Study on the Mechanism of CircDUSP16 Regulating the Proliferation and Apoptosis of Hepatocarcinoma Cells

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Research Article

Keywords: circDUSP16, hepatocarcinoma cells, miR-136-5p, Hippo/YAP signaling pathway

Posted Date: December 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1160989/v1>

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Abstract

Purpose: This study aims to explore the expression of circDUSP16 in liver cancer and its effect on the proliferation and apoptosis of liver cancer cells.

Methods: Real-timePCR was used to measure the expression of circDUSP16, miR-136-5p, and YAP1 in HCC tissues and cells. MTT, colony analysis, and apoptosis analysis were performed to determine the progress of HCC cells. The relationship between circDUSP16, miR-136-5p, and YAP1 was verified by using the luciferase gene experiment.

Results: The expression level of circDUSP16 in HCC tissue samples was significantly increased and can be used as an independent prognostic factor for the survival of HCC patients. Inhibition of circDUSP16 can inhibit HCC cell viability, colony formation, and invasion potential. Furthermore, inhibition of circDUSP16 can regulate the expression of YAP1 in the Hippo/YAP signaling pathway in HCC by targeting miR-136-5p, thereby affecting cell proliferation and apoptosis, and participating in the progression of HCC disease.

Conclusion: The ectopic expression of circDUSP16 can regulate the expression of YAP1 by competitively binding to miR-136-5p, therefore participating in the progression of HCC, which can provide a new therapeutic target for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor in the world and the third leading cause of cancer-related mortality, which seriously threatens people's health and lives [1–2]. Although there are a variety of treatments that can be used to treat HCC, such as surgical resection or targeted therapy, HCC is usually diagnosed as advanced and has a poor prognosis due to its tumor invasion. Recurrence and metastasis after surgical resection are often related to poor prognosis [3]. Therefore, it is essential to find new therapeutic targets for HCC [4].

A large amount of evidence has shown that the expression of non-coding RNAs (ncRNAs) is involved in the progression of HCC disease [5]. Circular RNAs (circRNAs) is a new subtype of ncRNA. It has a covalently closed loop structure and more conserved. Its reverse splicing site is located between the 5'-3'end and is more resistant to RNase R than the corresponding linear RNAs [6]. circRNA plays a key role in a variety of molecular mechanisms, including tumor biomarkers, regulating gene expression, and making miRNAs spongy [7–9]. As another subgroup of small ncRNA, microRNA (miRNA) negatively regulates its target genes and plays as an oncogene or tumor suppressor gene in HCC. Previous studies have shown that hsa_circ_0009910, hsa_circ_0049783, and hsa_circ_0089172 were up-regulated in HCC tissue and plasma, and their increased expression was associated with poor prognosis of HCC patients [10]. Another recent study also confirmed that hsa_circ_0003855 (circDUSP16) can stimulate miR-145-5p to promote the occurrence and invasion of gastric cancer [11], but its role in HCC has not been explored. Besides,

miR-136-5p has been reported to be down-regulated in HCC. As an anti-cancer miRNA, it affects the progression of liver cancer by regulating various pathways.

The overexpression of the yes-associated protein 1 (YAP1) is implicated in HCC vascular infiltration, cell differentiation, tumor size, and TNM tumor stage, and maybe a risk factor for HCC formation [12]. YAP1 is highly expressed in human HCC, which can promote the development and progress of HCC by up-regulating Jagged1 and activating the Notch pathway [13]. By using the Circular RNA Interactome and TargetsCan online software it was predicted that miR-136-5p has both circDUSP16 and YAP1 binding sites. Furthermore, previous studies have confirmed that circDUSP16 was significantly highly expressed in HCC tumor tissues, and its expression was positively correlated with YAP1. The dual-luciferase gene experiment also confirmed that miR-136-5p can interact with circDUSP16 and YAP1. Therefore, this study aims to explore the expression characteristics of circDUSP16 in liver cancer and explore its potential relationship with miR-136-5p and YAP1, in order to provide mechanism basis for investigating new therapeutic targets for HCC.

1 Material And Methods

1.1 Sample source

1.1.1 Clinical samples

Thirty pairs of HCC patient tumors and adjacent tissues were obtained from Hangzhou Fuyang Hospital of Traditional Chinese Medicine. All patients received adjuvant therapy before surgery, including chemotherapy or radiation therapy. All patients were provided with the informed consent, and the study was approved by the ethics committee of Hangzhou Fuyang Hospital of Traditional Chinese Medicine.

1.1.2 Cell lines

The LO2 cell line (human immortalized normal liver cell line) and human HCC cell line (MHCC97, Huh7, SK-Hep1, Hep3B, and HepG2) were purchased from the Chinese Academy of Sciences.

1.2 Experimental method

1.2.1 Cell culture

The cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific) containing 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific) and were kept at 37°C in a humid atmosphere with 5% CO₂.

1.2.2 Cell transfection

Lentivirus-mediated sh-circDUSP16 (target sequence: ACCTGCTTGCAGGCTTTCAGT), plasmid-mediated circDUSP16, miR-136-5p mimic, miR mimic control, miR-136-5p inhibitor, and miR-136-5p inhibitor control were purchased from Genomeditech (Shanghai, China). According to the manufacturer's instructions, before transfection, HCC cells were seeded in a 6-well plate for 24 h. Lipofectamine 2000 reagent (Invitrogen, USA) was then used for the cell transfection.

1.2.3 Real-time quantitative PCR (qRT-PCR) analysis

According to the manufacturer's instructions, the RNAiso Plus kit (Takara) was used to extract total RNA from HCC cells, and the RNAiso kit (Takara) was used to extract miRNA. qRT-PCR was used to evaluate the expression levels of circDUSP16, miR-136-5p, and YAP1 in HCC tissue samples and cell lines. Specifically, the PrimeScript RT Reagent kit (Takara) was used to synthesize complementary DNA (cDNA) by reverse transcription of total RNA. Mix-X miRNA First-Strand Synthesis Kit (Takara) was used to synthesize the cDNA for miRNA. SYBR Premix Ex Tap (Takara) was used to carry out the qPCR and amplification was performed in LightCycler 480II (Roche, Switzerland). GAPDH was used as an internal control and the relative expression level of genes was calculated using the $2^{-\Delta\Delta C_t}$ method.

1.2.4 Analysis of cell viability, colony formation, and apoptosis

According to the manufacturer's instructions, MTT was used to measure the cell viability at 0, 24, 48, and 72 h. A spectrophotometer was used to evaluate the spectrophotometric absorbance of each sample at 450 nm. To evaluate the formation of colonies, low-density cells were inoculated into Petri dishes 48 h after transfection, after visible colonies were formed, the colonies were counted after staining with 1% crystal violet. To determine the apoptosis, cells were plated in a 6-well plate at a density of 5×10^5 cells/well, and when the cells grew to the logarithmic growth phase, the cells were harvested and counted. After centrifugation, the cells were then resuspended by adding 195 μ L Annexin V-FITC binding solution. 5 μ L Annexin V-FITC and 10 μ L propidium iodide staining solution were then added to mixed thoroughly. The cells were then incubated in the dark for 10-20 min and then analyzed by flow cytometry.

1.2.5 Western blotting

RIPA buffer (Beyotime, Shanghai, China) was used to extract proteins from cells. The BCA kit was used to determine the protein concentration and the loading buffer was used to denature the protein. The protein sample (20 μ g) was electrophoresed on a 10% SDS-PAGE gel and after that transferred to a PVDF membrane (Millipore, USA). The PVDF membrane was blocked with 5% skim milk, then incubated with specific primary antibody overnight at 4°C. The antibodies used in the study were anti-YAP1 (1:1000; catalog number ab52771; Abcam) and anti-GAPDH (1:3000; catalog number 5174S; Cell Signaling Technology). Then the PVDF membrane and the secondary antibody were incubated for 2 h at room temperature. Enhanced chemiluminescence reagent (Pierce™ ECL, Thermo Scientific™, USA) was then used for protein detection.

1.2.6 Luciferase reporter gene experiment

The HCC cell line was seeded into a 24-well plate. After 24 h of incubation, the reporter vector containing wild-type (WT) or mutant (MUT) 3'UTR of circDUSP16 and YAP1 and miR-136-5p mimic were co-transfected into Huh7 and Hep3B cell lines. 48 h after transfection, Luc-Pair™ dual luciferase assay kit (Genecopoeia) was used to quantify the luciferase activity.

1.3 Statistical analysis

Statistical analysis was performed by SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism. The student independent or paired t-test and analysis of variance (ANOVA) were used to assess the statistical significance of the comparison between the two groups. Pearson correlation analysis was used to analyze the correlation. The Kaplan-Meier method and log-rank test were used to analyze the survival curve. $P < 0.05$ was considered statistically significant.

2 Results

2.1 The up-regulation of circDUSP16 was associated with the poor survival of HCC patients.

The qRT-PCR analysis showed that the expression of circDUSP16 in HCC tissues was significantly higher compared with neighboring normal tissues (Figure 1A; $P < 0.001$). Kaplan-Meier analysis showed that compared with patients with low circDUSP16 expression, patients with high circDUSP16 expression had a shorter survival time (Figure 1B). Furthermore, the expression level of circDUSP16 in different HCC cell lines was measured by qRT-PCR analysis, and the results showed that circDUSP16 was highly expressed in Huh7 and Hep3B cell lines (Figure 1C; $P < 0.001$).

2.2 Inhibition of circDUSP16 can inhibit the proliferation and promote the apoptosis of HCC cells.

The transfection efficiency of sh-circDUSP16 in Huh7 and Hep3B cell lines was determined by qRT-PCR analysis (Figure 2A; $P < 0.05$, $P < 0.001$, $P < 0.01$). By transfecting sh-circDUSP16 in Huh7 and Hep3B cell lines, it was found that sh-circDUSP16 significantly inhibited the viability and proliferation of Huh7 and Hep3B cell lines (Figure 2B; $P < 0.05$, $P < 0.01$), and inhibited colony formation (Figure 3C-D; $P < 0.01$), as well as promoting cell apoptosis (Figure 3E-F; $P < 0.01$).

2.3 circDUSP16 targets miR-136-5p in HCC

Compared with adjacent tissues, the expression level of miR-136-5p was down-regulated in tumors of HCC patients (Figure 3A; $P < 0.001$). Kaplan-Meier analysis showed that compared with patients with high miR-136-5p expression, patients with low miR-136-5p expression had a shorter survival time (Figure 3B). Pearson correlation analysis showed that circDUSP16 was negatively correlated with the expression level of miR-136-5p in HCC tissue samples (Figure 3C; $R^2 = 0.6954$, $P < 0.05$). The 3'UTR binding site of circDUSP16 and miR-136-5p was shown in Figure 3D. Besides, after the co-transfection of miR-136-5p mimic and WT or MUT circDUSP16 into Huh7 and Hep3B cell lines, it was found that miR-136-5p reduced the luciferase activity of WT circDUSP16 in Huh7 and Hep3B cell lines but is not for the MUT circDUSP16

(Figure 3E; $P < 0.001$). In addition, we found that both circDUSP16 and miR-136-5p could regulate the expression of each other (Supplementary figure 1).

2.4 circDUSP16 and miR-136-5p regulate the Hippo/YAP signaling pathway in HCC.

Compared with adjacent tissues, YAP1 expression was up-regulated in tumors of HCC patients (Figure 4A; $P < 0.001$). Kaplan-Meier analysis showed that compared with patients with low circDUSP16 expression, patients with high YAP1 mRNA expression had a shorter survival time (Figure 4B). Pearson correlation analysis showed that the expression of YAP1 was positively correlated with circDUSP16 expression (Figure 4B; $R^2 = 0.7944$, $P < 0.05$), while the expression of YAP1 was negatively correlated with miR-136-5p expression (Figure 4C; $R^2 = 0.5825$, $P < 0.05$). The 3'UTR binding site of YAP1 with miR-136-5p was shown in Figure 4D. Besides, after co-transfection of miR-136-5p mimic and YAP1 WT or YAP1 MUT into Huh7 and Hep3B cell lines, it was found that miR-136-5p reduced the luciferase activity of YAP1 WT in Huh7 and Hep3B cell lines, but not in YAP1 MUT (Figure 4E; $P < 0.001$). Furthermore, after overexpression or inhibition of miR-136-5p in Huh7 and Hep3B cell lines (Figure 4F; $P < 0.001$), western blotting results showed that overexpression of miR-136-5p can reduce the expression of YAP1 in cells (Figure 4G) while inhibiting miR-136-5p can increase YAP1 expression in cells (Figure 4G).

2.5 circDUSP16 regulates HCC cell proliferation and apoptosis through targeting miR-136-5p/YAP1.

In Huh7 and Hep3B cell lines, where the expression of circDUSP16 and miR-136-5p were interfered with. The results of western blotting showed that transfection of sh-circDUSP16 can reduce the expression of YAP1 in the cells, whereas the inhibition of miR-136-5p can raise YAP1 expression and reverse the inhibition of the YAP1 expression by circDUSP16 (Figure 5A). The MTT analysis showed that sh-circDUSP16 can reduce the cell viability and proliferation while inhibiting miR-136-5p could promote the cell viability and proliferation and reverse the functions of sh-circDUSP16 (Figure 5B; $P < 0.05$, $P < 0.01$). Furthermore, colony analysis and flow cytometry showed that sh-circDUSP16 can inhibit colony formation and promote cell apoptosis (5C-D; $P < 0.01$, $P < 0.001$), on the other hand, the inhibition of miR-136-5p could promote colony formation and inhibit cell apoptosis and reverse the functions of sh-circDUSP16 (5E-F; $P < 0.05$, $P < 0.01$).

3 Discussion

More and more evidence shows that circRNAs play vital roles the progression of various cancers, including HCC [14–16]. For example, in HCC patients with a higher body fat ratio, the circ-DB expression is up-regulated, and circ-DB can inhibit miR-34a, activate the USP7/Cyclin A2 signaling pathway, thereby accelerating tumor growth, and reducing DNA damage [17]. TWIST1 can promote the expression of EMT in HCC vimentin by increasing the expression of CIRC-10720 [18]. circ-ADD3 can promote EZH2 degradation through CDK1-mediated ubiquitination, thereby inhibiting the metastasis of HCC cells [19]. In this study, we found for the first time that circDUSP16 is highly expressed in liver cancer tissues and cells

and is significantly related to patient survival. The in vitro studies have also confirmed that inhibiting the expression of circDUSP16 can inhibit the proliferation and promote the apoptosis of HCC cells, possibly by competitively binding to miR-136-5p, therefore regulating the expression of YAP1, and participating in the progression of HCC.

Functionally, circRNA can act as an either oncogene or tumor suppressor gene in HCC. For instance, circ_HIPK3, circ_UBXN7, and circ-PRKCI can promote tumor cell proliferation, migration, and invasion, and accelerate the apoptosis of HCC cells [20–22]. While some circRNA, such as circZKSCAN1, can inhibit tumorigenesis and invasion of HCC cells [23]. However, the functional role of circDUSP16 in HCC is still unclear. In this study, we explored the role of circDUSP16 in HCC cells and found that inhibiting the expression of circDUSP16 can inhibit the proliferation and promote the apoptosis of Huh7 and Hep3B cell lines. These results indicate that circDUSP16 may be a tumor-promoting factor in HCC cells.

miRNA is involved in various physiological and pathological processes [24–26]. It has been reported that a variety of miRNAs regulate the malignant biological behavior of cancer cells, including the proliferation, migration, apoptosis, autophagy, drug resistance, and angiogenesis of a variety of cancers including HCC [27–29]. Mechanically, miRNAs usually regulate target genes by targeting the 3'-untranslated region of target mRNA, thereby inhibiting, or promoting tumor occurrence and development [30]. Previous studies have shown that miR-136-5p is down-regulated in epithelial ovarian cancer and glioma cell lines [31–32], while up-regulated in non-small cell lung cancer [33]. Also, miR-136-5p can promote chemotherapy-induced apoptosis of glioma cells [31]. According to the above research, miR-136-5p may be expressed heterologously in different cancers. In this study, we found that miR-136-5p expression was down-regulated in HCC samples and cell lines and was negatively correlated with the expression of circDUSP16 in HCC tissue samples. As expected, we also found that both circDUSP16 and miR-136-5p could regulate the expression of each other. This can be explained by the Argonaute 2 (Ago2)-mediated RNA decay, in which the binding of miRNAs on circRNAs is identified by Ago2 and executed by the RNA-induced silencing complex [34].

In the current study, YAP1 was identified as a target of miR-136-5p. YAP1 plays an important role in the Hippo signaling pathway, which is an essential pathway in cell proliferation, apoptosis, and organ growth regulation [35]. YAP1 has also been reported to be involved in the development of cancer because it can promote cell proliferation, inhibit cell apoptosis, and induce the epithelial-mesenchymal transition and drug resistance of cancer cells [36]. This study found that YAP1 is the target gene of circDUSP16 and miR-136-5p. Specifically, overexpression of miR-136-5p reduced the expression level of YAP1, while the inhibition of miR-136-5p increased the expression level of YAP1. In addition, transfection of circDUSP16 can increase the expression level of YAP1, and the ectopic expression of miR-136-5p can reverse this change and further affect the proliferation and apoptosis of HCC cells. These results indicate that circDUSP16 regulates HCC cell proliferation and apoptosis by targeting miR-136-5p/YAP1.

4 Conclusion

The ectopic expression of circDUSP16 can regulate the expression of YAP1 by competitively binding to miR-136-5p and participates in the progress of HCC. Our findings in this study may provide provide mechanism basis for investigating new therapeutic targets for HCC.

Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authorship Principles

Conception and design: All authors; Administrative support: Xiaofei Tang; Provision of study materials or patients: Yang Xiang; Collection and assembly of data: All authors; Data analysis and interpretation: Yang Xiang; Manuscript writing: All authors; Final approval of manuscript: All authors.

Data Availability

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

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Hangzhou Fuyang Hospital of Traditional Chinese Medicine.

Informed Consent

Informed consent was obtained from all individual participants included in the study. The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 3a, 3b, 3c, 4a, 4b, 4c, and 4d.

Compliance with Ethical Standards:

The authors declare that they have no conflict of interest.

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Supplementary

Supplementary Figure 1 is not available with this version

Figures

Figure 1

Up-regulation of circDUSP16 is associated with poor survival of HCC patients. (A) qRT-PCR analysis of circDUSP16 expression level in HCC tissue and adjacent tissues of 30 pairs of HCC patients. (B) Kaplan-Meier analysis of the relationship between high or low expression of circDUSP16 and the overall survival rate of HCC patients. (C) qRT-PCR analysis of the expression level of circDUSP16 in different HCC cell lines. ** $P < 0.01$ *** $P < 0.001$.

Figure 2

Inhibition of circDUSP16 can inhibit the progress of HCC. (A) qRT-PCR analysis of the transfection efficiency of sh-circDUSP16 in Huh7 and Hep3B cell lines. (B,C,D) Analysis of cell viability and proliferation, cell colonies, (E-F) cell apoptosis after transfection with sh-circDUSP16 in Huh7 and Hep3B cell lines. * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$.

Figure 3

circDUSP16 targets miR-136-5p in HCC. (A) The miR-136-5p expression in 30 pairs of HCC patients' tumors and adjacent tissues. (B) Kaplan-Meier analysis of miR-136-5p expression and survival rate of HCC patients. (C) Pearson correlation analysis of circDUSP16 expression and miR-136-5p expression. (D) 3'UTR binding site of circDUSP16 with miR-136-5p. (E) Luciferase activity of WT or MUT circDUSP16 after transfection with miR-136-5p mimic or miR-NC in Huh7 and Hep3B cell lines. *** $P \leq 0.001$

Figure 4

circDUSP16 and miR-136-5p regulate the Hippo/YAP signaling pathway in HCC. (A) The YAP1 expression in 30 pairs of HCC patients' tumors and adjacent tissues. (B) Kaplan-Meier analysis of the relationship between high or low expression of miR-136-5p. (C) Pearson correlation analysis of YAP1 expression and circDUSP16 expression in tissues. (D) Pearson correlation analysis of YAP1 expression and miR-136-5p expression. (E) 3'UTR binding site of YAP1 with miR-136-5p. (F) Luciferase activity of YAP1 WT or YAP1 MUT after transfection with miR-136-5p mimic or miR-NC in Huh7 and Hep3B cell lines. (G) The expression of miR-136-5p in Huh7 and Hep3B cell lines after overexpression or inhibition of miR-136-5p. (H) Western blotting was used to detect the expression level of YAP1 in Huh7 and Hep3B cell lines. *** $P \leq 0.001$

Figure 5

circDUSP16 regulates HCC proliferation and apoptosis by targeting miR-136-5p/YAP1. A) Western blotting was used to detect YAP1 expression in Huh7 and Hep3B cell lines after simultaneous interference of circDUSP16 and miR-136-5p. (B) Analysis of cell viability and proliferation after simultaneous interference with circDUSP16 and miR-136-5p in Huh7 and Hep3B cell lines. Analysis of cell colony number after simultaneous interference of circDUSP16 and miR-136-5p in Huh7 and Hep3B cell lines. (E-F) Analysis of cell apoptosis after simultaneous interference with circDUSP16 and miR-136-5p in Huh7 and Hep3B cell lines. ** $P \leq 0.01$, *** $P \leq 0.001$, compared with sh-NC group; # $P \leq 0.05$, ## $P \leq 0.01$, compared with sh-circDUSP16+inhibitor NC group.