification) to calculate the average number of invasion cells.

Wound healing assay

Wound healing assays were performed to evaluate the migration ability of HCC cells. Transfected and controlled cells (1×10^6 cells/well) were seeded onto 6-well plates and cultured at 37°C, 5% CO₂. After the cells were fully grown, cell monolayers were scratched with a 200- μ l pipette tip to draw a vertical line in the middle of the cell and then cultured in DMEM. After routine culture for 24h and 48 h, photographs were taken and the cell migration rate was calculated.

Apoptosis assay

To perform cell apoptosis assay, a FITC Annexin V Apoptosis Assay Kit (BD Biosciences, CA, USA) was used as follows. Cells in logarithmic growth phase were collected, resuspended and evenly spread in a 6-well plate. After 24 h of transfection, a total of 1×10^6 cells were collected and washed twice with pre-cold PBS. Thereafter, $1 \times$ binding buffer (100 μ L) was used to disperse these cells, 5 μ L Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and 5 μ L propidine iodide (PI) were added to the cells for 15 min in a dark at room temperature. Subsequently, the apoptosis rate was analyzed by flow cytometry.

Statistical analysis

All assays were conducted at least three times, quantitative data are presented as the means \pm standard deviation (SD). Differences between two groups or among multiple groups were compared via student's t test (two-tailed) or ANOVA with GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze the experimental data. Differences were considered statistically significant with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

CircITCH (hsa_circ_0001141) is downregulated in HCC cells

According to the circbase database (<http://www.circbase.org/>), has-circ-0001141, which is located at chr20:33095678–33095698[+], is derived from its parental gene ITCH, and its spliced length is 873bp (Fig. 1a). To verify the qRT-PCR results of hsa_circ_0001141 in HCC cells, we performed Sanger sequencing on the PCR products of hsa_circ_0001141. The result confirmed the head-to-tail splicing in the PCR products with the expected size and predicted splicing site (Fig. 1b). In addition, the stability of circITCH was demonstrated by RNase R treatment. qRT-PCR results showed that the levels of linear β -actin decreased sharply under RNase R treatment, while circITCH showed resistance to RNase R digestion (Fig. 1c-d), which confirmed that circITCH has a circular structure. To determine the intracellular distribution of circITCH, RNA FISH assay showed that circITCH mainly located in the cytoplasm (Fig. 1e), which might participate in HCC progression. To investigate whether circ-ITCH expression was changed in HCC cell lines, qRT-PCR was utilized to assess circITCH expression. As the data showed in Fig. 1f, circ-ITCH expression was remarkably declined in HCC cells (Huh7, HCCLM3, SMMC-7721, MHCC97H) compared with normal liver cell line (LO2), While circITCH expression of HepG2 in HCC cells was higher than normal liver cells. Thus, we chose HCCLM3 cells with the lowest expression and HepG2 cells with the highest expression for subsequent cell experiment.

Function of circITCH in HCC

To study the biological function of circITCH in HCC, the expression of circITCH was downregulated in the HepG2 cell line, three short interfering RNAs targeting the backsplice site of circITCH were constructed and the one that provided the most significant downregulation was chosen to access cell functions. In addition, HCCLM3 cell lines were selected to stabilize overexpression of circITCH. The efficiency and specificity of circITCH knockdown and overexpression in HepG2 and HCCLM3 cells were verified by RT-qPCR (Fig. 2a). CCK-8 and EdU assays were carried out to evaluate cell proliferation. As shown in Fig. 2b-c, the results showed that circITCH silencing notably promote the vitality and proliferative ability of HepG2 cells, while circITCH upregulation inhibited cell growth. Subsequent wound healing assay and

transwell invasion assay also confirmed that mobility of HCC cells was increased in the si-circITCH group and inhibited in the overexpression group (Fig. 2d-e). Moreover, the results of flow cytometry showed that silencing circITCH could significantly inhibit cell apoptosis (Fig. 2f). Taken together, these data suggest that circITCH exerts a tumor suppressor role in HCC cells.

CircITCH functions as a sponge of miR-184

The competing endogenous RNA (ceRNA) mechanism, which mainly regulates gene expression at the posttranscriptional level, is primarily the most widely reported circRNA regulatory mechanism. It has been reported that circRNAs usually act as miRNA “molecular sponges” to regulate the expression of downstream genes [25]. Therefore, we performed a cross-analysis using two databases: CircInteractome (<https://circinteractome.irp.nia.nih.gov/>) and starBase(<http://starbase.sysu.edu.cn/>) to predict the potential miRNAs that may bind to circITCH. It was found that hsa-miR-184, hsa-miR-665, hsa-miR-944 from the overlap of this databases (Fig. 3a). Among the three candidate miRNAs, only hsa-miR-184 (miR-184) was found to be significantly differentially expressed in HCC, and was reported as a carcinogen. Also, the binding sites between circITCH and miR-184 were predicted by starBase. qRT-PCR results showed that circITCH in HCCLM3 and HepG2 cells negatively regulated the expression of miR-184 after upregulated or downregulated (Fig. 3b), suggesting that miR-184 is a miRNA potentially sponged to circITCH. To further determine whether miR-184 could directly target circITCH in HCC cells, we performed a dual luciferase reporter assay. Based on the complementary base pairing prediction with the “seed” region of miR-184, plasmids containing the wild-type sequence (circITCH-WT) and the mutant binding sequence (circITCH-MUT) were constructed (Fig. 3c). HepG2 cells were cotransfected with miR-184 mimic or miR-NC. As shown in Fig. 3d, the luciferase activity in miR-184 mimics with circITCH-WT groups was significantly lower than other groups. However, miR-184 mimics or miR-NC showed no significant difference in luciferase activity when cotransfected with mutant reporter. These results confirmed that circITCH can directly and specifically interact with miR-184. Additionally, we investigated the expression of miR-184 in HCC cell lines, in contrast to circITCH, miR-184 was notably increased in HCC cell lines (Fig. 3e). According to the Starbase database (<http://starbase.sysu.edu.cn/>), the expression of miR-184 in HCC tissues was significantly increased (Fig. 3f). The Kaplan–Meier Plotter database (<http://kmplot.com/analysis/index.php>) revealed that the low expression of miR-184 had a significant advantage in the short-term survival rate of patients with HCC, while the high expression of miR-184 was associated with the long-term survival rate of patients, but with no significant statistical significance ($p = 0.26$) (Fig. 3g). Altogether, the above results revealed that circITCH functions as a “molecular sponge” for miR-184 might participate in HCC progression.

MiR-184 reverses the antitumor effects of circITCH on HCC progression

To elucidate whether circITCH functions by sponging miR-184 in HCC progression, rescue experiments were conducted with cotransfection of miR-184 mimics or inhibitor with circNEIL3 or si-circNEIL3. The

efficiency of miR-184 mimics and inhibitor on its expression level in HepG2 cells was verified by RT-qPCR (Fig. 4a). Then used to verify the expression of circITCH and miR-184 after transfection. The results demonstrated that after adding miR-184 mimics on the basis of circITCH overexpression, the expression level of miR-184 was significantly increased while the expression level of circITCH was not affected (Fig. 4b). As shown in Fig. 4c-f, miR-184 mimics partly reversed the suppressive effects on the proliferation, migration and invasion induced by circITCH upregulation in CCK-8, EdU, wound healing and transwell assays. In addition, miR-184 inhibitors reversed the inhibition of apoptosis induced by down-regulation of circITCH (Fig. 4g). Collectively, these data suggested that circITCH inhibited HCC proliferation, invasion and promoted cell apoptosis, at least partly depend on miR-184.

Prediction and discovery of circITCH/miR-184/mRNA related regulatory networks in HCC

CircRNA /miRNA/mRNA regulatory networks have been extensively reported in the literature and play an important role in various diseases and cancers. Therefore, we searched for and predicted the downstream targets of miR-184 from various biological information websites to determine the main functions and roles of relevant molecules in HCC. RNAInter database [27] shows the top 100 mRNA molecules that miR-184 may bind to (Fig. 5a). The WebGestalt (WEB-based Gene Set Analysis Toolkit) (<https://webgestalt.org/>) predicted 785 target genes of miR-184, and the related functions and effects of these genes enrichment are shown in Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. The results showed that many enriched GO terms were associated with biological regulation and metabolic process in biological process, membrane and nucleus in cellular component, protein binding and ion binding in molecular function (Fig. 5b). According to KEGG analysis, the highly enriched pathway was regulation of membrane permeability and the most differentially expressed pathway was cell-cell signaling by wnt (Fig. 5c-d). We then validated the association between these molecules and HCC against the Gene Set Cancer Analysis (GSCA) database [28]. For GSVA (Gene Set Enrichment Analysis) analysis, GSCA estimates variation of gene set activity (represents as GSVA score) over a specific cancer's sample population in an unsupervised manner. This module provides differential gene set activity analysis between tumor and normal samples. The GSVA score represents the integrated level of the expression of gene set, which is positively correlate with the expression of gene set. Therefore, As shown in Fig. 5e-g, the GSVA score in tumor group is higher than that in adjacent group, it can indicate that the overall expression of the gene set in the tumor group is higher. The correlation between GSVA score and activity of cancer related pathways indicated that GSVA score were highly expressed in Apoptosis and EMT pathways, while the expression levels were decreased in Hormone AR, Hormone ER, RASMAPK and RTK pathways. As for immune cells, GSVA score was negatively correlated with the expression of Th17, effector memory cells, cytotoxic cells and neutrophils, while positively correlated with the expression of naive CD8 cells, naive CD4 cells, n /iTreg cells, NKT, CD4-T, B cells and central memory cells.

Discussion

As novel noncoding molecules, increasing quality of circRNAs have been discovered and identified through next-generation sequencing technology (NGS) [29, 30]. It has been shown that circRNAs play important roles in the occurrence and development of multiple cancers and involved in pleiotropic regulation of cell functions [8].

Previous reports have identified a number of abnormally expressed circRNAs in HCC that are upregulated or downregulated in HCC tissues and cells function as oncogenes or suppressor genes might be prognostic and diagnostic biomarkers. For example, the circARPP21/miR-543/LIFR axis suppresses the proliferation, invasion, and migration of hepatocellular carcinoma cells [31]. circMEMO1 can promote the demethylation and expression of TCF21 and can be considered a crucial epigenetic modifier in HCC progression [32]. Hsa_circ_0110102 acted as a sponge for miR-580-5p and inhibited CCL2 secretion into tumor microenvironment by decrease the expression of PPAR α in HCC cells, then inhibited the pro-inflammatory cytokine release from macrophages by regulating the COX-2/PGE2 pathway [33].

In this study, we demonstrated circITCH as a significantly downregulated circRNA in HCC cell lines. For loss-and gain-of-function experiments, we revealed that overexpression of circITCH inhibits the proliferation, migration and invasion, promotes apoptosis of HCC cells in vitro, while the experimental group with silenced circITCH showed the opposite phenotype. Overall, these results elucidate the potential of circITCH as a new biomarker and tumor-suppressor molecule in hepatocellular carcinoma. The classic regulatory mechanisms of circRNA include ceRNA, bind to specific proteins to influence their functions and functional proteins coding [34]. Accumulating evidence has revealed that circRNAs primarily function as miRNA sponges. Considering that circITCH was primarily localized in the cytoplasm, we assume that circITCH may perform by sponging miRNA. To verify miRNAs targeted by circITCH, candidate miRNAs with sequences complementary to circITCH were screened through bioinformatic databases (starbase and circinteractome). We observed that miR-184 was the most highly enriched in the binding sites with circITCH, and its expression was also negatively regulated by circITCH in HCC cells. Furthermore, dual luciferase reporter assays indicated that circITCH could directly bind to the seed region of miR-184. In a rescue experiment, miR-184 blocked the suppression of tumour proliferation and invasion induced by overexpression circITCH. In addition, current research indicates that miR-184 acts as an oncogene in many types of cancers [35–37]. In short, our work suggests that circITCH might alleviate HCC progression via the circITCH/miR-184 axis (Fig. 6).

Finally, to further elucidate the underlying mechanism of miR-184, bioinformatic analyses were performed to identify target genes regulated by miR-184. The results identifying target gene set that miR-184 might bind to was analyzed by biological function analysis. GO functional annotation and KEGG enrichment analysis showed that the enriched function and pathway were mainly related to biological process of cancer. GSEA score showed that gene sets were mainly involved in tumor apoptosis and EMT process. In addition, it may influence the immune process by regulating the expression of relevant immune cells. However, the specific relationship between miR-184 and its target gene needs to be further illustrated to explore a brand-new idea for circRNA biogenesis regulation. We have also described for the first time the

relationship between miR-184 and circITCH. These findings offer a promising new therapeutic approach for treatment of HCC.

Trustily, there are several weaknesses in this research due to experimental condition limitations and schematic design insufficiency. First, we need clinical samples to further verify our study. Second, this study lacks the in vivo verification link of animal experiments. Finally, the regulatory network of ceRNA is not perfect, and the downstream further experiments are needed to provide valuable theoretical support for this finding. Hence, further research is essential to acquire a deep understanding of operation of molecular mechanisms in HCC biology.

Conclusion

Here, our findings reveal that circITCH served as a repressor to restrain HCC malignancy via miR-184. This novel finding not only improves our knowledge of the molecular mechanisms underlying HCC tumorigenesis, but also points to a promising therapeutic strategy against HCC.

Abbreviations

HCC: hepatocellular carcinoma; CircRNA: Circular RNA; miRNA: MicroRNA; Circ-ITCH: circular RNA itchy E3 ubiquitin protein ligase; RT-qPCR: real-time polymerase chain reaction; RNase R: Ribonuclease R; CeRNA: competing endogenous RNAs; SiRNA: small interfering RNA

Declarations

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Authors' contributions

XG were responsible for performing the experiments and drafted the manuscript. XG and CjW were responsible for designing the experiments and supervising the study. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table1 Primers for real-time PCR and probe for FISH

Genes	Forward (5'-3')	Reverse (5'-3')
CircITCH	AAGGAGCAATGCAGCAGTTT	AGTCACAACACTACTTCTTCAACCCAT
β -actin	CCTTCCTGGGCATGGAGTC	TGATCTTCATTGTGCTGGGTG
MiR-184	CGCGTGGACGGAGAACTGAT	AGTGCAGGGTCCGAGGTATT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
circITCH	ACAACACTACTTCTTCAACCCATCCAGGTGGCAA	

Table2 Overexpression and silent related sequences for transfection

oligo	sense	antisense
CircITCH-si-1	GCCACCUUGGAUGGGUUGAAGA	UCUUCAACCCAUCCAGGUGGC
CircITCH-si-2	ACCUGGAUGGGUUGAAGAAGU	ACUUCUUCAACCCAUCCAGGU
CircITCH-si-3	AUGGGUUGAAGAAGUAGUUGU	ACAACUACUUCUUCAACCCAU
CircITCH-si-NC	UUCUCCGAACGUGUCACGU	ACGUGACACGUUCGGAGAA
MiR-184	UGGACGGAGAACUGAUAAGGGU	ACCCUUAUCAGUUCUCCGUCCA
MiR-184-NC	UCACAACCUCCUAGAAAGAGUAGA	UCUACUCUUUCUAGGAGGUUGUGA
MiR-184-i		ACCCUUAUCAGUUCUCCGUCCA
MiR-184-i-NC		UCUACUCUUUCUAGGAGGUUGUGA

Figures

cells using qRT-PCR product. e. RNA FISH analysis of circITCH in HCCLM3 and HepG2 cells. Nuclei were stained with DAPI. Scale bars, 100 μ m. The samples were imaged at 1000 \times magnification. Scale bar = 10 μ m. f. The relative expression of circITCH in a normal liver cell line (LO2) and HCC cells (HepG2, MHCC97H, HCCLM3, Huh-7, SMMC-7721) was measured by RT-qPCR. The data are presented as the means \pm SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

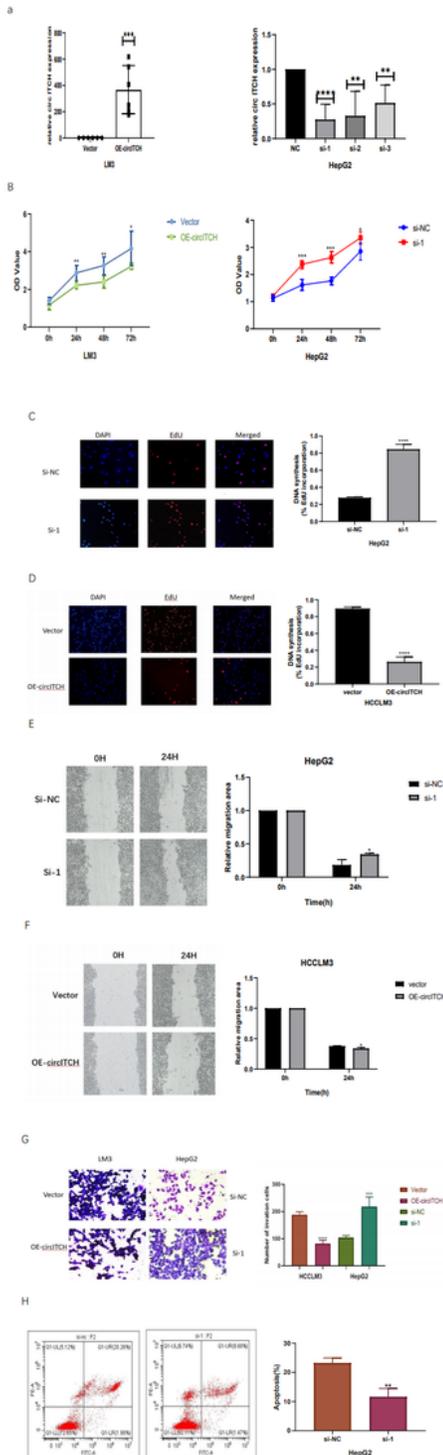


Figure 2

CircITCH suppresses HCC cell proliferation, invasion, and promoted apoptosis of HCC. a. qRT-PCR was conducted to determine the transfection efficiency of circITCH-overexpressing in HCCLM3 cells and siRNAs targeting circITCH (si-circ #1, si-circ #2 and si-circ #3) in HepG2 cells. b-d. CCK-8 and EdU assays was performed to evaluate the influence of circITCH overexpression or knockdown cell proliferation. The samples were imaged at 200× magnification. Scale bar = 50 μm e-g. Wound healing assay and transwell experiment was carried out to assess cell invasion capacity. The samples were imaged at 100×, 200× magnification. Scale bar =100um, 50 μm, respectively. h. The apoptosis rate was analyzed via flow cytometry. The data are presented as the means± S.D. of at least three independent experiments. * P < 0.05, **P < 0.01, ***P < 0.001

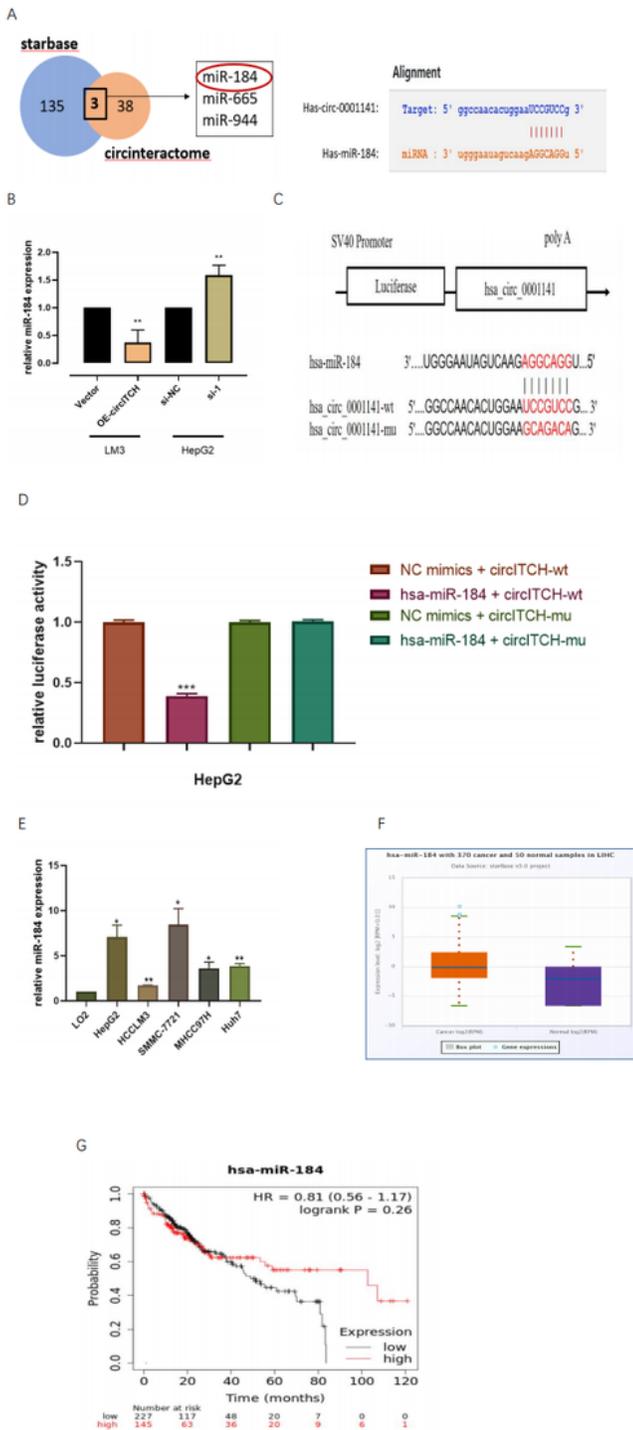


Figure 3

CircITCH acts as a sponge of miR-184 in HCC. a. Venn diagram showing the overlap of the target miRNAs of circITCH predicted by circinteractome and starbase online databases. A schematic model shows potential binding sites for miR-184 and 3'UTR of circITCH. b. The relative expression of miR-184 in HCC-LM3 and HepG2 cells transfected with circWAC or vector and si-circITCH or control was detected by RT-qPCR. c. A schematic of miR-184 and wild-type/mutant circITCH binding site sequences with

luciferase reporter vectors. d. Luciferase activity of the circITCH luciferase reporter vector (wt or mut) in HepG2 cells cotransfected with the miR-142 mimic or control. e. RT-qPCR analysis of the relative expression levels of miR-184 in LO2 cells and HCC cell lines. f. The relative expression of miR-142 in HCC tissues and adjacent nontumor tissues was analyzed by starBase Pan-Cancer Analysis Platform. g. Kaplan-Meier survival analysis of TCGA cohort with HCC stratified by the correlation between the overall survival and miR-184 expression. The data are presented as the means \pm SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

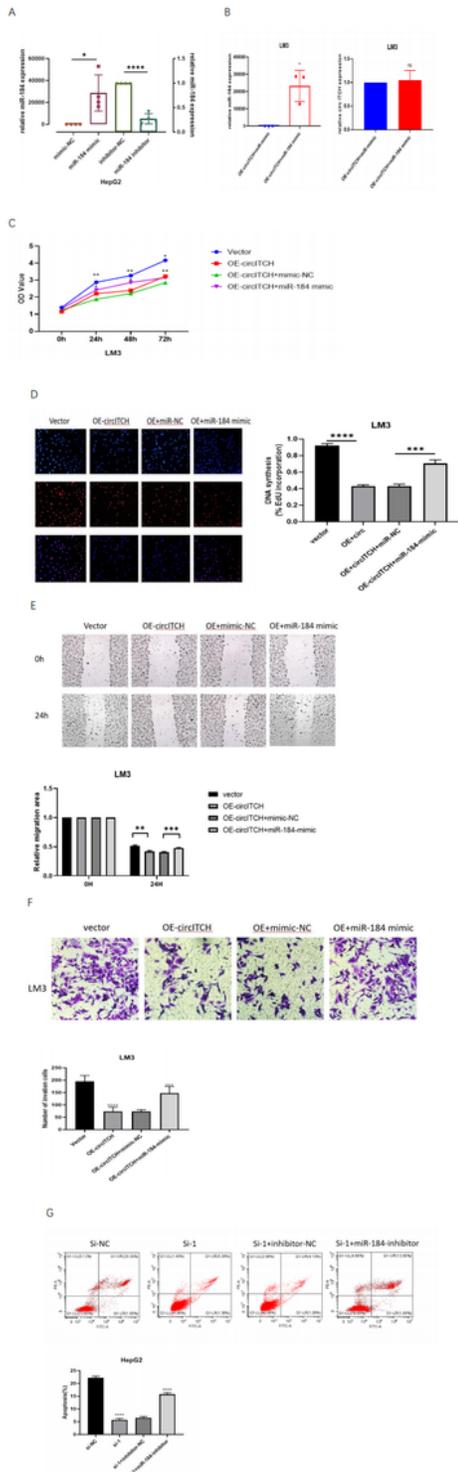


Figure 4

MiR-184 reverses the suppresses effect of circITCH in HCC. a. The expression of miR-184 in HepG2 cells transfected with miR-184 mimics, inhibitor and corresponding NC was detected by RT-qPCR. b. The expression of circITCH and miR-184 in HCCLM3 cells cotransfected with circITCH + miR-NC, or circITCH +miR-184 mimic was verified by qRT-PCR. c-f. HCCLM3 cells were divided into four groups (vector, OE-circITCH, OE-circITCH+mimic-NC, OE-circITCH+miR-184 mimic). The proliferation and invasion ability was analyzed through CCK-8, EdU, wound healing assays and transwell. The EdU and transwell samples were imaged at 200× magnification. Scale bar = 50 μm. The wound healing images taken at 100× magnification. Scale bar = 100 μm. g. Flow cytometry were conducted to examine apoptosis rates in HepG2 cells transfected with si-circITCH and miR-184 inhibitor. The data are presented as the mean± S.D. of at least three independent experiments. * P < 0.05, **P < 0.01, ***P < 0.001

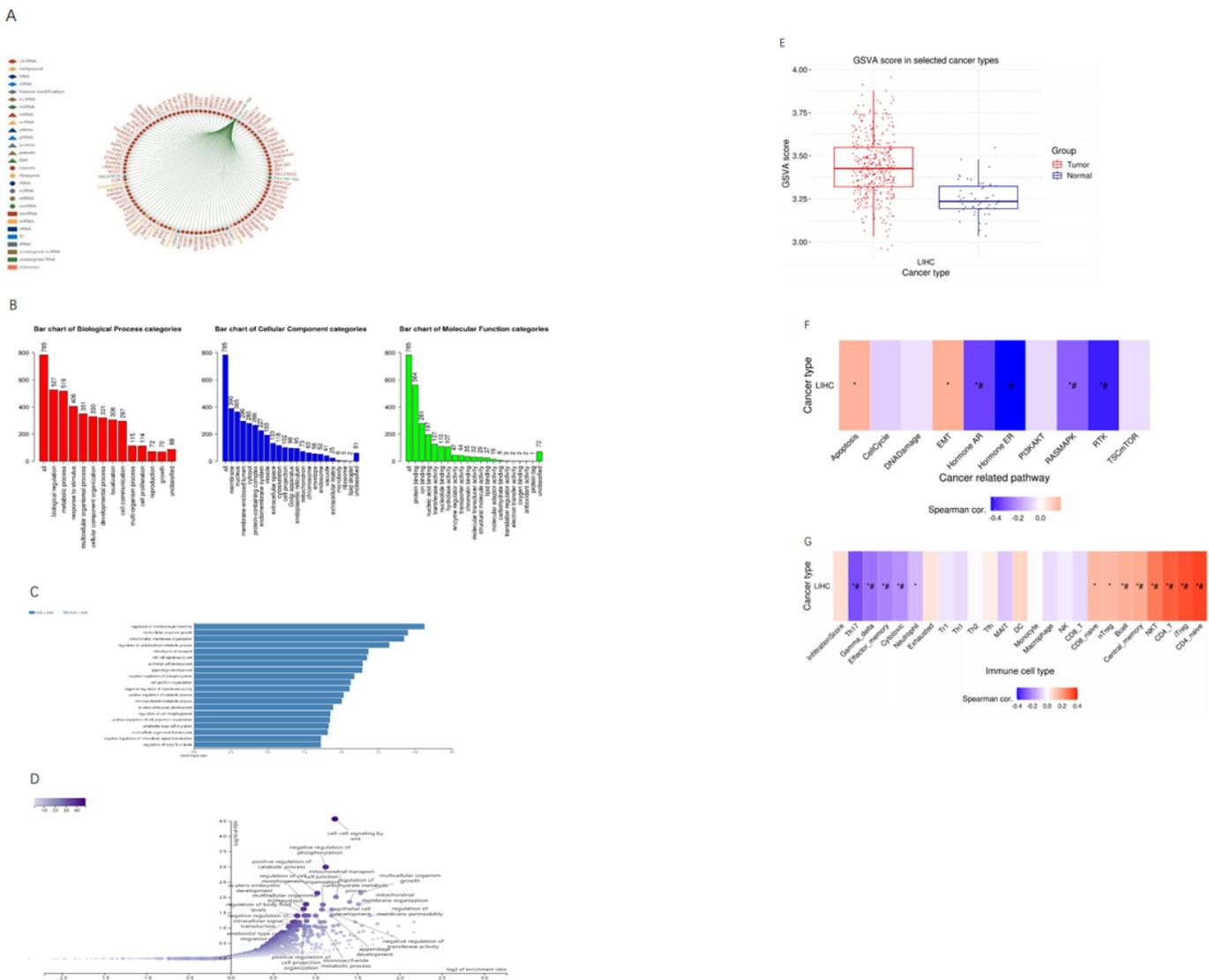


Figure 5

CeRNA regulatory networks and relative functions in HCC. a. The top 100 target genes bound to miR-184 were shown in the RNAInter database. b-d. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis show the related enrichment genes function in biological process and are involved in major functional pathways including bar charts and volcanic charts. e. GSEA (Gene Set Enrichment Analysis) analysis provides differential gene set activity analysis between HCC tumor and normal samples. f. The correlation between GSEA score and activity of cancer related pathways. g. The relationship between GSEA score and immune cells. *: P value < 0.05; #: FDR < 0.05

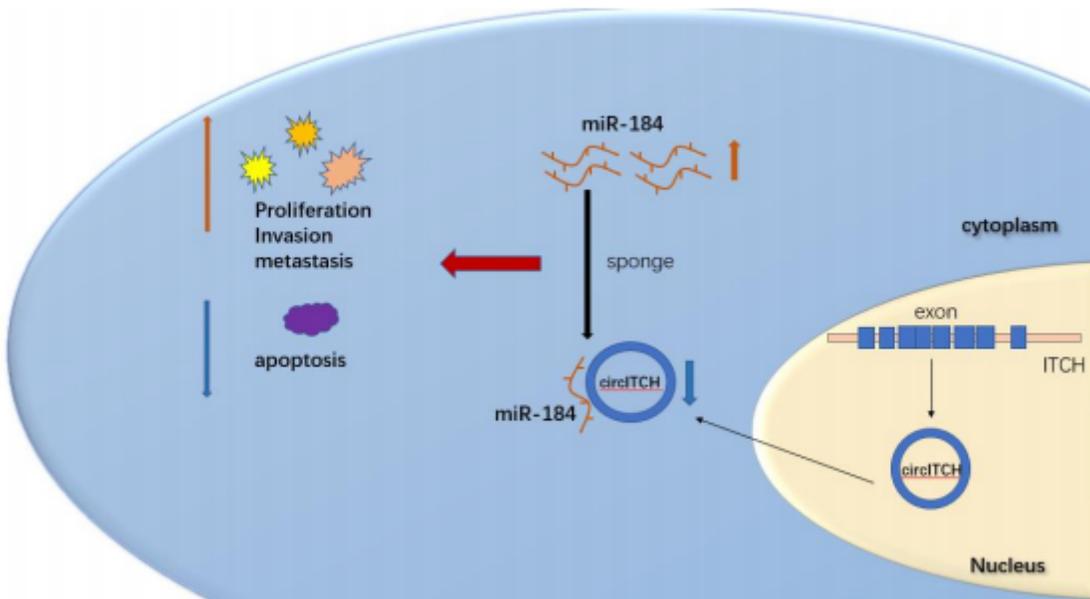


Figure 6

The schematic diagram illustrate the mechanism of circITCH in HCC.