



CircZKSCAN1 Suppresses Hepatocellular Carcinoma Tumorigenesis by Regulating miR-873-5p/Downregulation of Deleted in Liver Cancer 1

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Abstract

Background Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortality worldwide. CircZKSCAN1 (hsa_circ_0001727) was reported to be related to HCC development. The present study aims to elucidate the potential role and molecular mechanism of circZKSCAN1 in the regulation of HCC progression.

Methods CircZKSCAN1, miR-873-5p, and downregulation of deleted in liver cancer 1 (DLC1) in HCC tissues and cells were detected by RT-qPCR. Correlation between circZKSCAN1 expression and overall survival rate was measured by Kaplan–Meier survival analysis. The effects of circZKSCAN1, miR-873-5p, and DLC1 on proliferation, migration, and invasion were analyzed by CCK-8 and transwell assays, respectively. CyclinD1, Matrix metalloproteinase (MMP)-9, MMP-2, and DLC1 in HCC cells were detected by Western blot assay. The binding relationship between miR-873-5p and circZKSCAN1 or DLC1 was predicted by the Circinteractome or Starbase, and then confirmed by dual-luciferase reporter assays, respectively. Tumor volume and tumor weight were measured in vivo.

Results CircZKSCAN1 was downregulated in HCC tissues and cells. Kaplan–Meier survival analysis suggested that there was a positive correlation between circZKSCAN1 expression and overall survival rate. Functionally, circZKSCAN1 blocked proliferation, migration, and invasion of HCC cells. MiR-873-5p was a target miRNA of circZKSCAN1, and miR-873-5p directly bound with DLC1. Rescue experiments confirmed that miR-873-5p overexpression or DLC1 knockdown attenuated the suppressive effects of circZKSCAN1 on HCC tumor growth in vitro. Besides, circZKSCAN1 inhibited HCC cell growth in vivo.

Conclusions This study firstly revealed that circZKSCAN1 curbed HCC progression via modulating miR-873-5p/DLC1 axis, providing a potential therapeutic target for HCC treatment.

Keywords circZKSCAN1 · miR-873-5p · DLC1 · Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), a main subtype of primary liver cancer, is the third leading cause of cancer-associated mortality worldwide [1], affecting about 466,100 new cases

and 422,100 of HCC deaths in China every year [2]. Despite the substantial improvement in novel diagnostic approaches and therapeutic strategies, the cure rates and long-term survival of HCC patients remain unsatisfactory [3]. Therefore, it is imperative to explore the potential molecular mechanism of HCC tumorigenesis to figure out the novel therapeutic targets.

Circular RNAs (circRNAs), a new subgroup of noncoding RNAs, are characterized by a covalently closed continuous loop with neither 5' end caps nor 3' end poly (A) tail [4]. In recent years, circRNAs have been a hotpot research area for their function in regulating gene expression at the transcriptional or posttranscriptional level of mammalian cells [5]. Some studies have proved that circRNAs can exert the role as oncogenes or tumor suppressors in various cancers,

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including HCC [6, 7]. circZKSCAN1 (hsa_circ_0001727), an expression form of ZKSCAN1 gene, has been reported to be particularly abundant in the human hepatoma cell line Huh7 [8]. Furthermore, a previous study confirmed that circZKSCAN1 was downregulated and suppressed growth, migration, and invasion of HCC cells [9]. However, the molecular mechanism of circZKSCAN1 in the regulation of HCC progression is still unknown.

MicroRNAs (miRNAs) are a type of small noncoding RNAs that bind to the 3' UTR of mRNAs, resulting in mRNA degradation or inhibition of gene translation [10]. Increasing studies have reported that abnormally expressed miRNA played pivotal roles in tumor formation and progression of diverse tumors, including HCC [11, 12]. MiR-873-5p, a form of mature miR-873, has been identified to be a carcinogen in HCC. For instance, overexpression of miR-873 contributed to cell growth and metastasis of HCC by regulating the AKT/mTOR-mediated Warburg effect via targeting NDFIP1 [13]. Consistently, the upregulation of miR-873 facilitated the malignancy of HCC through promoting cell proliferation, migration, and invasion by enhancing TSLC1 expression [14]. Yet, the pathogenesis of miR-873-5p involved in HCC remains unclear.

Downregulation of deleted in liver cancer 1 (DLC1), encoded a GTPase-activating protein, served as a tumor suppressor gene in a variety of cancer, such as gastric cancer [15], breast cancer [16], and colorectal cancer [17]. A recent report demonstrated that DLC1 was frequently downregulated in HCC tissues, and upregulation of DLC1 could block proliferation, migration, and invasion of HCC cells [18]. All of these studies suggested that DLC1 was closely associated with HCC progression.

Therefore, in this paper, we aimed to expound that circZKSCAN1 hamper tumor growth of HCC via the miR-873-5p/DLC1 axis, providing a novel potential therapeutic strategy of HCC progression.

Materials and Methods

Clinical Specimens and Cell Culture

Human normal liver cell line (THLE-2) and HCC cell lines (SK-HEP-1, PLC/PRF-5, and Hep3B) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCC Huh-7 cells were purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). HCC LM3 cells were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). All cells were incubated in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C under an incubator with 5% CO₂.

HCC tumor tissues (n=60) and adjacent normal tissues were collected from HCC patients undergoing surgical treatment at the Second Affiliated Hospital of Wannan Medical College. Meanwhile, samples of serum from those HCC patients (n=60) and enrolled healthy volunteers (n=50) were collected from the Second Affiliated Hospital of Wannan Medical College. The comprehensive pathological and clinical data including gender, age, tumor size, distant metastasis, TNM stage, vascular invasion, histological differentiation, and serum AFP are summarized in Table 1. This research was approved by the Second Affiliated Hospital of Wannan Medical College ethics committee and informed consent was signed by each patient.

Cell Transfection

CircZKSCAN1 small interference RNA (si-circZKSCAN1), scrambled si-RNA control (si-NC), and DLC1 small interference RNA (si-DLC1) were purchased from Gen-eray Biotech Co., Ltd (Shanghai, China). CircZKSCAN1

Table 1 Relationship between circZKSCAN1 expression and the clinicopathologic feature of HCC patients

Clinicopathologic feature	n(60)	circZKSCAN1		p
		High expression (n=29)	Low expression (n=31)	
<i>Gender</i>				
Female	11	7	4	0.261
Male	49	22	27	
<i>Age (years)</i>				
≥55	24	11	13	0.752
<55	36	18	18	
<i>Tumor size</i>				
≥5 cm	32	12	20	0.073
<5 cm	28	17	11	
<i>Distant metastasis</i>				
Yes	26	8	18	0.017*
No	34	21	13	
<i>TNM stage</i>				
I–II	39	23	16	0.025*
III–IV	21	6	15	
<i>Vascular invasion</i>				
Yes	18	4	14	0.008*
No	42	25	17	
<i>Histological differentiation</i>				
Well and moderate	31	19	12	0.038*
Poor	29	10	19	
<i>Serum AFP (ng/mL)</i>				
≥20	43	18	25	0.111
<20	17	11	6	

*p < 0.05

overexpression plasmid (pcDNA3.1-circZKSCAN1 termed as circZKSCAN1), miR-873-5p mimic (miR-873-5p) and its negative control (miR-NC), and miR-873-5p inhibitor (anti-miR-873-5p) and its negative control (anti-NC) were obtained from GenePharma (Shanghai, China). Thereafter, SK-HEP-1 and Huh-7 cells were transfected with all these oligonucleotides and plasmids using Lipofectamine 2000 reagent (Invitrogen) following the operation manual.

RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from HCC tissues, cells, and serums using TRIzol reagent (Invitrogen) based on the supplier's direction. RNA reverse transcription assay was implemented to synthesize cDNA first strand with specific primers in accordance with the instructions of M-MLV reverse transcriptase reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA). The relative expression levels of circZKSCAN1 and DLC1 were detected using the GoTaq[®] qPCR Master Mix kit (Promega, Madison, WI, USA), and the quantitative analysis of miR-873-5p was measured using a miRNA-specific TaqMan MicroRNA Assays kit (Applied Biosystems, Foster City, CA, USA). GAPDH or U6 snRNA was used as the internal reference to normalize the expression of circZKSCAN1 and DLC1 or miR-873-5p, respectively. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

The primer sequences for this study were shown as follows:

circZKSCAN1: divergent primers: 5'-AGTCCCACTCAAACATTCGTCT-3' (sense), 5'-CACCTTCACTATTACGATACCATCC-3' (antisense); convergent primers: 5'-TACCGCCCCGATAGTGGAGA-3' (sense), 5'-TGAAGTGGGACTGGGTGGC-3' (antisense)
 GAPDH: 5'-TCCGGAAACCAGATCTCCCA-3' (sense), 5'-ACGTGAGGGTATGAAGGGGC-3' (antisense);
 DLC1: 5'-GCGTACCTGTGTCGCTTTAT-3' (sense), 5'-CTCCTCTGTGCAAACCTTTCT-3' (antisense);
 U6: 5'-CTCGCTTCGGCAGCACA-3' (sense), 5'-AACGCTTACGAATTTGCGT-3' (antisense).

Cell Counting Kit-8 (CCK-8) Assay

SK-HEP-1 and Huh-7 cells (2×10^3) were seeded in a 96-well plate, followed by incubation for 24 h at 37 °C with 5% CO₂. Afterward, each well was added with 10 μL CCK-8 solution (Beyotime Institute of Biotechnology, Shanghai, China). At 2 h after incubation, the absorbance at 450 nm was detected at various time points (0 h, 24 h, 48 h, and 72 h) with a microplate reader (Elx800, BioTek Inc, North Brunswick, NJ, USA).

Cell Migration and Invasion Assay

The migratory and invasive abilities of HCC cells were appraised with transwell chamber (Corning Costar, New York, NY, USA) according to the manufacturer's instructions. Briefly, 2×10^5 cells were plated in the upper chamber with non-coated (for migration) or pre-coated (for invasion) Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA) for migration and invasion evaluation, respectively. Next, cells in the upper chamber were resuspended in serum-free medium, and the medium supplemented with 10% FBS (as a chemoattractant) was added into the lower chamber. After incubation for 24 h at 37 °C, cells in the upper chamber were discarded with cotton swabs, while cells on the lower surface were fixed and stained. Finally, the number of migrated or invaded cells was counted and photographed under an optical microscope (Nikon, Tokyo, Japan).

Western Blot Assay

Total protein was lysed from cultured cells with RIPA buffer (Sigma Aldrich, St Louis, MO, USA). The quantization of proteins was determined by BCA kit (Thermo Fisher Scientific). Extracted proteins were separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Then the membranes were sealed with 5% skim milk (dissolved in TBST buffer) for 2 h at room temperature and then probed with primary antibodies against DLC1 (1:500, sc-32931, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CyclinD1 (1:1000, ab134175, Abcam, Cambridge, UK), Matrix metalloproteinase-9 (MMP-9, 1:1000, ab73734, Abcam), MMP-2 (1:1000, ab37150, Abcam), and GAPDH (1:1000, ab8227, Abcam) overnight at 4 °C. Whereafter, membranes were rinsed with TBST and incubated with horseradish peroxidase (HRP)-linked secondary antibody (1:2000, CWBIO, Beijing, China) for 2 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence reagent (GE Healthcare UK Ltd, Little Chalfont, UK). GAPDH was seen as an internal control to normalize DLC1 expression.

Dual-Luciferase Reporter Assay

The sequences of circZKSCAN1 and DLC1 were amplified by PCR. Mutant-type circZKSCAN1 without miR-873-5p binding sites and mutant DLC1 3'-UTR fragments were acquired by overlap extension PCR with mutant primers. Afterward, these sequences were subcloned into the luciferase reporter pISCHECK-2 vector (Promega), generating circZKSCAN1 WT, circZKSCAN1 MUT, DLC1 WT, and DLC1 MUT reporter plasmids. The constructed reporter

plasmids were co-transfected with miR-NC or miR-873-5p mimic into SK-HEP-1 and Huh-7 cells. Luciferase activities were detected with a dual-luciferase reporter assay kit (Promega) at 48 h post-transfection.

Tumor Xenograft Formation Assay in Nude Mice

For in vivo experiments, 4-week-old male BALB/C nude mice (National Laboratory Animal Center, Beijing, China) were kept in a normal environment free from a pathogen (SPF). The nude mice were randomly divided into two groups ($n = 5$ each group). SK-HEP-1 cells stably transfected with circZKSCAN1 or vector were chosen through G418 (Invitrogen). Then, 5×10^6 cells were subcutaneously injected into the left flank of the mice. Tumor volumes were measured every week. Four weeks later, mice were killed, and the tumors were carefully excised, weighed, and preserved at -80°C for the subsequent analyses. All animal studies were approved by the Animal Research Committee of the Second Affiliated Hospital of Wannan Medical College.

Statistical Analysis

All data are presented as the mean \pm standard deviation (SD) and analyzed using SPSS version 17.0 software (IBM, Chicago, IL, USA). Each assay was repeated independently at least three times. The overall survival curve of HCC patients was plotted by the Kaplan–Meier method and long-rank test. Pearson correlation analysis was performed with GraphPad Prism 6 software using linear regression. The differences were evaluated using Student’s t test or one-way ANOVA. $p < 0.05$ was considered significant.

Results

CircZKSCAN1 Was Downregulated in HCC Tissues and Cells

To investigate the function of circZKSCAN1 in HCC, its expression pattern was firstly detected by RT-qPCR assay. As displayed in Fig. 1a, circZKSCAN1 expression was obviously downregulated in 60 HCC tumor tissues relative to adjacent normal tissues. Moreover, to further prove the clinical significance of circZKSCAN1 in HCC, the patients were divided into two groups, with the median circZKSCAN1 expression level serving as the cutoff. Data exhibited that low circZKSCAN1 expression was significantly associated with distant metastasis ($p = 0.017$), TNM stage ($p = 0.025$), vascular invasion ($p = 0.008$), and histological differentiation ($p = 0.038$) (Table 1), implying that circZKSCAN1 might influence the clinical prognosis of HCC patients. Then, Kaplan–Meier curve analysis suggested that higher expression of circZKSCAN1 in HCC patients was related to lower survival rate (Fig. 1b). Furthermore, we further confirmed that circZKSCAN1 was lower expressed in HCC cell lines (SK-HEP-1, Huh-7, PLC/PRF-5, LM3, and Hep3B) than that in normal human liver cell line THLE-2 (Fig. 1c). Also, we found that circZKSCAN1 was decreased in serum from HCC patients compared with non-cancerous donors (Figure S1A). Then, receiver operating characteristic (ROC) curve was further utilized to assess the diagnostic value of circZKSCAN1. According to the ROC curve analysis, the area under the curve (AUC) and 95% confidence interval (CI) of circZKSCAN1 were 0.813 and 0.733–0.894 (Figure S1B), respectively, suggesting the underlying diagnostic significance of circZKSCAN1 in HCC. These results suggested the involvement of circZKSCAN1 in HCC progression, working as a prognostic and diagnostic biomarker.

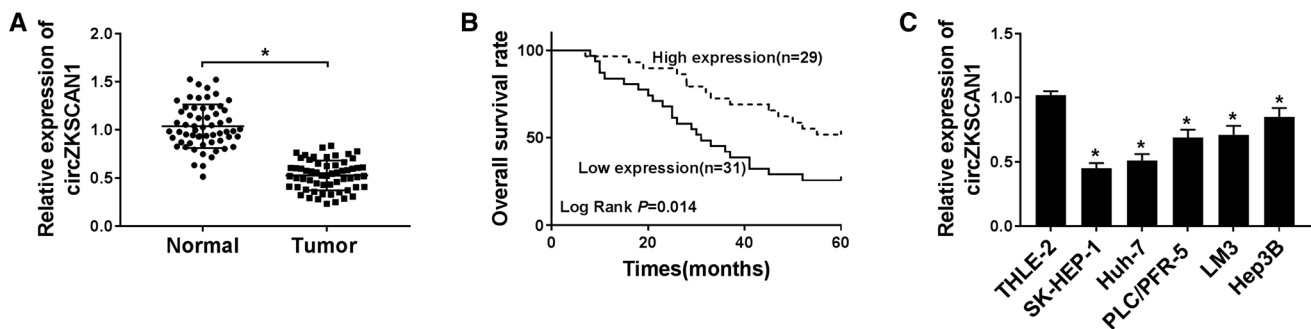


Fig. 1 CircZKSCAN1 is downregulated in HCC tissues and cells. **a** The expression of circZKSCAN1 in 60 pairs of HCC tissues and adjacent non-tumor samples is examined via RT-qPCR. **b** Relationship between the expression of circZKSCAN1 and overall survival in

HCC samples is determined. **c** Expression level of circZKSCAN1 in HCC cell lines (SK-HEP-1, Huh-7, PLC/PRF-5, LM3, and Hep3B) and normal human liver cell line (THLE-2) is detected by RT-qPCR. * $p < 0.05$

CircZKSCAN1 Repressed Proliferation, Migration, and Invasion of HCC Cells

Next, to ascertain the role of circZKSCAN1 in HCC cells, the overexpression vector of circZKSCAN1 was constructed. As presented in Fig. 2a, circZKSCAN1 level was markedly upregulated in SK-HEP-1 and Huh-7 cells transfected with pcDNA3.1-circZKSCAN1 in contrast to cells transfected with empty vector. Therefore, we used the overexpression systems to further explore the effect of circZKSCAN1 on proliferation, migration, and invasion of HCC cells. CCK-8 and transwell assays showed that overexpression of circZKSCAN1 remarkably weakened the proliferation (Fig. 2b, c), migration (Fig. 2d), and invasion (Fig. 2e) of SK-HEP-1 and Huh-7 cells. Moreover, decreased expression level of CyclinD1 (proliferation-related protein), MMP-9, and MMP-2 (migration- and invasion-associated proteins) were observed due to overexpression of circZKSCAN1 (Fig. 2f,

g), further confirming that the inhibitory effect of circZKSCAN1 on proliferation, migration, and invasion in HCC cells. All these results proved that circZKSCAN1 could repress tumorigenesis of HCC.

MiR-873-5p Is the Target of circZKSCAN1 in HCC Cells

In recent years, some studies have revealed that circular RNA could exert the role by interacting with miRNAs [4, 19]. Hence, we used the Circinteractome database (<https://circinteractome.nia.nih.gov>) to probe the underlying target miRNAs of circZKSCAN1. As exhibited in Fig. 3a, miR-873-5p was predicted to contain binding sites of circZKSCAN1. To further demonstrate the potential interaction of miR-873-5p with circZKSCAN1, circZKSCAN1 WT or circZKSCAN1 MUT reporter vector was firstly co-transfected with miR-NC or miR-873-5p mimic in SK-HEP-1 and Huh-7 cells. Afterward, the luciferase activity was measured

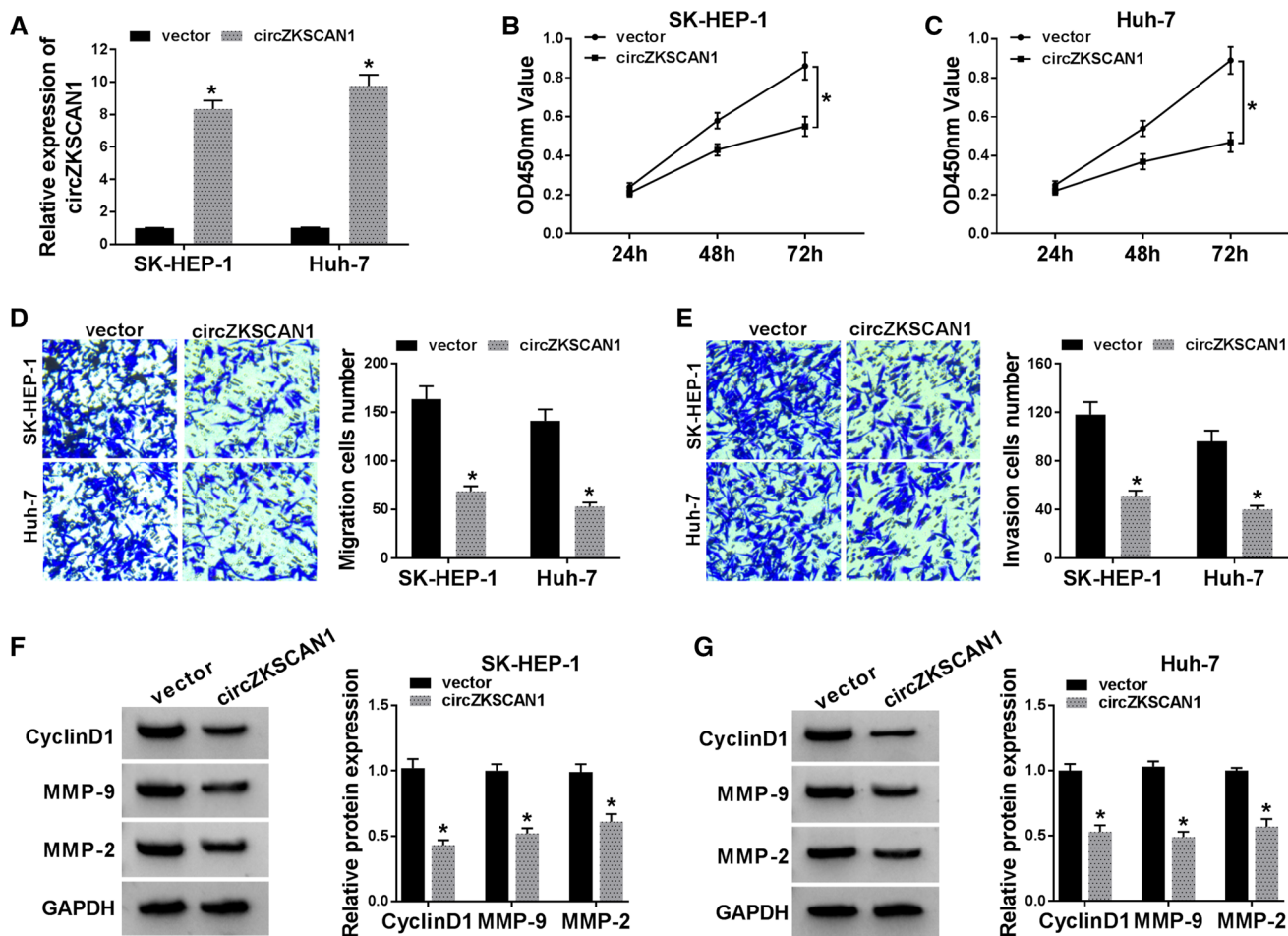


Fig. 2 CircZKSCAN1 inhibited proliferation, migration, and invasion of HCC cells. **a** Transfection efficiency of pcDNA3.1-circZKSCAN1 in SK-HEP-1 and Huh-7 cells is determined by RT-qPCR assay. **b–e** Proliferation (**b**, **c**), migration (**d**), and invasion (**e**) analysis in pcDNA3.1-circZKSCAN1-transfected SK-HEP-1 and Huh-7 cells are conducted. (**f**, **g**) The protein levels of CyclinD1, MMP-9, and MMP-2 in pcDNA3.1-circZKSCAN1-transfected SK-HEP-1 and Huh-7 cells are detected by Western blot assay. * $p < 0.05$

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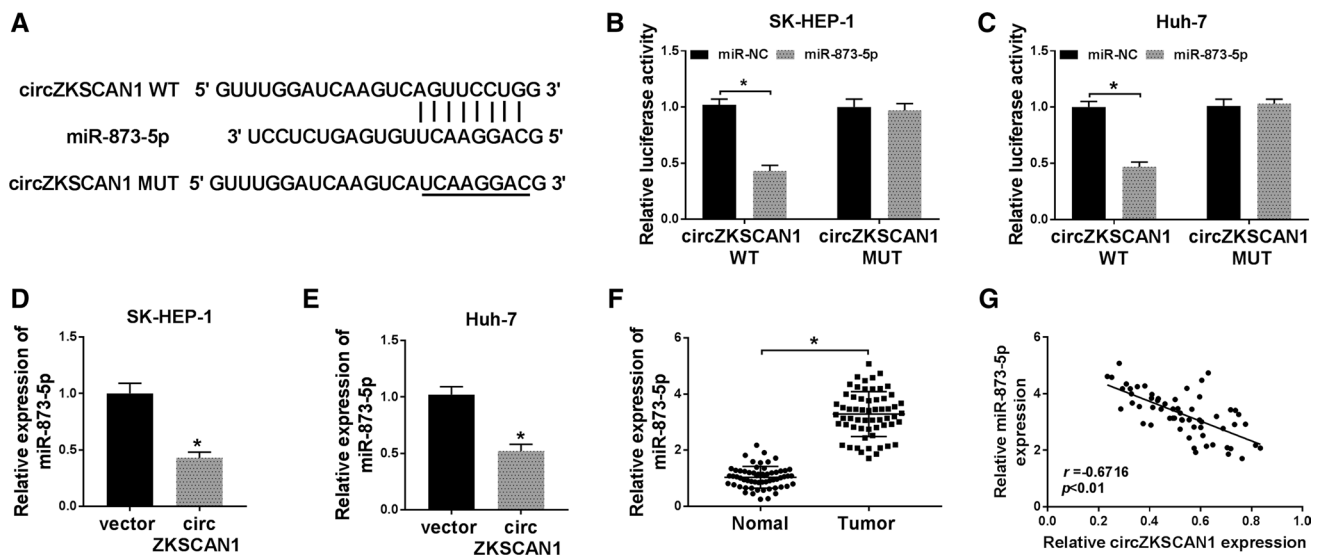


Fig. 3 MiR-873-5p is the target of circZKSCAN1 in HCC cells. **a** The predicted binding sequences between miR-873-5p and circZKSCAN1 as well as miR-873-5p and the mutants of circZKSCAN1 are shown. **b, c** SK-HEP-1 and Huh-7 cells are co-transfected with luciferase reporter plasmids, including circZKSCAN1 WT or circZKSCAN1 MUT and miR-NC or miR-873-5p mimic. Luciferase activity

is measured by the dual-luciferase assay. **d, e** miR-873-5p expression is detected by RT-qPCR in SK-HEP-1 and Huh-7 cells transfected with pcDNA3.1-circZKSCAN1. **f** MiR-873-5p expression is assessed by RT-qPCR in HCC tissues. **g** The correlation between expression of circZKSCAN1 and miR-873-5p is analyzed. * $p < 0.05$

at 48 h post-transfection using the dual-luciferase assay. As shown in Fig. 3b, c, miR-873-5p decreased the luciferase activity of circZKSCAN1 WT reporter, but had no great effect on the luciferase activity of circZKSCAN1 MUT. Next, we detected the effect of pcDNA3.1-circZKSCAN1-transfected or si-circZKSCAN1-introduced on the expression level of miR-873-5p in SK-HEP-1 and Huh-7 cells. RT-qPCR results disclosed that circZKSCAN1 overexpression abated the expression level of miR-873-5p in SK-HEP-1 and Huh-7 cells (Fig. 3d, e). Besides, we validated that miR-873-5p expression was upregulated in HCC cells in comparison with adjacent non-tumor samples (Fig. 3f). Taken together, these results proved that circZKSCAN1 interacted with miR-873-5p to inhibit its expression.

DLC1 Acted as the Target of miR-873-5p in HCC Cells

It is widely accepted that miRNAs could exert their functions via modulating the target gene expression. By using the online software program Starbase, we found that there existed some complementary bases pairing between miR-873-5p and DLC1 3'UTR (Fig. 4a). Subsequently, luciferase reporter assay was implemented to further prove the bioinformatics prediction. As shown in Fig. 4b, c, miR-873-5p mimic reduced the luciferase activity of DLC1 WT reporter vector, but not that of DLC1 MUT reporter vector, suggesting the interaction between miR-873-5p and DLC1. Simultaneously, transfection efficiency of miR-873-5p mimic or anti-miR-873-5p was examined and is illustrated in Fig. 4d,

e. Moreover, we viewed that DLC1 level was downregulated in SK-HEP-1 and Huh-7 cells compared with THLE-2 cell (Fig. 4f). And the level of DLC1 was negatively correlated with miR-873-5p level in SK-HEP-1 and Huh-7 cells (Fig. 4g, h). In parallel, DLC1 was expressed at the low level and inversely associated with the miR-873-5p expression in HCC tissues (Fig. 4i, j). All these data revealed that miR-873-5p directly bound with DLC1 in HCC.

Validation of circZKSCAN1/miR-873-5p/DLC1 Regulatory Axis in HCC Cells

Based on the above findings, we inferred that the circZKSCAN1/miR-873-5p/DLC1 regulatory axis may influence tumor growth of HCC. In order to confirm the hypothesis, we further carried out rescue assays in SK-HEP-1 and Huh-7 cells. As exhibited in Fig. 5a, b, circZKSCAN1 promoted DLC1 protein expression of HCC cells, which was counteracted by miR-873-5p overexpression or DLC1 knockdown. Functionally, the suppression of cell proliferation due to circZKSCAN1 upregulation was overturned by miR-873-5p mimic or DLC1 inhibitor in SK-HEP-1 and Huh-7 cells (Fig. 5c, d). Similarly, miR-873-5p overexpression or DLC1 silencing partly abrogated the negative effect of circZKSCAN1 upregulation on migration and invasion in SK-HEP-1 and Huh-7 cells (Fig. 5e, f). Furthermore, Western blot results suggested that the re-introduction of miR-873-5p or si-DLC1 obviously abolished pcDNA3.1-circZKSCAN1-triggered reduction in the protein levels of

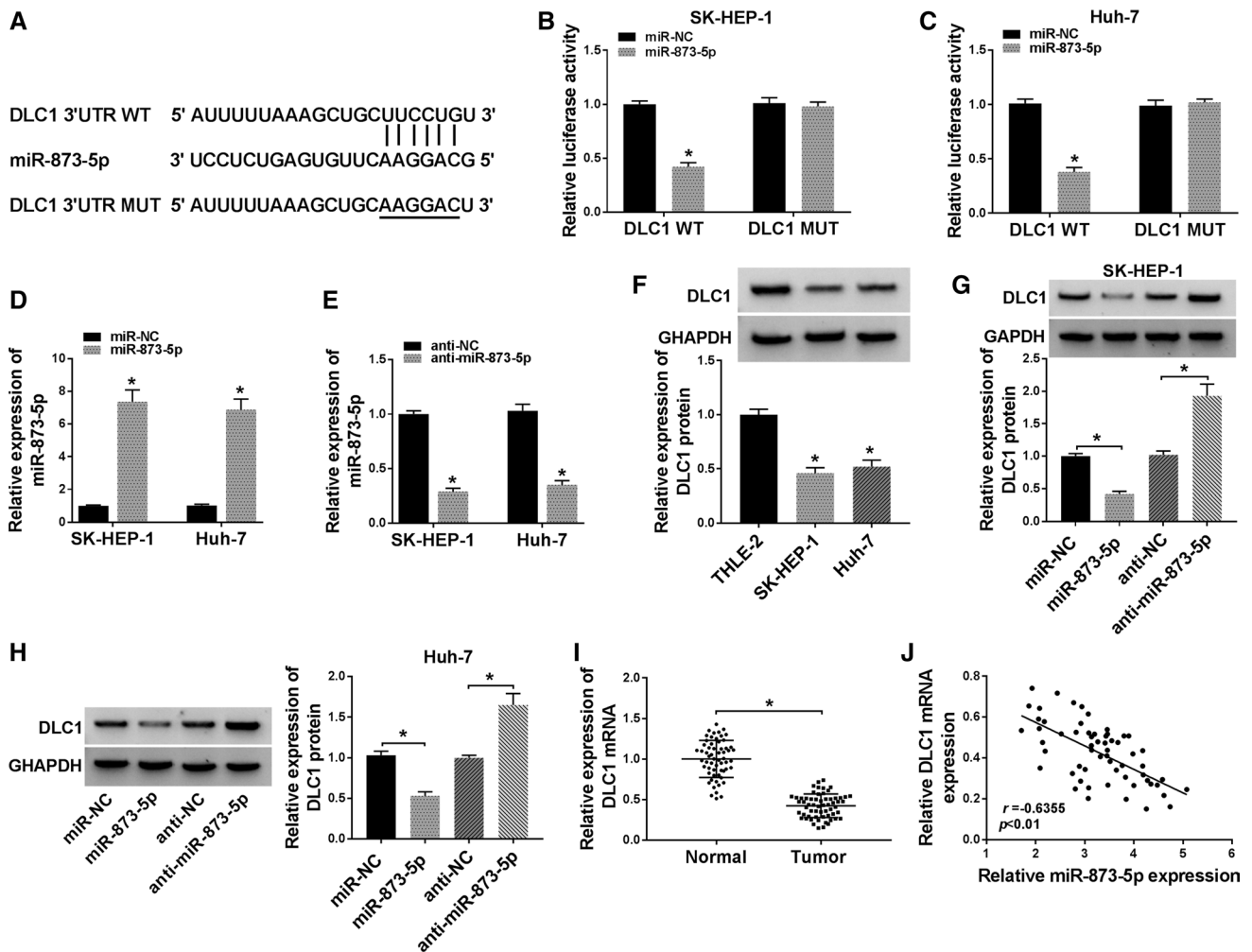


Fig. 4 CircZKSCAN1 regulated DLC1 expression via targeting miR-873-5p in HCC cells. **a** The putative binding sites between miR-873-5p and DLC1 as well as the sequences of DLC1 MUT are exhibited. **b, c** The interaction among circZKSCAN1, miR-873-5p, and DLC1 is confirmed by luciferase activity analysis. **d, e** The expression level of miR-873-5p is measured by RT-qPCR assay in SK-HEP-1 and Huh-7 cells transfected with miR-NC, miR-873-5p, anti-NC, and anti-miR-873-5p. **f** DLC1 protein level is tested by Western

blot assay in THLE-2, SK-HEP-1, and Huh-7 cells. **g, h** The protein level of DLC1 is assessed in SK-HEP-1 and Huh-7 cells transfected with miR-NC, miR-873-5p, anti-NC, and anti-miR-873-5p. **i** The expression level of DLC1 is measured by RT-qPCR in 60 pairs of HCC tissues and adjacent non-tumor samples. **j** Pearson correlation analysis is performed to assess the expression association between DLC1 and miR-873-5p in HCC tissues. * $p < 0.05$

CyclinD1, MMP-9, and MMP-2 in SK-HEP-1 and Huh-7 cells (Fig. 5g, h). In a word, all mentioned results implicated that circZKSCAN1 impeded HCC growth by regulating the miR-873-5p/DLC1 axis in vitro.

CircZKSCAN1 Hindered HCC Cell Growth In Vivo

Additionally, a xenograft tumor mouse model was established to further verify whether circZKSCAN1 could impact the growth of the tumor in vivo. As exhibited in Fig. 6a, b, xenograft formation assay disclosed that high expression of circZKSCAN1 hindered tumor size and weight, indicating that stable upregulation of circZKSCAN1 substantially repressed tumor growth of HCC in vivo. Moreover,

xenograft study also confirmed that circZKSCAN1 and DLC1 were highly expressed in tumors formed from mice injected with cells transfected with circZKSCAN1 (Fig. 6c, e), while miR-873-5p was lower expressed (Fig. 6d). All of these demonstrated that circZKSCAN1 curbed HCC tumor growth through regulating the miR-873-5p/DLC1 axis in vivo.

Discussion

With rapid development in RNA sequencing technologies, the biological role and molecular mechanism of circRNAs are gradually confirmed [20–22]. Increasing evidence

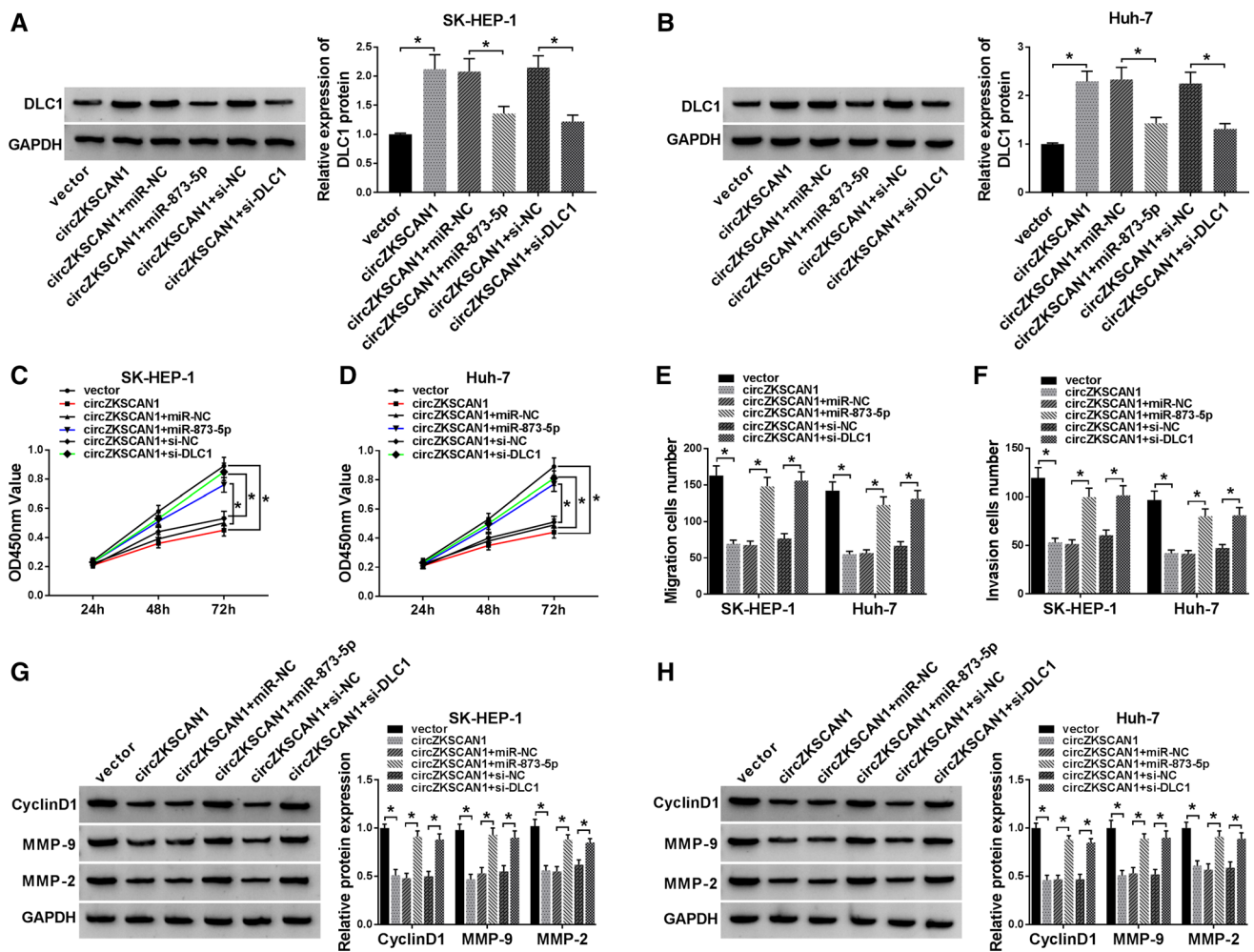


Fig. 5 Regulation of circZKSCAN1 on cell proliferation, migration, and invasion is mediated by miR-873-5p/DLC1 axis. **a, b** DLC1 protein level is detected in SK-HEP-1 and Huh-7 cells transfected with vector, circZKSCAN1, circZKSCAN1 + miR-NC, circZKSCAN1 + miR-873-5p, circZKSCAN1 + si-NC, and circZKSCAN1 + si-DLC1.

c, d Cell proliferation is measured in transfected SK-HEP-1 and Huh-7 cells. **e, f** Migration and invasion are examined in transfected SK-HEP-1 and Huh-7 cells. **g, h** Protein levels of CyclinD1, MMP-9, and MMP-2 are tested in transfected SK-HEP-1 and Huh-7 cells. * $p < 0.05$

indicates that circRNAs are potential molecular markers and attractive therapeutic targets in multiform types of tumors, including HCC [23, 24]. CircZKSCAN1 has been reported to be downregulated and circZKSCAN1 exerts its tumor inhibition effect by several cancer-related signaling pathways in HCC [9]. Nevertheless, the precise function and pathomechanism of circZKSCAN1 are still obscure.

In this study, circZKSCAN1 was identified as the low expression in HCC tissues and cell lines with respect to their corresponding controls. Previous studies have exhibited that there existed a correlation between circZKSCAN1 expression and the prognosis of cancer patients [9, 25]. Analogously, ROC curve analysis presented that circZKSCAN1 has certain diagnostic value for HCC, indicating that circZKSCAN1 could be used as a non-interventional tumor biomarker for HCC. Functionally, circZKSCAN1 retarded

proliferation, migration, and invasion of HCC cells. These data confirmed that circZKSCAN1 exerted the suppressor factors in the development of HCC, in agreement with former work [9].

Recently, some documents have stated that circRNAs can exert the function through the interaction with miRNAs. In the paper, bioinformatics analysis results and dual-luciferase reporter assay first proved that circZKSCAN1 is directly bound with miR-873-5p in HCC cells. Meanwhile, circZKSCAN1 level was negatively correlated with miR-873-5p in HCC tissues and cells. Interestingly, miR-873-5p as a tumor suppressor has been reported in various cancers, such as thyroid cancer, colorectal cancer, and endometrial cancer [26–28], but our results confirmed that miR-873-5p was the high expression in HCC, and receded the anticancer effect of circZKSCAN1 of HCC

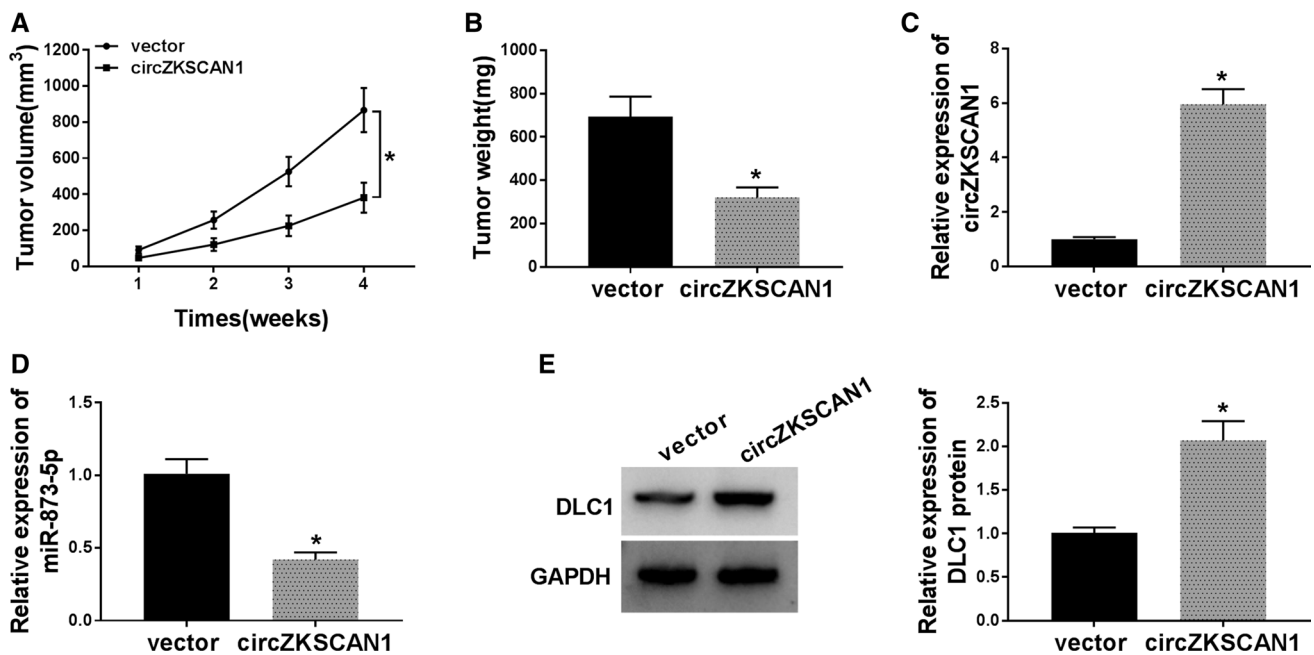


Fig. 6 CircZKSCAN1 inhibited HCC cell growth in vivo. **a, b** Tumor volume and weight are detected. **c, d** The expression levels of circZKSCAN1 and miR-873-5p in xenografts are measured by RT-qPCR. **e, f** DLC1 protein level in xenografts is detected by Western blot. * $p < 0.05$

cells. Our finding is in accordance with prior studies suggested that miRNAs, depending on which gene or pathway they regulate [29, 30], functioned as either oncogenes or tumor suppressors in diverse cancers.

Up to date, it has been widely recognized that circRNAs performed as ceRNAs or miRNA sponges to affect mRNA expression [31, 32]. In the manuscript, our research proved that DLC1 was a direct target of miR-873-5p, and miR-873-5p mitigated the promotion of circZKSCAN1 on DLC1 protein expression in HCC. Mechanistic analysis discovered that miR-873-5p overexpression or DLC1 knockdown abated the suppression effect of circZKSCAN1 on HCC tumor growth. In agreement with our results, the deficiency of DLC1 could accelerate HCC development [18, 33]. Based on the above findings, it is concluded that circZKSCAN1 functioned as a sponge of miR-873-5p to elevate DLC1 expression, thereby curbing the cell growth of HCC in vitro. In addition, we further confirmed that circZKSCAN1 could exert the same function in vivo. As anticipated, the size and weight of tumor decreased in the presence of circZKSCAN1 overexpression, suggesting that circZKSCAN1 impeded HCC tumor growth in vivo. Moreover, the expression levels of circZKSCAN1 and DLC1 were increased in tumors from mice injected with circZKSCAN1 transfected cells, and miR-873-5p was downregulated in xenografts. That is to say, circZKSCAN1 hampered HCC cell growth through the miR-873-5p/DLC1 axis in vivo.

Conclusions

Herein, our results discovered that circZKSCAN1 was decreased in HCC tissues and cells, and repressed proliferation, migration, and invasion of HCC cells. Moreover, bioinformatics analysis presented that circZKSCAN1 contained some complementary bases pairing with miR-873-5p. This study firstly revealed that circZKSCAN1 impeded HCC progression via modulating the miR-873-5p/DLC1 axis. Our finding elucidated a novel potential therapeutic target for the treatment of HCC.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interests.

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